USE OF THESSES

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Exercise-induced changes in neutrophil and erythrocyte functions

by

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A thesis submitted to the Australian National University for the degree of Doctor of Philosophy

June, 1993
The design, technical work, data analysis, and writing of all manuscripts submitted in this thesis were carried out by myself with the following exceptions:

(i) the one minute maximal exercise protocol was designed by Mr A.B. Gray;

(ii) the phagocytic and bactericidal assays were performed by Mr S.J. Mackenzie

(iii) the 45 minute treadmill running protocol and antioxidant supplementation program were designed by Mr I. Gillam (Australian Institute of Sport, Canberra). Mr Gillam also performed the measurements of α-tocopherol and ascorbate concentrations in plasma and erythrocyte membranes; and

(iv) the red blood cell density fractionation experiments (and the associated laser densitometry scanning) were carried out by Mrs M. Kolbuch-Braddon.

Preliminary experiments conducted during my BSc (Hons) year were the starting point for the experimental and conceptual studies described in this thesis: they are incorporated in the published manuscript presented as Chapter 2.1 and the unpublished manuscript presented as Chapter 6.2. Some of the statistical analyses were carried out by Ross Cunningham and Christine Donnelly (University Statistical Consulting Unit). This is acknowledged in the experimental manuscripts where appropriate.

John A. Smith
21 June, 1993
Outline of thesis presentation

The work presented in this thesis consists of two separate projects which addressed the effects of single episodes of both moderate and vigorous exercise (and the chronic effects of endurance training) on neutrophil microbicidal activity and erythrocyte susceptibility to osmotic and oxidative stress. The thesis is presented in two parts in the form of published and unpublished manuscripts. Part 1 reports the neutrophil studies and Part 2 the erythrocyte studies. Each Part contains an abstract, literature review (including summary and scope of thesis), experimental papers, and a general discussion. The relationship between the experimental papers in both parts of the thesis will be obvious from the introductions and discussions in each paper. The sequence of presentation of the experimental papers does not necessarily reflect the order in which the studies were carried out. Only the pages of text in the unpublished manuscripts are numbered in consecutive order. The figures and tables are not numbered (and thus not included in the page counts) but are inserted in between text pages in the results section of each manuscript after their first citation.
Acknowledgements

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Publications


Publications (to be submitted)


Presentations at Scientific Meetings


*Invited Keynote Address*
(Presented by Dr Weidemann at the Second international meeting of the Australian Behavioural Immunology Group, Newcastle, NSW, March, 1992).

**Academic Prize**

1990 Young Investigator Award [International Society for Free Radical Research (Pasadena, USA)]
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PART 1

NEUTROPHIL FUNCTION
ABSTRACT

Many reports suggest that, whilst moderate exercise may enhance resistance to infection, the effects of intensive exercise may be detrimental. Neutrophils are the “first-line-of-defence” against infectious agents (including viruses) and tumour cells but, paradoxically, these cells have also been implicated in tissue damage associated with inflammatory diseases. In this part of the thesis, I have examined the effects of single episodes of exercise (using both submaximal and maximal workloads) and athletic training on the “oxidative burst”, which is the best characterized pathway of the neutrophils’ microbicidal armoury (Chapter 2). Furthermore, I have investigated the humoral mechanisms which may regulate the intensity-dependent effects of exercise on the immune system (Chapter 3).

The microbicidal activity of neutrophils was assessed, initially, in cells isolated from untrained and highly-trained men both before and after one hour of cycling at 60% of maximum aerobic capacity (VO\textsubscript{2} max) and, on a separate occasion, before and after one minute of cycling at maximum effort. Luminol-amplified chemiluminescence was monitored to peak intensity following stimulation of the cells \textit{in vitro} with a phagocytic stimulus, [opsonized zymosan (OZ)] over a wide concentration range. These studies were followed up - with untrained subjects only - by measuring, using flow cytometry, the impact of submaximal exercise on the distribution of neutrophil “subpopulations” in the circulation and by determining the ability of the cells to kill acridine-orange-stained \textit{Staphylococcus aureus} on the basis of a green (living) to red (dead) transition as assessed by fluorescence microscopy. In association with these cellular studies, the effects of exercise on the plasma concentrations of potentially immunoregulatory cytokines and neuro-endocrine hormones (measured by specific immunoassays) were investigated.

The capacity of OZ-stimulated neutrophils to produce microbicidal reactive oxygen species in response to unit stimulus concentration (k) was “primed” (enhanced) significantly in response to one hour of cycling at 60% of maximum aerobic capacity (VO\textsubscript{2} max) but depressed after one minute of maximal exercise (P < 0.05). Compared to untrained controls, the specific activity of the neutrophil “oxidative burst” was 50% lower, both before and after exercise, in subjects undertaking long-term intensive training of about 25 hours/week (P < 0.075). This training trend was confirmed by a 13 month longitudinal study (P < 0.05). This work was completed by examining
possible mechanisms involved in the priming response in an attempt to elucidate its physiological relevance.

The bacterial killing capacity of neutrophils was primed substantially immediately after one hour of moderate cycling and this correlated with increased phagocytic activity ($P < 0.01$). The priming response also involved a small increase in the concentration of extracellular superoxide and the release of $\text{H}_2\text{O}_2$ from cells stimulated with either OZ or the protein kinase-C activator, phorbol myristate acetate (PMA) ($P < 0.05$). Mechanistic studies showed that, despite some time-dependent variability in some neutrophil responses, moderate exercise increased the expression of complement receptors by 20% ($P = 0.045$) and increased the capacity of neutrophils to generate $\text{H}_2\text{O}_2$ intracellularly in response to stimulation with PMA by three-fold ($P = 0.025$). A 10% increase in the rate of $\text{O}_2^-$ release extracellularly after stimulation with OZ ($P = 0.012$), as well as a two-fold increase in the plasma concentration of complexed elastase ($P = 0.003$), were also found after exercise.

In association with these cellular studies, I have examined whether the concentrations of humoral factors released into the circulation during exercise change sufficiently to be responsible for the intensity-dependent responses of neutrophils to exercise. Increases in the plasma concentrations of interleukin-1$\beta$, interleukin-6, tumour necrosis factor-$\alpha$, and granulocyte-macrophage colony-stimulating factor were not sufficiently different to the normal circadian fluctuations to be likely candidates for any putative neutrophil-priming role. Attention then turned to the neuroendocrine system as a possible source of such activity, since exercise is known to have profound acute and chronic effects on pituitary/adrenal secretions that have distinct intensity- and duration-dependant thresholds. Furthermore, evidence published during the course of this work showed that growth hormone and prolactin both displayed potent phagocyte-priming activity $\textit{in vitro}$. A neuroendocrine/cytokine hypothesis was developed to explain the intensity-dependent effects of exercise on neutrophil microbicidal activity and immunity. This hypothesis proposed that low to moderate levels of exercise may boost immunity through the action of growth hormone and, possibly, prolactin on cellular and humoral immune mechanisms. These beneficial effects may be negated once exercise intensity reaches a critical threshold where, through feedback mechanisms that may be initiated by cytokines (interleukin-1$\beta$ and tumour necrosis factor-$\alpha$),
adrenocorticotropic hormone (ACTH) release is activated; ACTH, in turn, induces the release of immunosuppressive glucocorticoids and opioids into the circulation which potentially impair a variety of immune responses.

One hour of moderate exercise increased the plasma concentration of growth hormone by 10- to 20-fold in both trained and untrained subjects but it did not alter the basal levels of prolactin, substance-P or endotoxin. In contrast to previous reports, we found increases in the plasma concentrations of adrenocorticotropic hormone (50%) and cortisol (20%) in the untrained subjects only (P < 0.05). While consumption of a glucose solution (5% w/v) every 15 min during exercise substantially attenuated growth hormone secretion, a three-fold increase was still found; glucose consumption did not influence the responses of any of the other hormones measured to exercise. However, while this modest rise in growth hormone was associated with priming of intracellular H₂O₂ production in response to stimulation with PMA, increased complement receptor expression, and the release of elastase into the circulation, the magnitude of all these responses declined progressively once plasma growth hormone concentration reached and increased beyond a critical threshold (> 20 ng/ml).

Overall, the cellular results suggest that the intensity-dependent effects of exercise on neutrophils may involve a shift in the proportion of low- to high-activity cells, which may due to priming of some cells and activation of others, the net response reflecting a balance between the two states. This may explain the variable but "real" intensity-dependent responses of neutrophils to exercise and offers some insights into why the putative effects of exercise on immunity has generated considerable controversy. This work indicates ways in which the controversy may now be resolved.

The neuroendocrine results are consistent with the hypothesis that moderate regular exercise may enhance resistance to infection by priming the phagocytic and microbial killing capacities of neutrophils through a growth hormone-dependent mechanism. In contrast, whilst intensive training may increase susceptibility to infection by diminishing neutrophil oxidative capacity below a critical threshold, it may also reduce inflammatory damage caused by individual episodes of intense exercise by limiting the extent of endogenous neutrophil activation. While acute immunosuppression may prevent exercise-induced inflammatory injury, chronic depression of the immune response - as observed in some athletes -
may also increase susceptibility to infection. The general discussion concludes with a revised neuroendocrine/cytokine hypothesis that attempts to explain the intensity-dependent effects of exercise on the human immune system and makes several suggestions for future work.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>APP</td>
<td>Acute-phase protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
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<tr>
<td>CD</td>
<td>Cluster designation</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>C3b</td>
<td>Complement factor 3b</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
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<tr>
<td>CRF</td>
<td>Corticotropin releasing factor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine 5'-monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>sn-1,2-Diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fc</td>
<td>&quot;Fragment crystallizable&quot; domain of Ig</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide regulatory protein</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>INF</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-tri-phosphate</td>
</tr>
<tr>
<td>LT</td>
<td>Leucotriene</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NK-cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OH•</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>phox</td>
<td>phagocytic oxidase</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PK-C</td>
<td>Protein kinase-C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SERPIN</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum rate of oxygen consumption</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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</table>
CHAPTER 1

LITERATURE REVIEW

This chapter includes a summary and outline of the work presented in this section of the thesis.
Natural and adaptive immune mechanisms

Figure 1. Natural and adaptive immune mechanisms.

The mechanisms mediating immunity consist of non-specific and specific processes that are each composed of cellular and humoral elements. Immune responses are also associated with inflammation. This simplified diagram illustrates the complexity and co-operativity of the cellular and humoral networks involved in the immune response.

Source: Playfair (1984)
1. INTRODUCTION

Whilst the health benefits of regular physical activity are well known, the relationships between exercise and immunity have only recently become a focus of intense research activity. At present, considerable evidence suggests that, while intensive training increases susceptibility to infection, moderate exercise may, in contrast, enhance some immune responses. The mechanisms by which these paradoxical responses to exercise are produced are poorly understood.

The human immune system consists of a complex network of interacting cells and humoral factors (Figure 1). The mechanisms mediating immunity can be divided functionally into innate (natural or non-specific) and adaptive (specific) elements. Whilst specific immune responses may take hours or even days to become effective (because of antigen processing by macrophages, T-cell and B-cell activation, and antibody synthesis), non-specific immune processes such as phagocytosis by neutrophils are activated immediately upon contact with infectious agents.

In this chapter, I have reviewed the literature (predominantly on human studies) associated with my own work on neutrophil microbicidal activity as a model of exercise-induced changes in immunity. The article begins by reviewing the biology of the neutrophil and the regulation of its microbicidal activities (Sections 2 & 3). This is followed by an overview of the influence of exercise on the immune system (Section 4) and its potential physiological consequences (Section 5). The summary and conclusions (Section 6) are followed by an outline of the hypotheses tested in this part of the thesis.
2. NEUTROPHILS

2.1 Role in immunity and inflammation

Neutrophils are the most abundant cell type in the human immune system (Huizinga et al., 1990a). They represent 50 to 60% of the total white blood cell (WBC) population in the circulation, and constitute the "first-line-of-defence" against infectious agents or "non-self" substances (Weissmann et al., 1980). Once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of infection or injury (Schleimer et al., 1989). Their targets include bacteria, fungi, protozoa, viruses, virally-infected cells, and tumour cells (Lehrer et al., 1988a; Ratcliffe et al., 1988). A severe impairment in neutrophil microbicidal function is normally fatal (Babior, 1987) and suppressed neutrophil functions are found in patients with AIDS and AIDS-related complications (Ellis et al., 1988). Thus, evasion of the first line neutrophil defences may provide a "window of opportunity" for local infections to be established unless the infectious agent is rendered harmless by interaction with memory components such as neutralizing antibodies (e.g. IgA, IgG) already present. Because neutrophils are the major circulating WBC type, they are readily accessible to experimental investigation.

The primary defensive role of neutrophils is to phagocytose and kill micro-organisms at sites of infection but these cells - by virtue of the potency of their microbicidal arsenal - may also contribute to the pathology of various inflammatory conditions and thus, to some extent, they are "double-edged swords". Since neutrophils lack specific receptors that recognise foreign antigens, they must rely on other arms of the immune system to select their targets (e.g. antibodies, complement factors and cytokines) (Weiss, 1989). The importance of neutrophils to host defence is shown by the frequency and severity of infections that occur in people who are deficient in neutrophil number or function (Lehrer and Ganz, 1990). Neutrophil microbicidal mechanisms consist of the formation of a combination of reactive oxygen and nitrogen species, and release of a number of hydrolytic enzymes and antimicrobial polypeptides (see Section 2.5). These mechanisms can be influenced - both positively and negatively - by a wide variety of soluble mediators which include cytokines, neuroendocrine factors, arachidonic acid (AA) metabolites and cyclic nucleotides (see Section 3). Neutrophils exist not only in dormant or
activated states but also in various intermediate stages. For example, priming is a mechanism whereby dormant neutrophils acquire a state of preactivation that enables a more powerful response to be generated once microbicidal activity is initiated (see Section 2.6). Neutrophils also interact reciprocally with other cells (e.g. T-cells, endothelial cells, and platelets) either through cell-to-cell contact or via humoral mediators (Zhang et al., 1992; Zhou et al., 1992). While neutrophils are thought, classically, to be effector cells, they also synthesize and secrete humoral mediators such as cytokines which may play a role in regulating the afferent limb of the immune response (Lloyd and Oppenhiem, 1992).

While the importance of neutrophils in fighting bacterial and fungal infections is well recognized, only limited attention has been paid to their involvement in viral infections. This is surprising considering that neutrophils are found in abundance in virally-induced lesions (Radcliffe et al., 1988; Van Strijp et al., 1989). Neutrophils are the primary cells responsible for protection against the influenza virus during the initial stage of infection (Tsudo et al., 1987), and they appear to play an important role in diminishing the severity of vaccinia and herpes infections (West et al., 1987). In contrast to these acute diseases, chronic influenza infections can diminish or exhaust the microbicidal potency of neutrophils (Hartshorn et al., 1990). The systemic depression of neutrophil activity could lead to secondary bacterial infections but the presence of priming agents such as cytokines (acting either locally or systemically) may, however, prevent this (J.S. Abramson et al., 1991) (see Section 3.1).

The rapid changes that occur in the antigenic determinants of some viruses such as influenza suggest that non-specific defences (e.g. neutrophils and natural killer cells) may, out of necessity, play an essential role in combating these infections (Hartshorn et al., 1990). In AIDS patients, for example, GM-CSF/G-CSF treatment primes the antibody-dependent cytotoxicity of neutrophils against HIV-infected lymphocytes in vitro, but neutrophils themselves are not susceptible to direct HIV infection (Baldwin et al., 1989). Neutrophils bind to opsonized viruses and virally-infected cells via Fc and C3b receptors (Ratcliff et al., 1988). The rate of virion uptake is increased following TNF-α-induced priming (Van Strijp et al., 1989). Viruses such as influenza can be inactivated by neutrophils through damage to viral proteins (e.g. haemagglutinin and neuraminidase).
mediated by the myeloperoxidase (MPO) released during degranulation (Yamamoto et al., 1991a; Klebanoff and Coombs, 1992).

Whilst neutrophils are critical to host defence, they have also been implicated in the pathology of a number of inflammatory disorders including rheumatoid arthritis, muscle damage (see Fantone and Ward, 1982) and exercise-induced asthma (Moqbel et al., 1986). This may be a side-effect of their function in removing damaged host cells, tissue or debris as a first step in tissue remodelling. Endogenous inhibitors and autoantibodies against some neutrophil components may protect host tissues, to some extent, from such gratuitous damage (Van der Woude et al., 1989). Tissue damage occurs when neutrophil microbicidal products are released into the extracellular milieu to such an extent that cellular defences (antioxidant and antiprotease screens) in the immediate vicinity are overwhelmed (see Section 2.5). Chlorinated oxidants, in particular, have been shown to cause tissue damage and to suppress lymphocyte function (El-Hag and Clark, 1987). Oxidants of neutrophil origin have also been shown to cause red blood cell damage and destruction in vivo (Hatherill et al., 1986). Elastase and other neutrophil hydrolytic enzymes such as collagenase and MPO may augment the tissue damage initiated by oxidants, especially if endogenous inhibitors of these enzymes are inactivated by the same oxidants (Weiss, 1989; Halliwell et al., 1992) (see Section 2.5.3).

The “double-edged sword” nature of neutrophil function is evident in inflammatory conditions such as the adult respiratory distress syndrome. For many years, neutrophils have been implicated in the pathology of this condition because of the associated damage to lung tissue by oxidants and hydrolytic enzymes released from activated neutrophils. The impairment of neutrophil microbicidal activity that occurs as this condition worsens may be a protective response on the part of the host, which is induced locally by inflammatory products (Martin et al., 1991). This “down regulation” of neutrophil function may explain why 60% of the patients eventually die from this condition, usually from overwhelming pulmonary infections (Martin et al., 1991; Tagen et al., 1991).

2.2 Biology of the neutrophil

Neutrophils are round cells that elongate upon activation. They are rich in cytoplasmic granules and contain a lobulated chromatin-dense nucleus with no nucleolus (Wandall, 1988). Mature neutrophils (which are derived from
myeloid progenitors) are terminally-differentiated cells that are unable to replicate and possess only limited capacity to synthesize proteins de novo (Lew, 1990). The cytosol is rich in secretory granules but contains only a low density of mitochondria, endoplasmic reticulum, and the Golgi complex (Bjerrum, 1993). Three major granule types [known as azurophilic (primary), specific (secondary) and tertiary] have been characterized by biochemical and morphological criteria as well as the sequence in which they appear during maturation (Sandborg and Smolen, 1988). These granules contain various preformed enzyme systems, opsonin receptors, adhesion proteins (integrins) and ions (Table 1). During phagocytosis, the granules fuse with the invaginating plasma membrane to form a phagolysosome into which they release their contents, thereby creating a highly toxic micro-environment (Wandall, 1988) (see Section 2.6.2.4). This prevents release of the components of their microbicidal armoury into the extracellular milieu. In fact, the intralysosomal pH is highly-acidic with the low pH (~5) being maintained by an active proton-translocating pump (Klempner and Styrt, 1983).

Neutrophils are produced in human bone marrow at the amazing rate of 2.5 billion cells per hour under the control of various colony-stimulating factors (CSF) (Weisbart et al., 1989) which direct the production and differentiation of bone marrow progenitor cells (Gregory et al., 1991). The rate of neutrophil differentiation can increase as much as ten-fold during states of stress and infection (Cannistra and Griffin, 1988). The serum concentration of colony-stimulating factor activity (in particular, granulocyte and macrophage CSFs) and the number of colony-forming cells in mouse spleen and bone marrow both increase during infection (Cheers et al., 1988). CSFs also amplify the activities of various neutrophil functions including the oxidative burst in vitro (Gregory et al., 1991). During differentiation of neutrophils in vivo, phagocytic capacity is acquired early. This is followed by the capacity for non-oxidative killing, while the acquisition of chemotaxis and oxidative killing capacity represents the terminal stage of differentiation (Glasser and Fiederlein, 1987).

Specific opsonin receptors on the neutrophil plasma membrane recognise the Fc domains of various immunoglobulins (e.g. IgA, IgG) and complement factors (Kerr et al., 1990). Several types of Fc-receptors for IgG (FcγR) have been characterized: FcγRI is expressed on activated cells only while FcγRII (CD 32) and FcγRIII (CD 16) are low-affinity receptors which are
Table 1  Neutrophil granules and their contents

<table>
<thead>
<tr>
<th>Azurophilic (primary) granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolyases: cathepsin-G</td>
</tr>
<tr>
<td>elastase</td>
</tr>
<tr>
<td>lysozyme</td>
</tr>
<tr>
<td>Azurocidin</td>
</tr>
<tr>
<td>Bactericidal/permeability increasing protein</td>
</tr>
<tr>
<td>Defensins</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
</tr>
<tr>
<td>Lysozyme</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specific (secondary) granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoferrin</td>
</tr>
<tr>
<td>Lysozyme</td>
</tr>
<tr>
<td>Specific collagenase</td>
</tr>
<tr>
<td>Vitamin B₁₂-binding protein</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tertiary granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatinase</td>
</tr>
</tbody>
</table>

* See text for further explanation and sources.
responsible for co-ordinated activation of the NADPH oxidase (FcγRII) and the control of degranulation (FcγRIII) (Huizinga et al., 1990b). Complement receptors-1 (CR 1) and -3 (CR 3) bind the opsonizing fragments of C3 - which are designated C₃b and C₃bi respectively - and, thus, ensure that binding and phagocytosis of complement-coated targets takes place (O’Shea et al., 1985).

During the inflammatory response, chemotactic factors generated by infectious agents themselves, as well as those released as a result of their initial contact with phagocytes and other immune cells, signal the recruitment of additional neutrophils to sites of infection (Sandborg and Smolen, 1988). This response begins when neutrophils adhere to vascular endothelium and, under the influence of the chemotactic gradient, penetrate this layer and move through connective tissue to sites of infection (diapedesis) where they finally congregate and adhere to extracellular matrix components (e.g. laminin and fibronectin) through a diverse array of integrins that serve as complementary receptors (Lehrer et al., 1988b; Nathan and Sanchez, 1990). T-cells may play a role in the regulation of neutrophil influx into sites of infection by secreting chemotactic cytokines (Appelberg, 1992).

Phagocytosis is triggered upon the binding of opsonized microorganisms through opsonin receptors (for C₃b and Fc) or through non-specific glycosylated receptors that recognise certain lectins on target microorganisms. Two microbicidal processes are activated concomitantly with phagocytosis: (i) the oxidative burst (so-called because of the 50- to 100-fold increase in O₂ consumption) which results in the production of cytotoxic reactive oxygen species (ROS) and, possibly - through a different mechanism - reactive nitrogen species (RNS); and (ii) degranulation, which corresponds to the release of contents of azurophilic and specific granules into the phagocytic vesicle to form a phagolysosome. These processes involve rearrangement of contractile proteins, triggered by Ca²⁺, which leads to internalization of that portion of plasma membrane that contains the receptor/target complex (Krause and Lew, 1988) (see Section 2.6.2.4).

### 2.3 Distribution of Neutrophils

In humans, mature neutrophils capable of microbicidal activity exist in four distinct pools: (1) bone marrow; (2) circulating; (3) marginated; and (4) tissue-localised (Metcalf et al., 1986). At any one time, more than 90% of the neutrophil population is contained within the bone marrow (Metcalf et al.,
1986). They mature at this site before being released into the circulation where they spend only four to ten hours before marginating and entering tissue pools; little is known about the size or fate of the individual tissue pools (MacNee and Selby, 1990). Neutrophils can also be found in saliva (Yamamoto et al., 1991b) and synovial fluid (Robinson et al., 1992). Senescent neutrophils are thought to undergo apoptosis (programmed cell death) prior to removal by macrophages (MacNee and Selby, 1990).

Neutrophils in marginated pools are bound to cells of the vascular endothelium at localised tissue sites (e.g. in the lungs and spleen). The circulating and marginated pools are thought to be in equilibrium with each other (Baglin and Pollock, 1987) and neutrophils in these pools have been considered by most groups to constitute an homogeneous population (Berklow and Dodson, 1987a) of functionally-equivalent cells (Hetherington and Quie, 1985). Marginated cells may show, however, enhanced phagocytic and killing capacity for some pathogens (Steele et al., 1987). Neutrophil demargination can be induced by either physical exercise or infection-associated stimuli. Exercise-induced demargination, for example, occurs primarily as a result of the action of adrenalin on β-adrenergic receptors which, through the resulting increase in intracellular cAMP, attenuates endothelial adherence (Baglin and Pollock, 1987).

2.4 Neutrophil “Subpopulations”

Although mature neutrophils have long been considered to be a homogeneous cell population, peripheral blood neutrophils have been shown by many criteria to be heterogeneous (Gallin, 1984). The majority of these so-called “subpopulations” have been identified on the basis of cell-surface markers, but heterogeneity within distinct neutrophil pools may also be due to subtle differences in levels of maturity and/or the state of activation of individual cells rather than to the presence of distinct subpopulations with relatively fixed characteristics (Brown et al., 1991). However, some subpopulations have been found (e.g. those identified by C10 antibodies) which are functionally homogeneous (Brown et al., 1991). Subpopulations have also been isolated on the basis of size and density by counterflow centrifugal elutriation (Glasser and Fiederlein, 1987; Berkow and Dodson, 1987b).

There are a number of reported examples of functional heterogeneity within the neutrophil population. Single cell assessment of
chemiluminescence in neutrophils isolated from healthy human subjects showed that, while 80% responded to phorbol myristate acetate (PMA), only 30% were activated by opsonized zymosan (OZ) (Fritzsche and De Weck, 1988). A subpopulation of primed neutrophils detected in the blood of patients with an acute bacterial infection was no longer detectable once these patients had recovered (Bass et al., 1986). Deficiency of lactoferrin in 6-10% of circulating neutrophils has also been reported (Esaguy et al., 1989).

The functional significance of alterations in cell surface proteins is not entirely understood but it may involve changes in the expression of adherence and chemotactic receptors which may, in turn, alter these receptor-dependent activities. Neutrophils isolated from patients with blunt trauma, for example, show reduced binding of the 31D8 antibody which correlates with the increased susceptibility of these people to infection (Krause et al., 1988). These “dull 31D8” neutrophils that dilute out the circulating population are normally located selectively in the bone marrow and are probably immature and functionally inactive (Krause et al., 1990). In patients suffering from severe burns, a strong correlation has been established between the onset of bacteraemia infection and reductions in the proportion and absolute numbers of neutrophils positive for FcR and CR3 receptors (Babcock et al., 1990).

Neutrophils appear to express specific complement receptors sequentially on the surface of the plasma membrane as maturation proceeds, with the fully -mature cells expressing C3b receptors exclusively (Ross et al., 1978). Neutrophils expressing the highest densities of Fc and C3b receptors may margnate preferentially, as circulating cells bear relatively few of these receptors compared to activated neutrophils (Berger et al., 1989). If the preferential margination of neutrophils is followed during clinical haemodialysis, the circulation becomes enriched with a subpopulation of non-marginating neutrophils that have a significant defect in the OZ-activated oxidative burst as detected by luminol-dependent CL (Cohen et al., 1982). As this reduced oxidative burst is not due to an impairment of MPO activity, the authors have suggested that the “down regulation” of C3b receptors may be the factor responsible for the loss of responsiveness to opsonized particles.

Heterogeneity dependent on subcellular structures also occurs within the neutrophil population. Most neutrophils contain subpopulations of azurophilic granules that are themselves heterogeneous in density and
composition (Rice et al., 1986) and specific granules that exhibit differential sensitivity to changes in cytosolic free calcium (Perez et al., 1987).

2.5 Microbicidal mechanisms

Neutrophil microbicidal mechanisms consist of a combination of oxidative and enzymatic (oxygen-independent) processes which appear to be activated simultaneously upon initiation of phagocytosis (Figure 2). Associated with the oxidative burst (involving stimulation of NADPH oxidase) is degranulation which results in the release of MPO from the azurophilic granules and hydrolytic enzymes and defensins from both the specific and azurophilic granules (Fantone, 1991).

2.5.1. Oxidative mechanisms

2.5.1.1 Reactive oxygen species

The oxidative or respiratory burst [i.e. production of microbicidal reactive oxygen species (ROS)] in neutrophils is triggered upon phagocytosis or when the pathway is activated by an appropriate synthetic stimulus in vitro. Phagocytosis occurs when neutrophils recognise foreign targets opsonized by non-specific complement components (e.g. C₃b), C-reactive protein and/or antigen-specific antibodies. The opsonized target binds to a complementary receptor on the surface of the plasma membrane specific for the particular class of opsonin. Neutrophils also possess high- and low-affinity receptors for the bacterial peptide formyl methionyl-leucyl-phenylalanine (fMLP) (Omann et al., 1987) which can activate the oxidative burst (see Section 2.6.1). The oxidative burst is characterised by a large increase in oxygen uptake that is non-mitochondrial, insensitive to cyanide inhibition and accompanied by accelerated [1-¹⁴C]-glucose metabolism via the oxidative segment of the pentose phosphate pathway (Fantone and Ward, 1982).

The oxidative burst results in the sequential production of a variety of microbiostatic and microbicidal ROS (Figure 2). Superoxide (O₂⁻) is formed, initially, by the reduction of molecular oxygen by single electrons which originate from NADPH generated via the pentose phosphate pathway (Rossi, 1986). Superoxide production is catalysed by the combined action of a plasma membrane NADPH oxidase and cytochrome b₅₅₈ (with an redox
Figure 2. PMN phagocytosis and microbicidal activity.

This diagram illustrates the sequential process of phagocytosis and the activation of the neutrophil microbicidal armoury. See text for further explanation.

Source: Playfair (1984)
potential of -245 mV) which appears to be the terminal electron acceptor of a short electron transport chain that conveys single electrons from NADPH to oxygen (Babior, 1987; Segal, 1989). H⁺ ions derived from NADPH oxidation are released into the extracellular milieu through an electrogenic membrane channel which thereby allows only a slight reduction in the membrane potential and internal pH (Henderson et al., 1988). The α- and β-subunits of cytochrome b558 incorporate the NADPH binding site and both the FAD and haem electron transfer moieties (Rotrosen et al., 1992). Two cytosolic proteins (p47phox, p67phox), quinone and a Rac-related GTP-binding protein are thought to be the other functional components of this electron transport system (Clark et al., 1990; Rotrosen et al., 1992) (see Section 2.6.2.3). The NADPH oxidase system appears to be dissociated and thus inactive in "dormant" (inactive or unstimulated) neutrophils (Rossi, 1986). Upon activation, the cytosolic components translocate to the plasma membrane to assemble the active oxidase (Babior, 1992) (see Section 2.6.2.5). The importance of the oxidative burst in the microbicidal activity of neutrophils is highlighted in patients with severe impairments in this pathway (e.g. chronic granulomatous disease (CGD); these people suffer from repeated infections that respond poorly to therapy and almost invariably lead to early death (Babior, 1987). Studies on neutrophils isolated from CGD patients have, in fact, enabled the roles of the various components of the complex NADPH oxidase to be defined (Gallin et al., 1991).

Subsequent ROS formed from O₂⁻ include: (1) hydrogen peroxide (H₂O₂), formed rapidly by either spontaneous dismutation and/or the catalytic action of superoxide dismutase (SOD); (2) MPO-dependent oxyhalides such as HOCl generated by the reaction of H₂O₂ with the abundant Cl⁻ ions taken up from extracellular fluid, and secondary chlorinated oxidants formed by the reaction of HOCl with amines (Weiss, 1989); (3) the hydroxyl radical (OH⁻) and; (4) possibly singlet oxygen (¹O₂). MPO is not toxic on its own but requires H₂O₂ and halide ions to exert its cytotoxic effect (Weiss, 1989). The importance of MPO oxyhalides in neutrophil microbicidal activity, however, is poorly understood because MPO deficiency has been reported to have little clinical significance (Nauseef et al., 1983). Higher NADPH oxidase activity in cells isolated from MPO-deficient people may partly compensate for this deficiency (McKenna and Davies, 1988). Chlorinated oxidants are thought to be an important component of defence against protozoan and eukaryotic parasites (Ferranti
et al., 1987) and some viruses (Yamamoto et al., 1991a). Furthermore, microorganisms cannot catalytically detoxify HOCl although some can detoxify O$_2^-$ and H$_2$O$_2$ (Eaton, 1993). The hydroxyl radical is the most reactive oxidant known (Halliwell and Gutteridge, 1990). Its putative formation in neutrophils occurs by H$_2$O$_2$ decomposition which is catalysed by Fe$^{2+}$ derived from the reduction of Fe$^{3+}$ by O$_2^-$; the so-called “Fenton reaction”. Fe$^{3+}$ is complexed to lactoferrin which is stored in the specific granules (Fantone and Ward, 1982). Singlet O$_2$ is thought to be generated via the MPO/H$_2$O$_2$/HOCl system in small amounts (Steinbeck et al., 1992). There is considerable debate, however, as to whether OH$^-$ and $^{15}$O$_2$ are produced by neutrophils at all under physiological conditions; lactoferrin may, in fact, prevent Fe$^{3+}$ from being used as a “Fenton” catalyst (Weiss, 1989; Halliwell and Gutteridge, 1990). Killing and degradation of some micro-organisms may also require the additional participation of certain non-oxidative processes (see Section 2.5.2).

During phagocytosis, the production of ROS is restricted to the “interior” space of invaginating phagolysosomes and/or the outer surface of the plasma membrane, but O$_2^-$ and H$_2$O$_2$ can be detected in the extracellular milieu (Blake et al., 1987). The oxidative burst can be activated in vitro by stimuli that either: (1) mimic a physiological challenge (e.g. opsonized zymosan, immune complexes); or (2) that stimulate the pathway directly [e.g. phorbol myristate acetate (PMA), fMLP] in the absence of phagocytosis. Whilst critical to neutrophil antimicrobial function, the exact mechanisms by which different oxidants contribute to microbial killing have not yet been elucidated (McKenna and Davies, 1988). Superoxide and H$_2$O$_2$ may augment phagocytosis independently of the MPO-halide system whilst, in contrast, chlorinated oxidants may limit this process by oxidatively inactivating opsonin receptors (Gresham et al., 1988). Oxidants also promote the margination of neutrophils by triggering the expression of integrins on endothelial cells (Patel et al., 1991). These oxidants are also involved in the regulation of MPO activity: HOCl production is sustained by O$_2^-$ [which maintains MPO in the active form (compound I)] while SOD is inhibitory because saturating levels of H$_2$O$_2$ promote the formation of the inactive form (compound II) (Kettle and Winterbourn, 1990). Compound I is regenerated by ascorbic acid, which reduces compound II (Marquez et al., 1990). The mechanisms by which the oxidative burst is terminated are not known, but inactivation of the oxidase may occur when activation factors
are consumed or when the enzyme is inactivated by oxidants and hydrolytic enzymes released by the neutrophil (Taubet et al., 1989) or as a result of specific dephosphorylation reactions (Ding and Badwey, 1992) (see Section 2.6.2.1).

MPO is the most abundant protein in the neutrophil (Kettle and Winterbourn, 1990) and it constitutes about 5% of the total (Suzuki et al., 1983). Interestingly, MPO gives pus and other purulent fluids their characteristic yellow-greenish colour (Weiss, 1989). Various isozymes of MPO that have different molecular weights and net electric charge have been identified (Miyasaki et al., 1991). Because degranulation can occur prior to oxidant production, the initiation of the oxidative burst and MPO release may be controlled by separate mechanisms (Tosi and Hammerschlag, 1988). Whilst neutrophils deficient in MPO can kill microbes, they do so at a greater metabolic cost than MPO-rich cells by prolonging H₂O₂ production, possibly because HOCl is 100 to 1000 times more effective than H₂O₂ (McKenna and Davies, 1988; Eaton, 1993). Neutrophils from infected animals are significantly deficient in MPO suggesting that neutrophils activated during infection may release MPO into the bloodstream whilst in transit through the circulation (Bradley et al., 1982).

Neutrophils are rich in endogenous antioxidants such as glutathione and ascorbate (Halliwell et al., 1987; Voetman et al., 1980). Their ability to maintain these antioxidants in the reduced state during phagocytosis (Voetman et al., 1980) may prevent premature death from oxidative suicide. A significant proportion of activated neutrophils are not, in fact, destroyed during the killing process, but a significant refractory period must elapse before these cells can be reactivated with a secondary stimulus (Prasad et al., 1991).

2.5.1.2 Reactive nitrogen species

While the importance of the neutrophil oxidative burst has been well documented, neutrophils have been shown recently to be substantial producers of reactive nitrogen species (RNS). These species may contribute to the microbicidal activity of neutrophils [and to the tumouricidal activity of activated macrophages (Keller and Keist, 1989)] by reacting with ROS to form secondary cytotoxic species such as peroxynitrite (McCall et al., 1989; Beckman et al., 1990). Recently, the production of RNS was reported to
contribute substantially to the killing of *Staphylococcus aureus* by neutrophils *in vitro* (Malawista *et al.*, 1992).

ROS and RNS are produced by pathways that appear to be independent of each other so that the oxidative burst is not involved directly in the synthesis of RNS (Iyengar *et al.*, 1987). Nitrite, a stable end-product, is derived from the guanido nitrogen of L-arginine by a reaction catalysed by nitric oxide synthase (Ding *et al.*, 1988). Short-lived nitric oxide (NO) is produced continuously by unstimulated neutrophils incubated at 37°C but stimulation by fMLP arrests this pathway in favour of the oxidative burst (Wright *et al.*, 1989). Thus, while the ROS and RNS pathways are independent, they may compete for common substrates.

The major physiological role of RNS appears to be in the maintenance of vascular tone by activating guanylate cyclase in target smooth muscle cells (Wright *et al.*, 1989). The main role of neutrophil-derived NO may be to facilitate the migration of neutrophils from blood vessels to surrounding tissues by causing vasodilation (Wright *et al.*, 1989; Mehta *et al.*, 1989). NO facilitates endothelial relaxation while ROS initiate vasoconstriction through the production of O$_2^-$ which inactivates NO (Mehta *et al.*, 1991; Csaki *et al.*, 1991). Activated neutrophils have been implicated in the development of inflammatory injury to the microvasculature through the release of ROS and hydrolytic enzymes (Smith *et al.*, 1991) but a shift in the balance in favour of RNS production may prevent this.

Hypertensive patients appear to have circulating neutrophils that are more oxidatively active than those of their normotensive counterparts (Pontremoli *et al.*, 1989). This suggests that some types of hypertension may be mediated by the collective failure of neutrophils and endothelial cells to produce sufficient RNS to maintain appropriate vascular tone. Further support for this hypothesis comes from a report suggesting that excessive formation of NO may mediate the hypotensive effects of TNF (Kilbourn *et al.*, 1990). Much more work, however, is required to unravel the interactions between the RNS and ROS pathways and the physiological consequences of their reciprocal activities. Recently, doubt has been cast as to the authenticity of RNS production assessed by arginine analogues because these inhibitors of NO synthase have also been reported to inhibit the activities of haem-containing enzymes (Peterson *et al.*, 1992). RNS production by neutrophils has not been addressed in the current project.
2.5.2 Non-oxidative mechanisms

While the role of the oxidative burst is well established in the microbicidal activity of neutrophils, the importance of the non-oxidative mechanisms is poorly understood in comparison. Acid hydrolases and antimicrobial defensins are contained within the cytoplasmic granules (Lehrer et al., 1988b). The azurophilic granules contain many proteolytic and saccharolytic enzymes capable of digesting microbial structural proteins and mucopolysaccharides, whereas the contents of the specific granules include binding proteins (e.g. lactoferrin), which deprive micro-organisms of essential nutrients, and lysozyme, which destroys cell wall components (Meers et al., 1987). Most of these proteins are positively charged, which enhances their binding to cell surfaces (Henson and Johnson, 1987).

Neutrophil hydrolytic enzymes (e.g. lysozyme and elastase) augment microbial damage initiated by ROS and participate in the digestion of killed microbes and damaged host cells (Roitt, 1984). Serine proteases such as elastase and cathepsin-G hydrolyse proteins in bacterial cell walls while lysozyme degrades the polysaccharide components. The enzymes may also limit the spread of inflammation within local microenvironments by degrading priming agents such as TNF-α and lymphotoxin (Scuderi et al., 1991). Bactericidal/permeability-increasing protein, a factor that is highly toxic to gram-negative (but not to gram-positive) bacteria, can also neutralize endotoxin, the toxic lipopolysaccharide (LPS) component of gram-negative bacterial cell walls (Marra et al., 1992). Azurocidin is also active against gram-negative bacteria and to a lesser extent gram-positive bacteria and fungi; its microbicidal mechanism is not known but proteolysis is not involved (Gabray and Almeida, 1993). Lactoferrin may function by sequestering iron, thereby preventing the growth of ingested micro-organisms which survive the killing process (Molloy and Winterbourn, 1990).

Defensins, which constitute 30-50% of azurophilic granule protein, are potent antimicrobial peptides that are cytotoxic to a broad range of bacteria and fungi and some viruses; their toxicity may be due to membrane permeabilization of the target cell (Lehrer and Ganz, 1990). Limited attention has been paid to microbial DNA damage by neutrophils, but endonuclease activity in human neutrophils has been reported (Chung et al., 1991). While neutrophils do not degrade the DNA of phagocytosed E coli, monocytes, in contrast, degrade chromosomal DNA but not plasmid DNA; this could have
important implications in antibiotic resistance (Rozenburg-Arska et al., 1984). Oxygen-independent processes were not investigated in this project.

2.5.3 Interactions between oxygen-dependent and oxygen-independent mechanisms

The co-operative interactions between neutrophil oxidative and non-oxidative mechanisms form a powerful microbicidal system that is capable of killing most types of infectious agents (Roitt, 1984). This system may, however, damage host tissues if these toxic agents are released into the extracellular milieu. While MPO activity does not appear to be essential for killing most infectious agents, it appears to be important in preventing bacterial growth within the neutrophil (McKenna and Davies, 1988). The release of MPO may be an important prerequisite that enables hydrolytic enzymes, subsequently, to kill and degrade phagocytosed microbes (McKenna and Davies, 1988). Gram negative bacteria, for example, are resistant to lysozyme unless they are subjected simultaneously to oxidants and/or complement factors (Lehrer and Ganz, 1990). Defensins and oxidants interact synergistically to lyse tumour cells in vitro (Lichtenstein et al., 1988). Chlorinated oxidants also sustain the activity of some proteases (Weiss, 1989).

Serine proteases such as elastase constitute part of a regulatory circuit that modulates the oxidative and phagocytic functions of neutrophils, the activation of lymphocytes, and the activities of complement factors (Kusner et al., 1991). Serine proteases can prime the responsiveness of NADPH oxidase to fMLP and PMA by modifying proteins present on the outer surface of the plasma membrane, thereby increasing the lateral mobility of membrane lipids (Kusner et al., 1991).

Hydrolytic damage to host tissue occurs only when the precise balance between proteolytic and antiproteolytic activities is perturbed. Many antiproteases are members of the serine protease inhibitor (SERPIN) family (Mast et al., 1991). Oxidative stress may initiate tissue damage by reducing the concentration of extracellular antiproteases to below the level required to inhibit released proteases (Weiss, 1989). Chlorinated oxidants produced by neutrophils can inactivate antiproteases like $\alpha_1$-protease inhibitor and $\alpha_2$-macroglobulin (which are endogenous inhibitors of elastase) but, surprisingly, simultaneously activate latent metalloproteases such as
Many antiproteases are susceptible to oxidative attack (Henson and Johnson, 1987), probably because of exposed thiol groups. These destructive processes may prolong the inflammatory response by transforming the immediate non-specific effects mediated by ROS into more-prolonged effects that depend on the activation of a complex array of endogenous, and perhaps more specific, proteolytic activities (Desrochers and Weiss, 1988). These reactions may be regulated by negative feedback because SERPINs like α₁-protease inhibitor, for example, block O₂⁻ production by activated neutrophils (Bucurenci et al., 1992; Kilpatrick et al., 1992), possibly by blocking the synthesis of second messengers such as platelet activating factor (Camussi et al., 1988) and/or by inhibiting NADPH oxidase activity by mechanisms not linked directly to their antiprotease activities (Kilpatrick et al., 1992).

2.6 Priming and activation

Neutrophils exist in various states of activation which vary from dormant to primed to fully-activated. While activation triggers the immediate expression of neutrophil microbicidal activity, priming stimuli amplify the magnitude of the response when it is activated subsequently and/or switch cells from a non-responsive to a responsive state. While priming and activation appear to be distinct processes, they are biochemically integrated and require at least two events (initiation and prolongation) which may be mediated by separate mechanisms (Bass et al., 1989). For example, subactivating concentrations of stimuli such as fMLP and PMA induce priming of the oxidative burst (Kusner et al., 1991). Stimuli at priming concentrations also regulate other neutrophil activities: for example, fMLP at nanomolar concentrations activates chemotaxis, whereas micromolar concentrations arrest cell movement and trigger secretory processes (Lew, 1989). Although the distinct multistep mechanisms responsible for the induction of priming and activation (and the interaction between them) are poorly understood, several levels of involvement have been identified. The transmembrane signalling processes involved share salient features with those which occur in other cell types. They begin with the binding of soluble or particulate mediators to a complementary cell surface receptor and the
transduction of this initial signal to the effector machinery. The signal transduction processes involved in coupling receptor-ligand binding vary according to the nature of the stimulus (Sibille and Reynolds, 1990; see Section 2.6.2.3).

The molecular basis of priming could include modification of the whole neutrophil population or a significant subpopulation in one or more ways (Johnston and Kitagawa, 1985): (1) the number and/or affinities of plasma membrane receptors for specific activating agents or opsonins may be increased (see Section 2.6.1); (2) the activities of one or more of the components of the signal transduction pathway involved in coupling receptor-ligand binding might be increased (see Section 2.6.2); (3) the activities of one or more of the enzymes of the physiological process under investigation (e.g. the neutrophil oxidative network) may be enhanced as a result of increased content or catalytic efficiency; and (4) the steady-state concentrations of positive effectors (i.e. elevation of the activator/inhibitor ratio) of specific enzymes might be increased. These mechanisms may not be mutually exclusive, and priming and/or activation may be directed at particular phenomena that do involve the entire cell (Davies et al., 1991).

2.6.1 Cell surface proteins

Priming and activation are associated with increased expression of a variety of proteins and receptors on the cell surface. Some of these, which may include oxidase components and integrins, are derived from cytoplasmic granules (Manara et al., 1991). As well as increasing receptor number via translocation of receptors to the plasma membrane, which would provide an extended surface area for phagocytosis, the receptors themselves may have increased affinity for the ligand.

Many investigators have reported that “up regulation” of complement receptors occurs in vitro in response to a variety of neutrophil priming and activating agents including cytokines (Berger et al., 1988; Ogle et al., 1990), arachidonic acid (AA) metabolites (Ogle et al., 1990) and bacterial chemoattractants (Fearon and Collins, 1983) as well as in vivo in patients with thermal injury (Moore et al., 1986). Under normal conditions, CR-1 and CR-3 expression is minimal on dormant cells but can increase up to 10-fold upon activation (Berger et al., 1991).
Dormant neutrophils do not express FcγRI under normal conditions, but expression of this protein can be induced by treatment with interferon-γ; in contrast, FcγRII expression is not affected by cytokines or glucocorticoids and FcγRIII expression is regulated permissively by the presence of FcγRII (Huizinga et al., 1990a). FcγRIII is a binding molecule that is thought, in fact, to present ligand to FcγRII (Huizinga et al., 1990a). In contrast to complement receptors, FcγRII and FcγRIII expression decreases by 50 to 80% in activated neutrophils (Leino and Lilius, 1992).

Several novel neutrophil activation markers have been described recently. CD 69, a lymphocyte activation marker, has been reported to be expressed on the surface of the plasma membrane of activated (but not dormant) neutrophils (Gavioli et al., 1992). Furthermore, CD 66 and CD 67, which are stored in the specific granules, and CD 63, a marker of azurophil degranulation, have also been reported to be neutrophil activation markers; the functions of these proteins are not known (Ducker and Skubitz, 1992; Niessen and Verhoeven, 1992). No marker that is exclusive to primed neutrophils has been described yet although expression of CD10 and binding of the monoclonal antibody 7D5 have been reported to be early activation markers in circulating neutrophils (Kuijpers et al., 1991).

Opsonin receptors are coupled to various signalling pathways (see Section 2.6.2.2) and some receptor systems may interact. While some complement and Fc receptors are linked to processes that activate phospholipase-C (Cockcroft, 1992), CR-3-mediated phagocytosis of OZ is coupled to generation of phosphatidic acid (PA) and sn-1,2-diacylglycerol (DAG) via phospholipase-D activity (Fallman et al., 1992). Ligation of Fc-receptors activates the release of free Ca²⁺ from cytoplasmic stores which is followed by an influx of Ca²⁺ from the extracellular milieu (Lew, 1989). Occupancy of complement receptors, however, does not activate phagocytosis in the absence of a second signal provided by the engagement of Fc receptors (Frank and Fries, 1991). Sustained generation of ROS requires continued occupancy of the triggering receptor for the duration of the burst (Haines et al., 1991).
2.6.2 Signal transduction

Multiple signal transduction processes are involved in priming and activation of neutrophil microbicidal activity. Stimuli such as PMA (a structural analogue of DAG), OZ and fMLP activate the oxidase through distinct signal transduction pathways (Takahashi et al., 1991). Primed neutrophil oxygenation activity may be the result of an alteration to one or more of the components of the transduction system (Figure 3) which may include fluxes of free cations (Na⁺, K⁺ and Ca²⁺), changes in membrane potential, activation of intracellular proteases, changes in AA and phospholipid metabolism, phosphorylation of specific proteins (i.e. oxidase components) and changes in the intracellular concentrations of cyclic nucleotides (Johnston and Kitagawa, 1985). At present, two distinct signalling pathways have been identified: one which is Ca²⁺-dependent and leads to the activation of the Ca²⁺/phospholipid-dependent protein kinase (PK-C) while the other is Ca²⁺-independent and does not involve phospholipase-C or PK-C; both pathways must be functional, however, for activation of the oxidative burst (Dewald et al., 1988). The presence of an adequate intracellular concentration of GTP appears to be essential for the activation of NADPH oxidase (Knaus et al., 1991).

Distinct pathways of activation of a common NADPH oxidase appear to be triggered by different classes of intracellular messengers, suggesting that the activation pathways converge at a common intermediate (McPhail et al., 1985). Interactions between PK-C, cAMP- and cGMP-dependent protein kinases may combine to regulate NADPH oxidase activity (McPhail et al., 1985; Pryzwansky et al., 1990). Some of the proximal steps of the Ca²⁺/PK-C pathways may be bypassed in vitro by using Ca²⁺-ionophores and/or PK-C activators (Clark, 1990).

The use of cell-free systems (where cytosolic as well as membrane and granule fractions can be separated and reconstituted in the presence and absence of various cofactors) and permeabilised cell systems [which enable small molecules (< 700 Daltons) to be transferred experimentally into the cytosol through small pores made in the plasma membrane] has enabled great progress to be made in understanding the mechanisms involved in the signalling mechanism, the requirement for specific cytosolic and membrane components in the assembly of the oxidase complex, and the roles of effector molecules involved in degranulation (Morel et al., 1991;
Binding of a ligand (L) [such as opsonized yeast cell walls] to a complimentary neutrophil receptor (R) triggers the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to generate inositol 1,4,5-tri-phosphate (IP\(_3\)) and diacylglycerol (DAG). This reaction is catalysed by phospholipase-C (PL-C) which is coupled to the receptor complex by a GTP-binding (G) protein. Binding of IP\(_3\) to cytosolic granules containing Ca\(^{2+}\) induces release of this cation, which, in combination with DAG, activates the protein kinase-C (PK-C) family of enzymes which catalyse the phosphorylation (P) of a number of cytosolic protein factors (CFs). Binding of these CFs to NADPH oxidase may activate the respiratory burst. MPO is released into a phagolysosome formed by the invaginating membrane as a result of fusion of the membrane with azurophilic granules (not shown). The molecular processes that trigger the release of myeloperoxidase (MPO) from the cytosolic azurophilic granules into the phagolysosome are not known. [See text for further explanation].
Cockcroft, 1992). However, the precise role(s) of second messengers and some individual oxidase components are still not known (Baggiolini and Wymann, 1990; Watson et al., 1991). Evidence for non-involvement of various enzymes such as PK-C has been inferred from experiments using inhibitors that are generally considered to be specific and totally inhibitory at the concentrations employed. The specificity of some PK-C and oxidase inhibitors has been questioned, however, because they also interact with components other than their recognised targets (Cross, 1990).

2.6.2.1 Triggering and Prolongation

The brief lag phase that precedes activation of the oxidative burst involves a sequence of complex intermediate steps which may include phosphorylation, translocation and, ultimately, assembly of the oxidase complex (Segal, 1989). For example, inositol triphosphate (IP$_3$) and DAG release are believed to activate the two independent but synergistic pathways that lead to activation of the oxidative burst; DAG activates PK-C directly while IP$_3$ triggers the release of free Ca$^{2+}$ from cytoplasmic granules (calciosomes) (Hurst, 1987). In unstimulated neutrophils, the cytosolic concentration of Ca$^{2+}$ is maintained at submicromolar levels by a Ca$^{2+}$-ATPase pump at the plasma membrane, but this increases to micromolar levels upon activation (Smolen, 1992). IP$_3$ and DAG are generated by the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP$_2$). The reaction is catalysed by phospholipase-C which is, in turn, activated by the binding of GTP to a linked guanine nucleotide regulatory protein (G-protein) (Koenderman et al., 1990). The coupling of receptors and enzymes to G-proteins can be confirmed positively through pertussis toxin-induced ribosylation (inhibition), but pertussis toxin-insensitive G-proteins have also been identified recently (Blackburn and Heck, 1989). These G-proteins include Rap1A which may be connected functionally to cytochrome b$_{558}$ (Quinn et al., 1992). Whilst the phospholipase-C catalysed generation of IP$_3$ and DAG was originally thought to be the only mechanism involved in activating the oxidative burst, Ca$^{2+}$-dependent phospholipase-A$_2$ and -D activities also appear to be involved downstream from phospholipase-C (Cockcroft, 1992).

PK-C is a family of monomeric polypeptide enzymes with single catalytic and regulatory domains that reversibly phosphorylate serine and threonine residues on target proteins (Azzi et al., 1992). PK-C is found in
both the cytosol and plasma membrane. Its distribution is in a state of dynamic equilibrium that appears to be controlled by the cytosolic concentration of free Ca\(^{2+}\) (Philips et al., 1989). PK-C isotypes that differ in location, substrate specificity and cofactor-dependence have also been identified (Majundar et al., 1991). The \(\alpha\) and \(\beta\) forms account for 35% and 60% of PK-C activity respectively in neutrophils (Pontremoli et al., 1990). PK-C activity is regulated by phosphatidylcholine, Ca\(^{2+}\) and DAG (Azzi et al., 1992). DAG may increase the affinity of the inactive form for Ca\(^{2+}\), thereby activating it at the low Ca\(^{2+}\) concentrations that prevail in the cytoplasm (Sandborg and Smolen, 1988). A Ca\(^{2+}\)-activated protease (calpain) cleaves PK-C which enables the catalytically-active domain to phosphorylate target proteins (Bjerrum, 1993). The magnitude of the PK-C-dependent oxidative burst is proportional to intracellular PK-C content in activated neutrophils (Salamino et al., 1991). Activation of neutrophils with fMLP or PMA also stimulates tyrosine-specific protein kinase activity which may trigger superoxide production independently of the classical PK-C; several cytosolic and membrane proteins are phosphorylated by tyrosine kinases (Berkow and Dodson, 1990). Phosphatases may reverse the enzyme activities which are activated by protein kinase-dependent phosphorylation (Ding and Badwey, 1992) but their involvement has not yet been proven (Steinbeck et al., 1991).

Priming and activation require not only triggering (initial generation of the signal) but maintenance of the signal to sustain the response (Reibman et al., 1988). Activation by PMA correlates with a biphasic rise in DAG which originates from at least two distinct pools; the first results in a rapid peak (< 15 sec) after receptor ligation and this is followed by a slow second peak that continues to rise after activation has been triggered (Haines et al., 1991). The generation of DAG via phospholipase-D may amplify the initial rise triggered by phospholipase-C (Perry et al., 1992). DAG may be directly involved in priming because its basal concentration appears to limit the amount of active oxidase that can be assembled in activated cells (Park and Babior, 1992). Priming mediated by GM-CSF has been reported to increase the availability of phosphatidylinositol phosphates which are otherwise rate-limiting for subsequent activation pathways (Macphee, 1992). With OZ and fMLP as stimuli, however, there is no correlation between DAG accumulation and oxidase activity (Koenderman et al., 1989a). This suggests that, with these stimuli, the activation of PK-C is not the sole
mechanism that triggers the oxidative burst, a step that becomes essential after one to two minutes to maintain oxidase activity (Koenderman et al., 1989a). While sustained activity of the oxidative burst may require continuous replacement of the oxidase by newly-activated enzyme, the mechanisms by which individual oxidase molecules are deactivated are not known. In principle, deactivation could involve dissociation of the receptor-ligand complex, accumulation of cAMP (which inactivates phospholipase-C through the action of a cAMP-dependent protein kinase), or dephosphorylation of the oxidase by protein phosphatases which would reverse signal transduction, or alternatively, the oxidase may be inactivated or damaged by its own end-products (Akard et al., 1988; Morel et al., 1991).

Priming by some stimuli (e.g. fMLP) causes a rapid rise in cytosolic Ca\(^{2+}\) which is followed by a smaller sustained increase and return to initial levels. Ca\(^{2+}\) may not activate the oxidase directly but may cause modification or release of a protein that must be translocated to the membrane before activation can occur (Davies et al., 1991) (2.6.2.3). Translocation of PK-C itself, however, appears to be independent of changes in cytoplasmic Ca\(^{2+}\) (O'Flaherty et al., 1990). As the lag phase can be virtually abolished in the presence of Ca\(^{2+}\)-ionophores (e.g. ionomycin), Ca\(^{2+}\)-induced priming may activate a calmodulin-dependent protein kinase. In this case, the resultant protein phosphorylations might generate a latent endogenous activator of NADPH oxidase that is stimulated upon receptor occupancy (Finkle et al., 1987).

2.6.2.2 Stimulus-specificity

Activation of the oxidative burst by opsonized zymosan (OZ) is mediated by bioactive lipids released through the stimulation of phospholipase-A\(_2\), -C & -D activities (Della Bianca et al., 1990). OZ has also been reported to activate a common NADPH oxidase through a Ca\(^{2+}\)-dependent phospholipase-A\(_2\)-mediated pathway that is independent of PK-C (Maridonneau-Parini et al., 1986), although activation of PK-C does participate in the overall phagocytic process (Twomey et al., 1990) and may be required to extend or sustain the duration of the oxidative burst (Watson et al., 1991; Koenderman et al., 1989a). The oxidative burst and microbial ingestion may be independent to some extent because phagocytosis of OZ occurs without changes in the cytosolic concentration of free Ca\(^{2+}\) and inositol triphosphate production (Fallman et al., 1989; Cooke et al., 1987). Platelet activating factor (PAF) may
also be important in OZ signal transduction because it is generated rapidly in OZ-stimulated neutrophils (Tool et al., 1989).

FMLP triggers phosphatidylcholine hydrolysis by phospholipase-D, suggesting that PA and not DAG is the major transducing agent (Rossi et al., 1990; Kessels et al., 1991; Agwu et al., 1991). PA can, however, be degraded to produce DAG by a PA phosphohydrolase (Morel et al., 1991). The mechanism of activation involving PA is not known, but it requires the presence of both membrane and cytosolic components that include PK-C (Olson et al., 1991). These processes may be co-operative, because fMLP triggers degranulation through DAG produced via phospholipase-C while the oxidative burst is activated by PA synthesized de novo (Haines et al., 1991). A recent review (Cockcroft, 1992) suggests that fMLP can stimulate phospholipase-A₂, -C & -D activities (which are all controlled by G-proteins). Activation of phospholipase-C is followed downstream by increases in the activities of phospholipases D and A₂ which are triggered by Ca²⁺ release (Cockcroft, 1992). Under normal conditions, phospholipase-D is poorly coupled to fMLP receptors but priming increases coupling and this leads to increased generation of PA and DAG in fMLP-stimulated neutrophils (Garland, 1992).

AA has been shown to activate NADPH oxidase directly, possibly through its action on a protein kinase distinct from PK-C. In a cell-free system, for example, AA activates the oxidase by increasing the binding of GTP to G-proteins (McPhail et al., 1985; S.B. Abramson et al., 1991). However, other workers have reported that AA is a potent direct activator of PK-C. AA and DAG interact synergistically, for example, to stimulate PK-C (Lester et al., 1991). In addition, AA metabolites such as leucotrienes and thromboxanes have been implicated in the signalling processes that lead to NADPH oxidase priming and activation (Zheng et al., 1991). New evidence suggests that AA may be the agent that activates NADPH oxidase, possibly, through promoting the translocation of p47phox to the membrane (Henderson et al., 1993). This group suggests that PK-C may phosphorylate phospholipase-A₂ which, in turn, generates AA release.

PAF, another phospholipase A₂ metabolite, also primes the oxidase (Maridonneau-Parini et al., 1986; Dewald and Baggio, 1985). PAF-induced priming can occur through either Ca²⁺-dependent or Ca²⁺-independent routes (Koendermann et al., 1989b). Furthermore, PAF also has the ability to stimulate, autocrinally, the cell it is released from (Dewald and Baggio,
PAF release requires the co-ordinated actions of G-proteins, Ca\text{2+} and some presently unknown factors (Gomez-Cambronero et al., 1989). PAF is also involved in the activation process because PMA is twice as potent as OZ in initiating PAF synthesis in neutrophils, and enhanced PK-C activity is involved in the response to both stimuli (Leyravaud et al., 1989). GM-CSF also primes PAF and leucotriene synthesis in response to secondary stimuli such as fMLP (Wirthmueller et al., 1990). This cascade of second messengers (derived from activated phospholipase A\text{2}) is also involved in other neutrophil functions such as degranulation, chemotaxis and actin assembly (Morel et al., 1991). Clearly, much more work is required to piece together the signalling jigsaw and discriminate between these processes.

2.6.2.3 Protein and enzyme changes

An obvious explanation for priming is the enhancement of the activity of the ROS-producing enzyme network itself. The catalytic efficiency of either NADPH oxidase and/or MPO may increase and priming can induce selective exocytosis of MPO with increased catalytic activity (Pember and Kinkade, 1983). LPS has been shown to prime human neutrophils by increasing the V\text{max} of NADPH oxidase without altering its K\text{m} (Guthrie et al., 1984). The authors showed that this was not accompanied by increased protein synthesis, which suggested that the cellular content of the enzyme itself was not increased; the question of phosphorylation by protein kinases was not addressed.

Upon neutrophil activation, NADPH oxidase complex is assembled from various phagocytic oxidase (phox) protein components derived from distinct cytosolic locations or granules and integral membrane-spanning proteins (Babior, 1992). A flavoprotein complexed to the large β- (gp91phox) and small α- (p22phox) subunits of cytochrome\text{b558} makes up the integral membrane-spanning proteins that combine with the cytosolic proteins to form the active oxidase complex (Babior, 1992). The cytosolic proteins include the NADPH-binding subunit (p32phox) and two other proteins (p47phox & p67phox) which have been shown to be essential components of the oxidase complex (Clark, 1990). CGD results, in fact, from defects in or the absence of some of these proteins (Babior, 1991), with absence of p47phox determining the autosomal recessive condition while the X-linked disorder is due to the presence of an abnormal β-subunit of cytochrome b\text{558} (Segal, 1989). These proteins, which are located in the cytosol of normal (inactive)
neutrophils, translocate, upon activation, to the plasma membrane where they function as either catalytic or regulatory elements (Clark, 1990). P47\textit{phox} is a substrate of PK-C (Koenderman \textit{et al}., 1989a).

Phosphorylation is involved in oxidase activation. Phosphorylation of p47\textit{phox} and p67\textit{phox} by PK-C may initiate their linkage with cytochrome b$_{558}$ in the plasma membrane (Nauseef \textit{et al}., 1991). P67\textit{phox} undergoes a continuous cycle of phosphorylation/dephosphorylation for the duration of the oxidative burst in PMA-stimulated neutrophils (Ding and Badwey, 1992). Protein phosphorylation alone, however, does not lead to activation of the oxidase (Steinbeck \textit{et al}., 1991). Rapid production of DAG triggers phosphorylation of p47\textit{phox} but this does not initiate the oxidative burst without the involvement of an unknown additional step (Haines \textit{et al}., 1991). In the case of fMLP, the unknown step may involve carboxyl methylation of one or more Ras-related G-proteins (Philips \textit{et al}., 1993). Translocation of P47\textit{phox} and the NADPH-binding component to the membrane occur concomitantly (Umei \textit{et al}., 1991) but p47\textit{phox} does not appear to be required for sustained O$_2^-$ production once the oxidase is activated (Woodman \textit{et al}., 1991). Nearly all of the p67\textit{phox} is closely associated with elements of the cytoskeleton, suggesting that it is an active part of the oxidase complex (Woodman \textit{et al}., 1991). The actin microfilament network plays an important regulatory role in NADPH oxidase assembly and activity (Woodman \textit{et al}., 1991). Recent work has shown that p47\textit{phox} and p67\textit{phox} form a complex with cytochrome b$_{558}$ which interacts with a third cytosolic GTP-binding protein, p21\textit{rac1} (Gallin \textit{et al}., 1991). The functional role of this G-protein is not known (Gallin \textit{et al}., 1991) but it may initiate activity and control the duration of electron transport to the oxidase (Segal and Abo, 1993). Deactivation of the oxidative burst results in dissociation of the oxidase and cytochrome components (Morel \textit{et al}., 1991) and, presumably, dephosphorylation of the phosphorylated components (Segal and Abo, 1993).

Cycloheximide inhibition of priming suggests that priming and activation induced by GM-CSF and growth hormone, for example, depend, in part, on the expression and synthesis of proteins \textit{de novo} (Wirthmueller \textit{et al}., 1990; Fu \textit{et al}., 1991). For instance, the ingestion of opsonized yeast by neutrophils induces the production of mRNA for TNF-\alpha and TNF-\alpha protein secretion may lead to the sequential priming of other neutrophils at infection sites before macrophages appear (F. Bazzoni \textit{et al}., 1991).
Exocytosis of the contents of various neutrophil granules is associated with phagocytic activation of the oxidative burst. Neutrophils are now considered to contain three major types of granules (see Section 2.2) and secretory vesicles. Exocytosis of each granule type is controlled by their differential sensitivity to free Ca\(^{2+}\), which is stored in IP\(_3\)-sensitive vesicles known as calciosomes (Sengeløv et al., 1993). Activation of the oxidative burst and exocytosis of granules are independent to some extent because ligation of FcγRIII activates exocytosis from both the specific and azurophilic granules without stimulating the oxidative burst (Huizinga et al., 1990b). Specific and azurophilic degranulation appear to be controlled by different mechanisms that share, however, Ca\(^{2+}\) dependency and a requirement for the presence of Mg\(^{2+}/ATP\) and control via G-proteins (Cockcroft, 1992). The main difference is that the cytosolic concentration of free Ca\(^{2+}\) required to reach the activation threshold for azurophilic degranulation is about five-fold higher than that required for exocytosis of the contents of specific granules (Lew, 1989). The secretory vesicles and tertiary granules are the most sensitive to Ca\(^{2+}\), perhaps, because they contain components involved in priming and early activation (Sengeløv et al., 1993). ATP may increase the affinity for Ca\(^{2+}\) of proteins involved in granule exocytosis and extend the duration of this process (Boonen et al., 1992). GTP lowers the Ca\(^{2+}\) requirement for azurophil degranulation but has no effect on specific granule exocytosis (Niessen and Verhoeven, 1992). In contrast, in the presence of very low cytosolic Ca\(^{2+}\), GTP and Mg\(^{2+}\) can promote assembly of a microfilament matrix which prevents specific degranulation by restraining granules from fusing with the plasma membrane (Smolen et al., 1991). A threshold, but still low, concentration of cytosolic Ca\(^{2+}\) may partially destabilize this matrix - thereby permitting specific degranulation - while a high Ca\(^{2+}\) cytosolic concentration may allow azurophilic degranulation to occur by activating full dissociation of the matrix (Smolen et al., 1991).

The phagolysosome is formed upon fusion of these granules with the invaginating plasma membrane (i.e. phagocytic vesicle) (see Section 2.2). This delivers the microbicidal contents of the granules to the vicinity of ingested micro-organisms without releasing them into the extracellular milieu (Meers et al., 1987). While the factors that mediate fusion between granules and the plasma membrane during phagolysosome formation are
not known, proteins known collectively as annexins appear to be involved in a Ca\(^{2+}\)-dependent process (Francis et al., 1992). DAG lowers the Ca\(^{2+}\) requirement of the fusion process (Francis et al., 1992). Annexins aggregate neutrophil granules and promote fusion by binding to membrane phospholipids (Ernst, 1991). Thus, granule/membrane fusion involves a multifaceted signalling system which includes G-proteins, Ca\(^{2+}\), DAG and PK-C (Jaconi et al., 1990).

### 2.6.2.4 Physiological examples of neutrophil priming

Various infectious and inflammatory conditions trigger the priming response. Neutrophils isolated from patients with acute bacterial infections show primed oxidative responsiveness (Bass et al., 1986; Anton et al., 1988), enhanced Fc-mediated phagocytosis (Simms et al., 1989) and OZ-stimulated-CL (Barbour et al., 1980). Primed neutrophils have been found in people with essential hypertension (Pontremoli et al., 1989), Hodgkin’s disease (Tullgren et al., 1991), inflammatory bowel disease (Suematsu et al., 1987), psoriasis (Bloomfield and Young, 1988), rheumatic fever (Naik et al., 1987), sarcoidosis (Barth et al., 1988), and septicaemia, where priming correlates with high concentrations of circulating TNF-α (Trautinger et al., 1991). In all cases, the mechanisms involved are not known.

This brief overview of priming and activation suggests that additional pathways and oxidase components remain to be identified. Future work needs to address the interactions between these pathways and to distinguish between pathways involved in priming and activating the oxidase and those involved in other components of the phagocytic process including degranulation, chemotaxis and integrin expression. The compartmentation of the burst suggests that oxidase heterogeneity may exist and that these separate activities may be regulated differentially. More specific inhibitors of signalling and oxidase components are required to resolve the present uncertainties.
2.7 Microbicidal assays

This brief overview of techniques used to study neutrophil microbicidal activity will focus mainly on assays used to monitor the oxidative burst but it will also refer to assays of non-oxidative mechanisms and to direct microbial killing assays. The reader is referred to the manual by Metcalf and colleagues (1986) and the review by Weber (1990) for a general overview of current techniques.

Superoxide production, measured by superoxide dismutase-inhibited ferricytochrome-c reduction, is the classical method for assessing the neutrophil oxidative burst. This technique relies on the ability of superoxide released extracellularly to reduce ferricytochrome-c to the ferrous form which can be examined spectrophotometrically using either continuous or discontinuous assays. In contrast, the production of intracellular \( O_2^- \) can be measured qualitatively by identifying microscopically the percentage of neutrophils that convert the colourless water-soluble acceptor dye, nitroblue tetrazolium, to the insoluble blue compound formazan. Recently, the fluorochrome hydroethidine has been claimed to be an \( O_2^- \)-sensitive agent which is suitable for use in flow cytometric assays (see below) (Rothe and Valet, 1990). The results obtained from extracellular and intracellular methods do not correlate directly in many cases (Weber, 1990).

The production of \( H_2O_2 \) can be measured extracellularly by a variety of spectrophotometric, fluorometric and polarographic techniques which are sensitive to catalase inhibition and boosted by the inhibition of \( H_2O_2^- \)-consuming enzymes by azide (Weber, 1990). \( H_2O_2 \) can be detected intracellularly (at the single cell level by flow cytometry) by loading the cells with \( H_2O_2^- \)-sensitive dyes that are trapped within the cytoplasm and become fluorescent upon oxidation by \( H_2O_2 \) (Bass et al., 1983).

The intracellular production of MPO-dependent oxyhalides and \( H_2O_2 \) can be detected in vitro - without destroying the function of the cells - by chemiluminescence (CL) using chemiluminogenic probes to amplify the signal (Allen, 1986). Amplified-CL is 100-1000 times more sensitive than conventional spectrophotometric and fluorometric assays (Campbell et al., 1985) and can be used accurately to detect the signal produced by a small number of cells. Luminol-amplified CL monitors both oxidant generation and degranulation because of its dependence on MPO activity while lucigenin-amplified CL has been claimed to measure extracellular \( O_2^- \) independently of degranulation (Allen, 1986; Edwards, 1987). Furthermore,
luminol diffuses freely into the cells and can be used to measure the intra-
and extracellular generation of luminol-reactive ROS (Robinson et al., 1992).
There is a close positive correlation between amplified CL and other
methods used to assess neutrophil microbicidal activity including bacterial
killing, phagocytosis and enhanced oxygen consumption (Hosker et al.,
1989). OZ-stimulation of neutrophils produces luminol-amplified CL with
kinetics similar to that generated in response to opsonized gram-positive
bacteria including S. aureus and some gram-negative micro-organisms
(e.g. Enterobacter, Salmonella, Proteus and Shigella) (Robinson et al., 1984).

The direct separate contributions of H$_2$O$_2$ or HOCl to luminol-
amplified CL cannot be determined when luminol is used as a CL probe
because light is produced as a result of the “co-oxidation” of luminol by both
H$_2$O$_2$ and HOCl; the light output in this case is 100 times greater than that
generated by either oxidant alone (Brestel, 1985). RNS such as peroxynitrite
(Radi et al., 1993) may also be involved in the excitation of luminol during
the phagocytic oxidative burst because arginine (the substrate of nitric oxide
synthase) enhances LCL while some arginine analogues (e.g. N-methyl-
arginine) are strongly inhibitory (Wang et al., 1991). Luminol may react with
a variety of radicals and thus, is only useful as a “non-specific” indicator of
ROS generation (Vilim and Wilhelm, 1989). H$_2$O$_2$ can be detected by this
system extracellularly, however, when horse-radish peroxidase is present
and when MPO and catalase activities are blocked with azide (Wymann
et al., 1987). Horse-radish peroxidase can also be used to discriminate
between the extracellular and intracellular production of oxidants in CL or
fluorimetric assays (Lock et al., 1988). A large amount of the O$_2^-$ produced
may be converted to H$_2$O$_2$, however, before a sufficiently high steady-state
concentration is reached that can be detected extracellularly by
ferricytochrome-c reduction or lucigenin-amplified CL. Numerous assays
are available to detect and quantify MPO activity by halide-dependent
activities (Metcalf et al., 1986) but no flow cytometric method has been
reported yet.

Many of the microbicidal functions of neutrophils can be studied at the
single cell level by flow cytometry (see Bjerknes et al., 1989 for review).
These functions include: the expression of cell surface receptors; membrane
potential; attachment and phagocytosis of micro-organisms; phagosomal
pH; generation of superoxide and H$_2$O$_2$; degranulation and enzyme
activities; intracellular Ca$^{2+}$; and intracellular killing and degradation of ingested micro-organisms (Bjerknes et al., 1989).

Neutrophil microbicidal activity is usually studied with neutrophil suspensions (Rebut-Bonneton et al., 1988) but some workers have suggested that physiologically-meaningful results can be obtained only when adherent neutrophils are used (Nathan, 1987; 1989). The results obtained with cell suspensions cannot be extended directly to adherent cells (Rebut-Bonneton et al., 1988; Nathan and Sanchez, 1990). Neutrophils are thought to function in vivo by adhering to host cells or matrix proteins at sites of infection. However, adherence is not an obligatory step in the activation of the oxidative burst (Bellavite et al., 1992). The oxidative burst produced by adherent cells in vitro shows a substantial lag phase but its peak intensity and duration with some stimuli are increased 10 to 100 times compared to results obtained with cell suspensions (Nathan and Sanchez, 1990). In contrast, decreased responses to fMLP and PMA have been found with adherent cells (Rebut-Bonneton et al., 1988). Physiological adherence is difficult to reproduce in vitro because non-biological surfaces can themselves activate the burst while some biological surfaces can suppress it (Nathan, 1987). Further work is required to resolve this dilemma.

Because, to some extent, the specificity and sensitivity of all oxidative assays currently in use is questionable, possibly because of competition between indicator reagents and endogenous reactants (Weber, 1990), I have used several independent techniques to assess the oxidative burst in the work reported in this thesis. Workers in this field will have to determine, in future, a normal reference range for neutrophil responses assessed by these various methods to determine whether neutrophils isolated from individuals -where the values fall below or above this range - are vulnerable to infection, on the one hand, or to inflammatory disease on the other.
3. **IMMUNOREGULATION**

The activities of immune cells such as neutrophils are regulated by a complex balance of stimulatory and inhibitory pathways which are controlled by humoral mediators or direct cell-to-cell contact (see Tables 2 & 3). The immune system is not autonomous; cellular and humoral immune activities are influenced by soluble mediators secreted from the endocrine, nervous and cardiovascular systems as well as by those produced by other immune cells. These mediators include hormones, neurotransmitters and cytokines, many of which are secreted in response to stress. Immune cells express plasma membrane receptors for many classical neuroendocrine messengers [e.g. catecholamines, some pro-opiomelanocortin (POMC)-derived peptides [i.e. β-endorphin and adrenocorticotropic hormone (ACTH)], thyrotropin, gonadotropin, growth hormone and prolactin]; they have also been shown *in vitro* to synthesize and release small amounts of most of these factors which therefore have the potential to function in autocrine and paracrine amplification networks (Blalock, 1989; Johnson *et al.*, 1992). A large number of these mediators have been reported to be potent neutrophil-priming agents *in vitro* and this list is expanding rapidly (Table-2). These mediators include cytokines, neuroendocrine hormones and AA metabolites which are biologically active in their “free” forms only (Ekins, 1992). The activities of most hormones and cytokines are masked or inhibited by carrier and inhibitory proteins. Discussion will focus mainly on mediators whose plasma concentrations increase in response to exercise (see Section 4) and on their putative roles in regulating immune responses, particularly neutrophil function.

### 3.1 Cytokines

Cytokines are immunoregulatory proteins produced and secreted in different combinations and at different rates by most immune cells including macrophages, neutrophils and lymphocytes (Lord *et al.*, 1991). Cytokines have powerful and multiple overlapping (pleiotropic) regulatory actions on their target cells; they can, in appropriate concentrations, amplify or diminish all responses of the immune system (Ricci *et al.*, 1989). Cytokines were first reported as interleukocyte hormones (interleukins) but they are now known to be produced by, and to act on, virtually all nucleated cells (Blalock, 1989; Johnson *et al.*, 1992). They are normally present in
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<th>Table-3. Endogenous inhibitors of neutrophil microbicidal activity*</th>
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<td><strong>Cytokines</strong></td>
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* See text for further explanation and sources.
plasma in minute concentrations, except when greatly increased under pathological conditions, and may act locally at picomolar concentrations (Bocci, 1988; Arai et al., 1990). Cytokines have been shown to act both alone and/or synergistically to regulate the proliferation and activation of immune cells in vitro (Arai et al., 1990), but their exact functional roles in vivo and especially their interactions with neuroendocrine hormones are not known. Many cytokines have been shown to be potent neutrophil priming agents in vitro (Table-2) (Steinbeck and Roth, 1989) and many reports indicate that this increases their killing capacity against bacteria (Blanchard et al., 1989), fungi (Djeu et al., 1986), and protozoa (Ferranti et al., 1989) by enhancing ROS and lysosomal enzyme release. Elevated circulating TNF-α has been detected in patients with bacterial infections, cancer and thermal injury (Fong and Lowry, 1990).

The discussion here will focus mainly on the pyrogenic cytokines (IFN-α, IL-1, TNF-α, and IL-6) because their circulating activities have been reported to increase after exercise (Viti et al., 1985; Cannon et al., 1986; Dufaux and Order, 1989). This elevation can be triggered by mild endotoxaemia caused by local hypoxia in the splanchnic circulation and/or heat-stress, both of which lead to the release of endotoxin (derived from gut bacteria) into the portal circulation (Bosenberg et al., 1988). Endotoxaemia induced experimentally in human subjects triggers the release of IL-1, TNF and IL-6 into the circulation (Michie et al., 1988; Fong et al., 1989) and all of these cytokines have been reported to prime neutrophil microbicidal activity (Shalaby et al. 1987; Klebanoff et al., 1986; Ozaki et al., 1987; Sullivan et al., 1989; Dularay et al., 1990; Borish et al., 1989). Endotoxin may prime neutrophils directly (Worthen et al., 1988), but this does not occur with cells in suspension, possibly because additional serum factors are required (Aida and Pabst, 1991). IFN-α and IL-4, on the other hand, diminish O$_2^-$ production in phagocytes (Einhorn and Jarstrand, 1984; Abramson and Gallin, 1990). LPS activity can be neutralized by anti-LPS antibodies (Bosenberg et al., 1988) and by enzymes of phagocytic origin (e.g. acyloxyacyl hydrolase and bactericidal/permeability increasing protein) following their release into the circulation (Erwin and Munford, 1991; Marra et al., 1992). The stimulatory effect of LPS is enhanced when it is complexed to the LPS-binding protein found in the plasma of healthy humans in trace concentrations (Schumann et al., 1990). This complex binds to CD 14 which is expressed on the external surface of the plasma membrane of phagocytes.
plasma concentration of LPS-binding protein can increase 10-fold during an acute-phase response (Raetz et al., 1991; see Section 3.3). Thus, cytokine release may be initiated once anti-LPS defences are overcome, or through LPS-independent pathways (Webb et al., 1990).

Although TNF and IL-1 share a number of identical biological activities (i.e. they are pleiotropic), distinct membrane receptors exist for each of the individual cytokines (Dinarello, 1989). In addition, IL-1 and TNF both exist in two separate molecular isoforms (i.e. α & β). Studies with each cytokine in recombinant form have shown that, although the α and β isoforms share limited amino-acid sequence homology, they each possess similar biological properties and compete for the occupation of a common receptor (Dinarello, 1989). High affinity receptors for IL-1 (Rhyne et al., 1988) and TNF (Shalaby et al., 1987) have been characterized on human neutrophils. Both IL-1 and TNF induce processes central to the development of all inflammatory reactions. They can stimulate and/or augment phagocytosis and the oxidative-burst in neutrophils in vitro. TNF-α also primes HOCl production in these cells both in vitro (She et al., 1989) and in vivo (Wewers et al., 1990). MPO and O₂⁻ release are also primed by IL-1β (Dularay et al., 1990; Ozaki et al., 1987).

While most neutrophil priming studies in vitro have involved cell suspensions, some cytokines can stimulate the burst directly in adherent cells but not in cell suspensions (Nathan, 1989). Neutrophils must adhere to matrix proteins via CD 11/CD 18 integrins for cytokines to activate the oxidative burst directly; this may prevent premature or “accidental” activation of circulating neutrophils by cytokines (Nathan, 1989) but without preventing priming. Adherence may also lower the steady-state concentration of cytosolic cAMP which could prepare neutrophils for the onset of the oxidative burst by driving the concentration of this potent inhibitor below its effective threshold (Nathan and Sanchez, 1990).

The mechanisms by which TNF-α primes neutrophils are not known but they could involve the generation of PA through activation of phospholipase-D (Schleiffenbaum and Fehr, 1990). Priming by TNF-α does not appear to act through phospholipase-A₂ which suggests that AA itself, AA metabolites or pertussis toxin-sensitive G-proteins (Schleiffenbaum and Fehr, 1990) are not involved initially (Bauldry et al., 1991). Cytokine-induced priming may not involve the pre-synthesis of any of these intracellular messengers but it may enhance their production rapidly once the cells are
activated (Worthen et al., 1988). PAF, for example, appears to mimic many of the roles ascribed to TNF-α and IL-1 and, thus, it may be a direct second messenger for these cytokines. PAF induces the synthesis and release of IL-1 in a positive feedback manner, which may be an important factor in the amplification of the immune response (Braquet et al., 1987). Priming by endotoxin, GM-CSF and TNF-α does, for example, boost the production of AA metabolites (Atkinson et al., 1990) by neutrophils that have been prestimulated with fMLP (Worthen et al., 1988; DiPersio et al., 1988; Bauldry et al., 1991). The AA metabolites detected include PAF and LTB₄, both of which may act as secondary messengers (Camussi et al., 1989).

TNF can amplify its own physiological actions by inducing its release autocrinally as well as stimulating, paracrinally, the de novo synthesis and release of IL-1 (Perlmutter et al., 1986). Low concentrations of TNF may sensitize neutrophils to the synergistic effects of IL-1 and other mediators (e.g. interferons, complement factors) (Billingham, et al., 1987). The synergistic effects of IL-1 and TNF may augment neutrophil microbicidal activity substantially above the level of activity produced by either cytokine acting alone (Shalaby et al., 1987). This has physiological implications because priming of neutrophils by non-toxic doses of TNF-α and IL-1 appears to be responsible for the increased resistance of mice to bacterial infection (Cross et al., 1989) and for the enhanced Candidicidal activity of human neutrophils that accompanies the priming of the oxidative burst (Jupin et al., 1989). These positive effects may be “down regulated”, however, by endogenous inhibitors of cytokines or the presence of carrier proteins (including soluble receptors shed from neutrophils) and the cytokine autoantibodies which have been isolated in plasma (Jeffes et al., 1989; Porteu and Nathan, 1990). Individual macrophages and monocytes may be either immunostimulatory (i.e. secrete cytokines) or immuno-suppressive (i.e. secrete cytokine inhibitors), so that their net effect on the immune response reflects a homeostatic balance between the activation states of the two cell populations. Recent evidence suggests that neutrophil oxidative activity may be enhanced by direct cell-to-cell contact with activated T-lymphocytes which may or may not involve the direct presentation of cytokines to the neutrophil (Zhang et al., 1992). This provides further evidence that neutrophil-priming mediated by cytokines may occur in a paracrine manner.
3.2 Neuroendocrine factors

The rapidly accumulating evidence that some pituitary hormones are potent activators of immune mechanisms has been reviewed recently (Kelley, 1989; Gala, 1991). The importance of the pituitary gland in immunity is shown by the detrimental effect of hypophysectomy on the immunological status of experimental animals and, conversely, the ability of hormone replacement therapy to restore immunocompetence (Gala, 1991; Edwards et al., 1991a).

The major "stress hormones" involved in immunoregulation are growth hormone (GH) and prolactin (PRL), which are immunostimulatory, and adrenocorticotropic hormone (ACTH), adrenalin, and cortisol, which are generally immunosuppressive. The plasma concentrations of these hormones fluctuate throughout the day because of pulsatile secretion and rapid metabolic clearance (Baumann et al., 1987; Winer et al., 1990) both of which are partially regulated by negative feedback (Ader et al., 1990). The bidirectional interactions of cytokines and neurotransmitters with neurons and immune cells respectively provide a means of indirect chemical communication between the neuroendocrine and immune systems (lmura et al., 1991; see Section 3.4).

GH is essential for the development of the immune system and the maintenance of immunocompetence (Berczi, 1986; Gala, 1991). GH-deficiency, which reduces the potency of virtually all immune mechanisms, leads to increased vulnerability to infection. Specifically, GH is essential for thymus development, but it also enhances lymphocyte proliferation (Berczi et al., 1991), T-cell and NK-cell cytotoxicity, TNF-α synthesis by rat macrophages (Edwards et al., 1991b) and is as potent as γ-interferon in priming the microbicidal and tumouricidal activities of macrophages (Kelley, 1989). Furthermore, three independent groups have shown that GH (in its physiological concentration range) also primes the oxidative burst of human neutrophils (Fu et al., 1991; Wiedermann et al., 1991; Spandoni et al., 1991). This is initiated by GH binding to the PRI (and not the GH) receptor on neutrophils in a zinc-dependent process (Fu et al., 1992). Zinc is also required for maximum microbial uptake and killing by murine macrophages (Wirth et al., 1989). The mechanisms underlying GH-induced priming have not been identified, but protein synthesis appears to be involved (Fu et al., 1991). GH has been co-administered with an inactivated flavivirus vaccine to act as an adjuvant to
stimulate the clonal expansion of antigen-specific T-cells (Stephenson et al., 1991). Administration of GH to rats enhances the ability of isolated macrophages to kill Salmonella typhimurium bacteria in vitro and increases the animals' chances of surviving this infection (Edwards et al., 1991a). The secretion of GH is regulated by negative feedback: when high plasma concentrations of GH are achieved, the release of somatostatin, which opposes the activity of GH-releasing hormone, is triggered within the hypothalamus (Lanzi and Tannenbaum, 1992).

The growth-promoting effects of GH are mediated through insulin-like growth factor-1 (IGF-1). This hormone, which is synthesized mainly in the liver, also enhances some immune activities including lymphocyte proliferation (Roldan et al., 1989) and thymic growth (Binz et al., 1990). IGF-1 is also a strong neutrophil-priming agent (Fu et al., 1991) which may act synergistically with GH to amplify the microbicidal potential of these cells.

PRL - which shares considerable functional and structural similarities with GH - is also a strong immunopotentiating agent (Gala, 1991). Treatment of dwarf and hypophysectomized experimental animals with either GH or PRL can restore optimum immune function (Gala, 1991). This hormone also augments tumoricidal activity and interferon synthesis in macrophages and appears to be essential for maximum T-cell responsiveness to antigenic stimulation in vitro (Hiestand et al., 1986; Bernton et al., 1988). PRL also stimulates thymulin production by thymocytes in vitro (Dardene et al., 1989). The immunosuppressive action of cyclosporin-A, which is prescribed regularly for transplant patients, may be due in part to its ability to displace PRL from its lymphocyte receptor (Hiestand et al., 1986). PRL primes the oxidative burst of macrophages and neutrophils to the same intensity as that induced by GH (Fu et al., 1992). GH and PRL are not necessarily released from the pituitary gland in a coordinated manner; endotoxin and interleukin-1 both stimulate GH release, for example, but inhibit that of PRL (Berczi, 1986).

The neuropeptide substance-P also potentiates many cellular and humoral immune responses (McGillis et al., 1987). Substance-P potentiates a variety of immune cell responses including neutrophil phagocytic and microbicidal activities (Perianin et al., 1989), B-cell differentiation and T-cell proliferation; it can also stimulate IL-1 release from monocytes (Lotz et al., 1988; Laurenzi et al., 1990). Substance-P blocks the secretion of ACTH (Chowdrey et al., 1990) and it can inhibit many functional disorders caused
by chronic stress (Oehme et al., 1985). At nM concentrations substance-P primes NADPH oxidase activity through a mechanism involving tyrosine phosphorylation that is independent of Ca\textsuperscript{2+}; activation, in contrast, which is caused by μM concentrations of substance-P, is associated with elevated cytosolic concentrations of Ca\textsuperscript{2+} and actin polymerization (Lloyd's and Hallet, 1993).

Atrial natriuretic peptide (ANP) has also been reported to be a potent neutrophil-priming agent (Wiedermann et al., 1992). ANP may play a role in neutrophil activation in heart tissue during the ischaemia/reperfusion injury phenomenon (Wiedermann et al., 1992). The immunological role of ANP has not been addressed to my knowledge prior to this study. Consistent with its newly-defined immunopotentiating role, ANP has been reported to be an potent inhibitor of ACTH and cortisol secretion in rats (Fink et al., 1991; 1992) and humans (Kellner et al., 1992).

While pro-opiomelanocortin- (POMC) derived peptides, glucocorticoids and prostaglandins of the E series may enhance some immune responses at very low concentrations, they are generally considered to be immunosuppressive (Ader et al., 1990; Farmer et al., 1991). At low concentrations, glucocorticoids and PGE\textsubscript{2} may amplify the stimulatory effects of priming agents while, in contrast, high concentrations may prevent "overshoot" of the immune response which could otherwise lead to autoimmune tissue damage (Munck and Guyre, 1986). These contrasting responses may be controlled by the presence of multiple receptors for the same mediator that are coupled to stimulatory and inhibitory pathways. Saturation of the stimulatory receptor may eventually trigger expression and activity of the inhibitory receptor-linked pathway. In fact, containment of the "stress response" may be the principal role of glucocorticoids (Munck and Guyre, 1986). For example, single doses of glucocorticoids to human subjects potentiate both basal and stimulated GH secretion, whereas long-term treatment is inhibitory (Burguera et al., 1990). Although adrenalin and, possibly, opioids may initiate transient perturbations in immune responses following stress, glucocorticoids appear to be the major mediators of sustained immunosuppression.

Glucocorticoids inhibit virtually all functions of cells of the monocyte/macrophage lineage, including cytokine secretion (Kern et al., 1988), and severely impair the phagocytic and microbicidal activities of neutrophils (Fuenfer et al., 1975; Debets et al., 1989; Petroni et al., 1988;
Berczi, 1986). Cortisol inhibits macrophage differentiation and attenuates cytokine secretion from LPS-stimulated cells (Baybutt and Holsboer, 1990). The mechanisms by which glucocorticoids produce their inhibitory action are not known unequivocally, but direct/indirect inhibition of phospholipase activity (Webb and Roth, 1987), and mechanisms involving gene transcription, have been reported *inter alia* (Cronstein et al., 1992). The ability of glucocorticoids to suppress every major T-cell function, including helper and killer activities, is quite well documented, but their effects on B-cell responses are variable (Berczi, 1986). The cytotoxic activity of NK-cells is inhibited by glucocorticoids but enhanced by β-endorphin (Berczi, 1986; Kay et al., 1990). Glucocorticoids also inhibit GH secretion by activating somatostatin secretion within the hypothalamus (Wehrenberg et al., 1990).

The physiological relevance of many studies is questionable, however, because of the pharmacological concentrations of glucocorticoids used (Schleimar et al., 1989). The release of glucocorticoids into the circulation is inhibited by negative feedback mechanisms which switch off ACTH secretion in response to high concentrations of glucocorticoids in an unknown manner (Sapolsky et al., 1990).

Opioid addiction increases susceptibility to a variety of infections. This may be due to suppressed T-cell proliferation and neutrophil microbicidal activity (Morley et al., 1987). A small proportion of the major immune cell populations (discrete subpopulations) may escape these suppressive effects by "down regulating" the appropriate receptors and/or uncoupling specific signal transduction mechanisms. In the case of opioids, conflicting reports of immunostimulation or immunosuppression may be due to expression of multiple receptors for opioids on immune cells (Van den Bergh et al., 1991). β-Endorphin has positive effects on lymphocytes when it acts through non-opioid receptors, but it is strongly inhibitory when it binds to authentic opioid receptors (Van den Bergh, 1991). Oxidant production by neutrophils is also inhibited by β-endorphin activity mediated via non-opioid receptors (Diamant et al., 1989).

Catecholamines also suppress a variety of immune activities (Berczi, 1986; Felton et al., 1992). Adrenalin treatment of isolated cells *in vitro* inhibits mitogenesis of lymphocytes (Keast et al., 1988), the oxidative burst of macrophages and neutrophils (Berczi, 1986; G. Bazzoni et al., 1991) and the tumouricidal and antiviral activities of macrophages (Dantzer and Kelley, 1989). While α-adrenergic stimulation enhances T-cell proliferation and
augments the synthesis of TNF-α by macrophages (Berczi, 1986; Spengler et al., 1990), β-receptor occupation is inhibitory, possibly because it elevates intracellular cAMP (Weicker and Werle, 1991) (see Section 3.4). Noradrenalin has been reported to enhance cytotoxic T-cell activity but to inhibit the neutrophil oxidative burst and thymocyte proliferation (Felten et al., 1992). However, the effect of noradrenalin on neutrophil microbicidal activity has received little experimental attention. The importance of noradrenalin in immunoregulation is highlighted by the widespread presence of noradrenergic autonomic nerve fibres in lymphoid tissue (Ader et al., 1990).

The secretion of pituitary hormones such as GH, PRL and ACTH is controlled normally by the complex interaction of releasing and inhibiting factors within the hypothalamic and pituitary axis (Hochberg et al., 1991). These hormones all show considerable circadian variation in their blood concentrations, with the greatest release occurring during sleep (Winer et al., 1990). Furthermore, regular sleep has an important role in general health and recovery from disease (Krueger and Majde, 1990). The signal, for example, that causes a rise in the hypothalamic concentration of corticotropin release factor (CRF) above the threshold required to stimulate ACTH release from the anterior pituitary is not known with certainty but the rapidity with which it responds to episodes of intense stress suggests that local (hypothalamic/pituitary), rather than systemic, factors are responsible. In this context, IL-1, IL-6 and TNF (which can be produced within the hypothalamus) are potent activators of CRF and ACTH release in vitro (Lumpkin, 1987; Lyson and McCann, 1991). Cytokines may, thus, play a pivotal role as mediators between the immune and neuroendocrine systems (see Section 3.4).

Other hormones that influence immune responses include oestrogen, which induces MPO release from resting neutrophils and primes the oxidative burst, while, in contrast, 2-hydroxy-oestrogens are inhibitory (Jansson, 1991). Vasoactive intestinal peptide inhibits the oxidative burst by increasing cytosolic cAMP (Wiik et al., 1990).

3.3 Other mediators

Histamine, adenosine, β-adrenergic agents (Fantozzi et al., 1985), lipoxins A₄ & B₄ and prostaglandins of the E series (Conti et al., 1990) and platelet-derived adenine nucleotides (McGarity et al., 1988) are potent inhibitors of
neutrophil microbicidal activity. The majority of these mediators use cAMP as a second messenger (Renz et al., 1988; Eppell et al., 1989). Increased intracellular cAMP in neutrophils is associated with decreases in a number of microbicidal functions (C.D. Wright et al., 1990; Krause and Lew, 1988). Phagocyte priming and activation may, in fact, be controlled by shifts in the intracellular ratio of cGMP to cAMP (Renz et al., 1988) since cGMP is stimulatory (Coffey et al., 1988).

Adenosine, a vasodilator, is a potent anti-inflammatory agent released from damaged host cells (Cronstein et al., 1983). Inhibition of the oxidative burst may be initiated by adenosine occupancy of A2 receptors on immune cells, leading to inhibition of PK-C activation (Walker et al., 1990; Cronstein et al., 1990). Adenosine will suppress the oxidative burst only if it is added before the triggering agent (de la Harpe and Nathan, 1989), but it has no effect on the initiation or progress of degranulation (Cronstein et al., 1983). Circulating adenosine, at physiological concentrations, may prevent the premature activation of peripheral blood neutrophils by cytokines without preventing priming (de la Harpe and Nathan, 1989). Adenosine can also potentiate the inhibitory action of adrenalin on neutrophil microbicidal activity (G. Bazzoni et al., 1991), but its suppressive effects are abolished by ADP (de la Harpe and Nathan, 1989). Furthermore, ATP may be a neutrophil priming agent because it stimulates PK-C activity (Belazovich and Boxer, 1990).

Some amino acids are also immunostimulatory. Arginine is a potent stimulatory agent that enhances the following processes: phagocyte cytotoxicity, by increasing RNS generation (Reynolds et al., 1988); NK-cell and T-cell mitogenesis, by increasing intracellular polyamine levels (Reynolds et al., 1988); and GH release, by “down regulating” the inhibitory action of somatostatin on GH secretion (Ghigo et al., 1991). Polyamines also have immunoregulatory roles; polyamine depletion in T-cells impairs mitogenesis by blunting the responsiveness of the cells to IL-2 (Bowlin et al., 1987); while, in contrast, spermine inhibits the receptor-dependent activation of the neutrophil oxidative burst (Ogata et al., 1992). Decreases in plasma glutamine may contribute to immune dysfunction in overtrained athletes (Parry-Billings et al., 1990a; 1992) and burns patients (Parry-Billings et al., 1990b) by impairing lymphocyte proliferation. Glutamine - which appears to be derived from skeletal muscle and dietary sources - is an essential major fuel (along with D-glucose) of quiescent and proliferating
lymphocytes (Parry-Billings et al., 1990a). In this context, it is somewhat paradoxical that glucocorticoids are potent triggering agents of glutamine synthesis and release by skeletal muscle (Ardawi and Jamal, 1990). The roles of glutamine and most other amino acids in neutrophil function are not known.

3.4 Interactions between the immune and neuroendocrine systems

The communication between the neuroendocrine and immune systems and its mediation by hormones and cytokines is one the most active areas of current biological research (Imura et al., 1989; Blalock, 1992). These interactions are now thought to be important not only for the regulation of immunity and inflammation, but because they may also trigger behavioural changes associated with the stress response (Sternberg et al., 1992). Immune cells possess receptors for many hormones associated classically with the neuroendocrine system (Carr et al., 1992) and they are able to synthesize and secrete small amounts of a variety of these hormones (Blalock, 1989; 1992).

Immune cells also synthesize and release hypothalamic regulatory factors such as somatostatin which may modulate hormone secretion from immune cells in a manner analogous to the regulation of pituitary hormone secretion by hypothalamic hormones (Aguila et al., 1991). The physiological significance of cell communication at this level is not known, but hormone secretion by immune cells may participate in the paracrine amplification of afferent pituitary signals in micro-environments such as infection sites, and immune cells in these regions may secrete cytokines and other mediators that feed back to the pituitary gland and the brain (Blalock, 1992). Cytokines, for example, stimulate parts of the brain, including the hypothalamus, to secrete CRF which, in turn, activates ACTH secretion. ACTH triggers, in turn, glucocorticoid secretion from the adrenal cortex; when glucocorticoids achieve a threshold plasma concentration, they inhibit immune responses and therefore complete the negative feedback loop (reviewed by Sternberg et al., 1992). In contrast to hormones of neuroendocrine origin, hormones secreted by leukocytes are not stored and must be synthesized de novo; the quantities produced by leukocytes are much smaller than those produced by neuroendocrine cells, but immune cells are mobile and can concentrate the hormone at distant targets (Blalock, 1992). While antigenic stimuli are not recognized directly by the central
nervous or endocrine systems, leukocytes may convey information delivered by antigens, via humoral mediators, to these systems (Blalock, 1992) which triggers, in turn, a physiological and/or behavioural response (Sternberg et al., 1992).

Cytokines have been shown by many authors (using animal models) to have differential stimulatory affects on hormone secretion by the hypothalamic/pituitary axis under both in vivo and in vitro conditions (reviewed by Imura et al., 1991; Sternberg et al., 1992). Intravenous injection of IL-1 (Uehara et al., 1987; Watanabe et al., 1990), TNF-α (Bernardini et al., 1990; Sharp and Matta, 1993) or IL-6 (Matta et al., 1992) into rats stimulates ACTH secretion through a CRF-dependent mechanism that can be blocked with anti-CRF antibodies or indomethacin, an inhibitor of prostaglandin (PG) synthesis. Infusion of IL-1α into the hypothalamic circulation stimulates CRF, but not ACTH, release (Sapolsky et al., 1987). It is not known whether circulating cytokines interact with cytokine-secreting cells of hypothalamic or pituitary origin, or whether the two systems are independent. It is possible that circulating IL-1 interacts with the hypothalamus at regions where the blood-brain barrier is weak (e.g. median eminence, preoptic nucleus) (Arnason et al., 1991).

In cultured anterior pituitary cells, IL-1β stimulates ACTH, GH, luteinizing hormone and thyrotropin release (Bernton et al., 1987) and TNF-α has been reported to activate, after a two hour lag period (Milenkovic et al., 1989), or inhibit (Walton and Cronin, 1989; Gaillard et al., 1990), the basal and stimulated release of ACTH, GH and PRL. ILs-1 and -6 stimulate the release of CRF and ACTH from hypothalamic explants in vitro in a dose-dependent manner, which is inhibited by indomethacin, while ILs -2, -8, TNF-α, and IFNs have no effect on this system (Navarra et al., 1991). In fact, physiological concentrations of IFN-γ have been shown to inhibit the secretion of ACTH, PRL and GH by cultured anterior pituitary cells (Vankelecom et al., 1990). AA metabolites may mediate the effects of cytokines in a paracrine or autocrine manner because exposure of explanted rat hypothalami to PGF2α, LTs -B4 and -C4, or thromboxane-A2 stimulates CRF secretion (Bernardini et al., 1989).

Cytokines may have concentration-dependent effects on immunoregulation. For example, injection of small doses of IL-1 into the brains of freely moving rats stimulates GH and PRL release, without causing fever, but high doses abolish hormone secretion and trigger the febrile response
(Rettori et al., 1987). Using a similar protocol, Lyson and McCann (1991) reported that small doses of IL-6 induced the release of ACTH, but not GH or PRL, in conjunction with a delayed mild fever. The efferent affects of cytokines may not be confined to the hypothalamic/pituitary axis because IL-1 has been reported to stimulate cortisol secretion from cultured bovine adrenal cells in a PG-dependent mechanism (Winter et al., 1990). In general, the effects of cytokines on glucocorticoid secretion have not been well characterized.

While the related abilities of cytokines (IL-1 and TNF) to stimulate neuroendocrine hormone release and of glucocorticoids to inhibit cytokine release from immune cells in vitro is well established, the influences of hormones and neurotransmitters on cytokine production and secretion has received limited attention. Substance-P stimulates the production and secretion of IL-1 and other cytokines from preactivated monocytes in vitro (Laurenzi et al., 1990). Arginine vasopressin and oxytoxin trigger γ-interferon release from mouse lymphocytes in vitro (Johnson and Torres, 1985). The diverse range of immunostimulatory effects documented for GH and PRL extend to cytokine production and release. Preliminary reports suggest that prolactin is required for the normal secretion of γ-interferon by antigen-stimulated mouse lymphocytes in vitro (Bernton et al., 1988) and GH treatment in vivo augments the capacity of endotoxin to induce greater necrosis of Meth A tumours in mice (Edwards et al., 1991b). These results suggest that GH, and possibly PRL, may prime macrophages for greater TNF release in vivo (Edwards et al., 1991b) and they correlate with previous findings of this group that GH and PRL also prime superoxide release from neutrophils and macrophages (Fu et al., 1991).

Taken together, these studies show unequivocally that some cytokines, particularly IL-1, have the ability to stimulate ACTH release from the pituitary gland via a CRF-dependent process that involves cyclo-oxygenase metabolites. These animal models may apply to humans because IL-1, for example, has been detected in cerebrospinal fluid from head-injured humans and in the nerve fibres of the human hypothalamus (reviewed by Scarborough, 1990). Furthermore, the administration of endotoxin to healthy men causes a substantial increase in the circulating concentrations of TNF-α and ACTH (Michie et al., 1988). The bidirectional communication between the immune and neuroendocrine systems is evidently an
extremely complex phenomenon and the physiological significance of these interactions in humans is an important challenge for future studies.

3.5 Acute-phase and stress proteins

The acute-phase response represents a generalised host reaction to infection, inflammation and trauma (Kushner, 1988). It may also represent a general, albeit much smaller, physiological response to exercise (Cannon et al., 1986). Acute-phase proteins (APP) function to restore physiological homeostasis perturbed by these stressors (Kushner, 1988). The proteins, some of which can increase in plasma concentration 1000-fold above normal include: protease inhibitors, including some SERPINs (Kilpatrick et al., 1992); complement components; a number of metal-binding proteins (e.g. lactoferrin, transferrin, ceruloplasmin); and C-reactive protein (CRP), which is considered by some authorities to act as a non-specific opsonizing agent (Dufaux et al., 1984). Cytokines like IL-6 also act in association with glucocorticoids to activate the synthesis of APP's in hepatocytes (Heinrich et al., 1990). They appear to regulate APP synthesis at the level of gene transcription (Perlmutter et al., 1986). Individual APP genes may respond to different concentrations and species of circulating cytokines and synergistic glucocorticoids (Jamison et al., 1987).

The role of the whole spectrum of APP's in host defence is not entirely clear, yet the response is common to all vertebrates (Dinarello, 1989). The role of the acute-phase response may be to restore homeostasis when this has been disturbed by conditions that cause local inflammatory tissue damage. Induction of APP synthesis contributes to a fall in the plasma concentrations of a number of free transition metal ions (e.g. iron, copper, zinc). Transition metals are: (1) essential for microbial growth; and (2) catalytic for the formation of a number of toxic ROS (Halliwell, 1987). Some APPs prevent inflammatory damage to body tissues by "down-regulating" the immune response. At inflammatory sites, for example, peptides derived from C-reactive protein (CRP) are generated when CRP binds to neutrophils and these fragments are potent inhibitors of the oxidative burst (Shephard et al., 1990). In contrast, aggregated CRP potentiates Fc-mediated neutrophil oxygenation activity (Zeller et al., 1986). Low levels of CRP are immunostimulatory, while the high levels achieved during an acute-phase response may prevent "over activation" of immune responses through negative
feedback (Buchta et al., 1987). The metal-binding proteins ceruloplasmin and haptoglobin also inhibit a number of neutrophil microbicidal functions (Broadley and Hoover, 1989; Oh et al., 1990).

Stress (heat-shock) proteins - a family of highly-conserved proteins - are believed to protect host cells from damage during inflammation and thermal stress (Dobrinich and Spagnuolo, 1991). While APPs are released into the circulation during stress, these proteins may represent a class of intracellular APP with a longer evolutionary history that maintain or restore intracellular homeostasis (Polla, 1988). Oxidative stress in phagocytes triggers the synthesis of stress proteins under conditions where they may function as antioxidants (Donati et al., 1990) and may be responsible for heat-shock-induced inhibition of NADPH oxidase (Maridonneau-Parini et al., 1988). Mature neutrophils, for example, synthesize stress proteins in response to hyperthermia (Eid et al., 1987).
4. EXERCISE AND IMMUNITY

Whilst the health benefits of regular exercise (e.g. reduced susceptibility to cardiovascular disease) are well known, the relationship between exercise and immunity has only recently become a focus of intense research activity. This is surprising considering that anecdotal evidence linking these two phenomena has been cited in reports that date back to 1920 (Mackinnon, 1986; 1992). At present, some reports suggest that moderate exercise may increase resistance to infection; beyond this point of moderation, however, the daily exercise undertaken by athletes during intensive training periods appears to depress immunity (see reviews by Mackinnon, 1992; Fitzgerald, 1988; Keast et al., 1988). The mechanisms by which these paradoxical responses to exercise are produced are poorly understood.

Numerous anecdotal reports suggest that intensive training (and overtraining) is associated with increased susceptibility to common infections (Fitzgerald, 1991). The psychological stress associated with intensive training and competition may exacerbate this tendency. Exercising during illness may worsen the symptoms of the infection (Fitzgerald, 1991). Transient immunosuppression induced by intensive exercise or stress may create a "window of opportunity" for infectious agents to evade host defences and establish infections (Fitzgerald, 1991). This may even apply to normally harmless commensals and useful symbionts (Eichmann, 1991).

4.1 Epidemiological evidence

Despite the dearth of wide-ranging epidemiological studies, several more specific investigations have shown that, compared to untrained individuals, athletes in training show increased incidence of upper respiratory tract infections (Linde, 1987; Peters and Bateman, 1983; Heath et al., 1991), infectious hepatitis and aseptic meningitis (Fitzgerald, 1988). The majority of infections that affect athletes are viral in origin but fungal skin infections are also common (Roberts, 1986). Highly-stressed individuals are also more susceptible to respiratory infections (Graham et al., 1986) and to some cancers (Kiecolt-Glaser et al., 1987; Shephard, 1990). Moderate exercise programs and/or physically active jobs may, in contrast, reduce susceptibility to colon and breast cancers (Kohl et al., 1988), upper respiratory
tract infections (Nieman et al., 1990a) and the severity and duration of depression-related illness (Moses et al., 1989).

Moderate training (26-30 miles/week of running) reduced the occurrence of self-reported infectious episodes compared with those experienced by individuals running less than 15 miles/week (Nieman et al., 1989a). In contrast, running more than 90 km/week doubled the odds of acquiring an infectious episode compared to those running less than 30 km/week (Nieman et al., 1990a). These workers also reported that running 42 km marathons at competition speed increased the odds of infection five-fold compared to a group who trained for the race but did not compete. High training mileage is, in fact, a significant risk factor for upper respiratory tract infections (Heath et al., 1991; 1992).

4.2 The leucocytosis of exercise

Submaximal (aerobic) and maximal (anaerobic) exercise cause substantial but transient increases in circulating leucocyte (immune cell) numbers due to their release from marginated vascular pools and the bone marrow (McCarthy and Dale, 1988). Blood leucocyte counts can remain significantly elevated for up to eight hours after prolonged moderate exercise but they return to pre-exercise (resting) levels within one hour after a brief episode of exercise at maximum capacity. The magnitude and period of the leucocytosis depends upon the intensity and duration of the workload; it is mediated by increased blood flow and the substantial elevations that occur in circulating catecholamines and cortisol (McCarthy and Dale, 1988).

Lymphocytes and neutrophils contribute jointly to the leucocytosis induced by submaximal and maximal exercise, but increased neutrophil numbers become predominant once cortisol release is activated (McCarthy and Dale, 1988). In general, exercise of moderate and maximal intensities lowers both the T-cell to B-cell and the helper T-cell (TH) to suppressor/cytotoxic T-cell (TS) ratios among the circulating cells that are transiently elevated (Keast et al., 1988; McCarthy and Dale, 1988) The TH/TS ratio is, in fact, reduced progressively as the intensity and duration of submaximal exercise increases (Kendall et al., 1991). Repeated bouts of exercise for five days showed, however, that, while NK cell numbers increased, the changes in T-cell subsets were variable (Hoffman-Goetz et al., 1990). Acute maximal exercise triggers the mobilization of all classes of white blood cells (WBC) in both trained and untrained human subjects.
while chronic bouts of submaximal exercise enhance neutrophil mobilization at the expense of lymphocytes (Ferry et al., 1990). Regular training status does not affect the magnitude of these acute responses to exercise, nor does it affect neutrophil and lymphocyte numbers in the circulation chronically (Oshida et al., 1988). The potential immunological consequences of these quite marked changes in circulating WBC numbers are not clear because they do not correlate positively with functional changes and may intersect with the circadian variations in leucocyte haemodynamics that also occur (Levi et al., 1988).

4.3 Exercise and functional changes in immunity

The majority of studies that have attempted to assess the effects of exercise on cell-mediated immune responses have focused on lymphocytes and NK cells but there are some recent reports in which neutrophil and macrophage functions have been examined. Many independent investigations have reported transient reductions in the responsiveness of isolated lymphocytes to T-cell and B-cell mitogens after exercise at maximum intensity while variable results have been documented for submaximal exercise (reviewed by Mackinnon, 1992; Nehlsen-Cannarella et al., 1991a; Field et al., 1991). This variability may be due to differences in the intensity and duration of the submaximal exercise protocols employed. For example, exercise at maximum intensity and marathon running both cause significant reductions in NK-cell cytotoxicity while moderate exercise is stimulatory (Berk et al., 1990; Watson et al., 1986; Pederson et al., 1990; Pederson, 1991). Prolonged submaximal exercise and brief maximal exercise both lower serum and salivary immunoglobulin levels, complement components and neutrophil microbicidal activity (Nieman et al., 1989b; Lewicki et al., 1987). Surprisingly, tissue macrophage phagocytic capacity has been reported to increase immediately after a bout of exhaustive exercise but this may be associated with the roles that these cells have in remodelling damaged tissues (Fehr et al., 1989).

In contrast to intensive exercise, individual episodes of moderate exercise potentiate antibody-dependent cytotoxicity and NK-cell cytotoxicity (reviewed by Keast et al., 1988; Pedersen et al., 1990) but have no effect on salivary IgA levels (McDowell et al., 1991). Moderate exercise also causes small increases in the serum concentration of immunoglobulins that are
independent of changes in plasma volume (Nehlsen-Cannarella et al., 1991b).

Several striking immunodeficiencies have been reported in "cross-sectional" studies of athletes undergoing intensive training programs. These include reductions in serum complement factors, salivary IgA, NK-cell cytotoxicity, and lymphocyte mitogenesis (reviewed by Mackinnon, 1992; Nieman et al., 1989c). Intensive training does not alter the baseline concentrations of serum immunoglobulins but falls have been reported in some athletes during periods of intense competition (Nehlsen-Cannarella et al., 1991a). Longitudinal studies have not been undertaken, however, to determine at what stage of the training program these diminished responses become significant.

Moderate training, in contrast, produces significant chronic increases in the following parameters: lymphocyte mitogenesis in vitro (Watson et al., 1986); NK-cell activity (Nieman et al., 1990b); serum immunoglobulins (Nehlsen-Cannarella et al., 1991a) and salivary IgA (Tharp, 1991). The beneficial effects of regular training on NK-cell activity were lost within 15 weeks (Nieman et al., 1990b), perhaps because the intensity of the exercise undertaken was no longer sufficient to induce the priming response, presumably because of adaptation to the higher fixed workload.

Trauma causes similar defects to those produced by intensive endurance exercise in T-cell, macrophage and neutrophil functions, which may explain the high infection rates observed in these individuals (Green and Faist, 1988). Transient perturbations of immune responses after intense exercise or trauma may be a protective response that prevents severe inflammatory damage to body tissues, particularly at injury sites. For instance, elevated concentrations of neutrophil elastase (which may damage body tissues) have been detected in plasma after prolonged running and cycling (Kokot et al., 1988; Dufaux and Order, 1989; Pincemail et al., 1990; Hanson et al., 1991).

These observations show that the intensity and duration of exercise may exert, via humoral mechanisms, a functional influence on cellular immunity. The depression of some immune cell functions in response to psychological and traumatic stress shows a remarkable similarity to that induced by intensive training. Impaired mitogenic responsiveness of lymphocytes and reductions in NK-cell cytotoxicity have, for example, been reported following bereavement and in response to the psychological stress.
of college examinations (Ader et al., 1990). The variability of the exercise effect may be due to the failure of some investigators to express their results in terms of a defined cell number (to take account of any coincident leucocytosis for example) or to the tendency of others to restrict sampling times to the periods just before and immediately after exercise. NK-cell cytotoxicity, for example, may increase immediately after moderate to intense exercise, only to fall substantially below pre-exercise values two hours later, with recovery to normal levels occurring over the next 21 hours (Berk et al., 1990; Pederson et al., 1990); administration of indomethacin, which blocks prostaglandin synthesis, abolishes this decrease to some extent (Pederson et al., 1990). Some immune responses may also show endogenous diurnal fluctuations in their activities (Levi et al., 1988).

4.4 Exercise and immunoregulation

In this section, I will consider the range of interactions between hormones, cytokines and immune cells that may be induced by exercise and the potential immunological consequences that may flow from such wide-ranging interactions.

The intensity and duration of exercise influences the extent to which many neuroendocrine hormones are released into the circulation (see reviews by Galbo, 1983; Viru, 1992). The magnitude of the response is substantially lower in trained subjects exercising at the same absolute workload as their untrained counterparts (Galbo, 1983). Training may, however, increase cellular responsiveness to some hormones (e.g. insulin) (Viru, 1992). There may also be individual differences in the kinetics of hormone secretion in response to exercise (Viru, 1992; Viru et al., 1992) that are independent of training status. Stress hormones such as GH, catecholamines and cortisol are thought to be secreted in response to the direct metabolic and physiological demands of exercise. Circulating and margined immune cells with receptors for these hormones are highly susceptible targets. With some hormones, the plasma concentrations peak immediately after a critical threshold is reached in workload intensity, but with some others (e.g. cortisol) this may not occur until several hours after the episode of exercise has ceased. There may also be a substantial lag time between the attainment of peak plasma concentration of a particular hormone and the appearance of a functional cellular response.
Exercise-induced increases in the plasma concentrations of cytokines and neuroendocrine hormones have been reported widely. The release of cytokines into the circulation has been reported following submaximal and eccentric exercise (Cannon et al., 1986) and strenuous running (Dufaux and Order, 1989; Espersen et al., 1990). Endotoxin, a potent stimulus of cytokine secretion, has also been detected in plasma after an ultratriathlon (Bosenberg et al., 1988). Furthermore, mononuclear cells isolated from human subjects after exercise have been reported to secrete significantly greater quantities of cytokines than mononuclear cells isolated before exercise when stimulated with LPS (Lewicki et al., 1988; Haahr et al., 1991). Sprenger et al. (1992) have reported, in contrast, that cytokine concentrations do not change significantly in the plasma of well-trained runners after a 20 km road race. They were able to demonstrate, however, quite significant increases in IFN-γ, TNF-α, IL-1β, IL-6 and soluble IL-2 receptors in the urine of exercised subjects, which they claimed to be indicative of enhanced production and/or release of these factors (Sprenger et al., 1992).

The circulating concentration of GH is elevated significantly after 30 minutes of exercise of very low intensity (10-15% VO₂ max) and it rises progressively with increasing workload (Galbo, 1986). PRL, which shares considerable structural and functional homology with GH (Kelley, 1989), does not increase in plasma until the exercise intensity exceeds 50% VO₂ max. The magnitude of the increase is much smaller than that of GH (Galbo, 1983; Farrell et al., 1986). The plasma concentration of substance-P may also be increased by moderate exercise (Galbo, 1986) but, in general, its response to exercise has not been examined. Thus, further studies are warranted. Moderate exercise also activates the secretion of ANP into the circulation (Follenius and Brandenberger, 1988; Schmidt et al., 1990; Freund et al., 1991). This hormone (which regulates blood pressure and fluid electrolytes) responds almost instantly to exercise and is cleared from the blood very quickly as soon as exercise ceases (Follenius et al., 1989; Schmidt et al., 1990). Exercise does not produce significant changes in plasma gonadotropins or thyrotropin (Galbo, 1983).

The immunostimulatory properties of GH and prolactin are antagonised by ACTH and glucocorticoids (Berczi, 1986). ACTH - which stimulates cortisol release from the adrenal gland and is secreted concomitantly with β-endorphin - does not rise in the plasma until exercise intensity exceeds 60% VO₂ max (Farrell et al., 1983; Goldfarb et al., 1990). This
threshold must be exceeded before cortisol (the major human glucocorticoid) and opioid release are triggered (Farrell et al., 1983; Langenfield et al., 1987; Goldfarb et al., 1990). These increases are not affected significantly by prolongation of the workload because exercise at maximum intensity causes the plasma concentrations of ACTH and \( \beta \)-endorphin to rise within 30-60 seconds (Buono et al., 1986; Rahkila et al., 1988). As already discussed, some hormones have concentration-dependent effects on immune cell function. For example, a transient 15 minute increase in NK-cell cytotoxicity induced by prolonged submaximal exercise may be mediated by low concentrations of endorphins whereas the post-exercise decrease in this cellular response - sustained for 24 hours - may be due to excessive release or slow catabolism of endorphins (Fiatarone et al., 1988; Berk et al., 1990).

Catecholamine secretion during exercise is also intensity-dependent. The rise in the plasma concentration of catecholamines is five-fold larger after maximal exercise than the small increase induced by moderate exercise (Deuster et al., 1989). Noradrenalin secretion is triggered at much lower workloads than those required to raise adrenalin (Galbo, 1986) and noradrenalin may, in turn, initiate GH release by activating hypothalamic GH-releasing hormone (Malozowski et al., 1990). Adrenalin may mediate the inhibitory effects on lymphocyte proliferation and IL-2 receptor expression \textit{in vitro} found immediately after single episodes of maximal exercise because non-selective blockage of \( \beta \)-receptors prevents this (Murray et al., 1992).

Nearly all immunosuppressive responses induced by intense exercise correlate with increases in circulating cortisol, the plasma half-life of which is 60-90 minutes at rest. Cortisol release is slower than that of catecholamines and ACTH, and may not peak until 30 minutes after exercise has ceased. Sustained cortisol secretion, such as the 200% increase observed after a marathon run (McCarthy and Dale, 1988), may represent failure of this glucocorticoid to trigger, under these conditions, the feedback signals that shut down ACTH release (Sapolsky et al., 1990). While the plasma concentrations of opioids and adrenalin usually return to resting values 10 to 20 minutes after exercise, these hormones may also contribute to transient suppression of immune responses after intense exercise (Berk et al., 1990; Sforzo, 1988; Kappel et al., 1991). The return of their plasma concentrations to pre-exercise values may not coincide with the
disappearance of their immunosuppressive effects. Increases in plasma histamine (Dufaux et al., 1991), prostaglandins (Pederson et al., 1990) and vasoactive intestinal peptide (Øktedalen et al., 1983) after intense exercise may also contribute to suppressed immune responses (see Section 3.3). Some of these changes may be prolonged and may require the recruitment of recently-matured cells from the bone marrow to reverse them. The effects of training on the rate of leucocyte division, differentiation and release have not been investigated.

Physical training may generate a homeostatic shift that involves changes in the concentrations of inflammatory mediators and their endogenous inhibitors (Liesen et al., 1977). Ultramarathon running and triathlons induce substantial elevations in plasma CRP levels that peak 24 hours after the race; CRP synthesis may be triggered by muscle damage or endotoxaemia (Taylor et al., 1987; Bosenberg et al., 1988). Elite athletes show chronic elevations of some APP's, including serum protease inhibitors (e.g. α1-antitrypsin, C1-inhibitor), yet training appears to progressively reduce the magnitude of the acute-phase response to regular episodes of acute exercise at the same intensity (Liesen et al., 1977). Attenuation of the production of CRP may increase the susceptibility of some individuals undergoing such an intense training program to bacterial infections (Dufaux et al., 1984).

Strenuous exercise of rats induces the production of several stress (heat-shock) proteins in lymphocytes and soleus muscle (Locke et al., 1990). This may be due to a form of exercise-induced heatstroke because exhaustive exercise of rats increases the liver and muscle contents of a 70 kD stress protein three-fold (Salo et al., 1991). In fact, this group suggested that exercise-induced hyperthermia may trigger mitochondrial uncoupling, resulting in oxidative stress (through leakage of O2−) that may be sufficient to stimulate mitochondrial biogenesis. Exercise at intensities which cause the body temperature to rise above 40°C also reduce the amount of a 70 kD stress protein synthesized by leucocytes when they are incubated at 41°C in vitro (Ryan et al., 1991). This is indicative of a post-exercise refractory period in which a leucocyte subpopulation may fail to respond to secondary stimulation in vitro and, perhaps, further evidence of immunosuppression induced by vigorous exercise.

Psychological stress is also associated with elevated cortisol activity and impaired immune responses (Vickers, 1988). Decreased phagocyte activity
was observed as early as 1920 in emotionally-stressed people (Khansari et al., 1990). Stress caused by trauma, infection, pain and fear also stimulates the secretion of cortisol (Munck and Guyre, 1986). These responses have functional consequences because psychological stress increases susceptibility to Rhinovirus infections (i.e. the common cold) (Cohen et al., 1991). Because the neuroendocrine responses to intensive physical exertion and psychological stress bear striking similarities, it is not surprising that similar changes in the responses of immune cells also occur. In contrast, moderate training reduces the basal circulating concentration of β-endorphin and this correlates with greater emotional stability and reduced incidence of depression (Lobstein et al., 1989).

Overtraining may induce immunosuppression through neuroendocrine dysfunction; thus immune and neuroendocrine factors may be useful in diagnosing overtraining (Fry et al., 1991). At present, overtraining is diagnosed clinically by a large fall in the testosterone/cortisol ratio (caused by increased cortisol) (Hackney, 1991). However, this ratio may fluctuate diurnally. Overtraining research is, in general, hampered by small sample sizes (Hackney, 1991) and the present lack of reliable diagnostic markers. Profiles of immune and neuroendocrine parameters every two to four hours over a twenty four hour period may provide more reliable results.
5. THE ROLE OF EXERCISE IN HEALTH CARE

While the previous discussion has presented evidence that moderate exercise may boost the activity of some of the major components of the immune system in vitro, we do not know whether these observations are clinically significant. Regular exercise is advocated as one of the lifestyle factors promoting good health and longevity, while low physical activity has been implicated as an important risk factor in morbidity, all-cause mortality from cardiovascular disease and cancer (Blair et al., 1989; McAuley et al., 1991). Thus, participation in regular exercise programs may, in fact, reduce overall medical care costs.

Infectious disease was the leading cause of death up to the early 1900’s. Today, it still ranks fourth behind chronic degenerative diseases and cancer, and immune dysfunction may be a major contributor in all of these conditions (Chandra, 1989; 1992). Many cancers may occur as a result of impaired immunity. Infants and the elderly are particularly vulnerable to immune dysfunction but this may be ameliorated to some extent by moderate exercise programs and nutritional intervention (Chandra, 1989). While the incidence of infection and cancer increases in the elderly, no correlating immunological changes which may coincide with the onset of these diseases have been found (Lehtonen et al., 1990).

Studies on neutrophil function in elderly individuals have produced conflicting results (MacGregor and Shalit, 1989; Lehtonen et al., 1990). The majority of neutrophil functions studied in cells isolated from healthy elderly individuals are similar to those found in normal young controls, but neutrophils from healthy elderly subjects do show impaired migration to skin window abrasions (MacGregor and Shalit, 1989). Recently, neutrophils isolated from elderly human donors were reported to show impaired ability to migrate towards chemotactic stimuli, and an attenuated degranulation response in vitro (Shoham-Kesari and Gershon, 1992). The conflicting results obtained in immunological studies of elderly subjects may be due to differences in study design, assay sensitivity and the general health of the subjects.

While immunological differences between the elderly and healthy young subjects may be minor, the stress of illness in the elderly may amplify the effects of minor dysfunctions to the point where they become clinically significant (MacGregor and Shalit, 1989). Respiratory infections are the most
common reason for hospitalization of the elderly (Chandra, 1989). Moderate exercise programs may significantly delay some of the manifestations of deterioration of immune system function associated with aging (Chandra, 1989; Fitzgerald, 1991). For instance, moderate exercise enhances NK-cell activity to the same levels detected in young subjects (Fiatarone et al., 1989), and it increases NK-cell-mediated tumour cytotoxicity in elderly women (Crist et al., 1989). Thus, both young and elderly subjects may derive the same positive benefits from moderate training programs.

Because of a favourable link with immunity, AIDS patients are now being prescribed exercise programs as part of their treatment (Fitzgerald, 1991) and some long-term AIDS survivors have reported favourably on participation in exercise programs (Soloman, 1991). Moderate exercise also reduces the emotional distress and decrements in NK cell activity of the subjects that occur following notification of positive serologic status for HIV-1 (LaPerriere et al., 1990). This may be due to increases in circulating $T_H$ cells triggered by exercise - which are similar to those induced by azidothymidine (AZT) - but without the deleterious side-effects (LaPerriere et al., 1991).

Nutritional deficiencies are also associated with impaired immune responses (Chandra, 1991; 1992). Nutritional deficiency of immunological significance includes: vitamins A, B₆ & C; trace metals such as zinc and iron; amino acid imbalance; and essential fatty acids. Ageing, when it is combined with malnutrition, does produce significant impairments in neutrophil function (Lipschitz and Udupa, 1986). Excessive intake of these micronutrients, on the other hand, may impair immune responses (Chandra, 1991). Therefore, a combination of different lifestyle factors may be necessary to contribute favourably to the maintenance of effective immunity.
Neutrophils are an important cellular component of the "natural" immune system that play an important role in host defence against all classes of infectious agents, on the one hand, but, paradoxically, have been implicated in the pathology of inflammatory disease on the other. The microbicidal mechanisms employed by these cells consist of oxidative and non-oxidative processes that are activated simultaneously upon phagocytosis. These cells exist not only in a dormant (inactive) and overtly activated state but also in several intermediate stages. In fact, various humoral mediators may "prime" neutrophils, which enables them, in turn, to mount a more powerful microbicidal response once the cell is activated. The biochemical mechanisms involved in the priming response are poorly understood but may involve increases in receptor expression and/or affinity, and/or enhanced effectiveness of the signal that couples receptor-ligand binding to the effector machinery. The cell surface receptors include various opsonin and adhesion receptors, while post-receptor changes may involve inter alia, cation fluxes, phospholipid hydrolysis, activation of protein kinases, changes in cyclic nucleotide metabolism, and translocation of NADPH oxidase components from the cytosol to the plasma membrane. These biochemical responses have physiological consequences because neutrophil priming has been reported in many situations in vivo including infectious and inflammatory disease. Neutrophil microbicidal activity can be studied by a variety of independent techniques that measure the responses of a cell preparation, either collectively or at the single cell level.

The activities of immune cells such as neutrophils are regulated locally and systemically by a variety of humoral mediators secreted by other immune cells and/or cells from the endocrine, nervous, and cardiovascular systems. These mediators include cytokines, "classical" neuroendocrine hormones, nucleotides, eicosanoids, amino acids and some acute-phase proteins. The net response depends on the complex balance of stimulatory and inhibitory pathways which are regulated differentially by a combination of these immunopotentiating and immunosuppressive mediators. It has now become clear, in fact, that the immune system is not autonomous but that it communicates actively with other physiological systems, including the brain, in a bidirectional manner. These interactions may have important consequences for host defence and behaviour.
Neuroendocrine responses to physical exercise may influence the immune system. Many reports suggest, in fact, that whilst moderate physical exercise may enhance resistance to infectious disease, the effects of repeated intensive exercise appear to be detrimental. These reports have been supported by several epidemiological studies and many studies of cellular and humoral immune responses in vitro. Exercise is an ideal and well-characterized “stressor” to impose on human subjects in an experimental situation as it provides an opportunity to examine the physiological mechanisms which regulate the immune system. If related to the person's own VO$_2$ max, exercise is completely reproducible and can be varied in intensity, type, duration and frequency. It has, furthermore, clearly-defined biochemical and neuroendocrine sequelae, that appear at different thresholds of exercise intensity and which have the potential to condition the milieu within which the circulating components of the immune system operate. If the hypotheses presented in this review are borne out, it may be possible to recommend suitable exercise regimes as part of a therapeutic program to immunodeficient individuals, on the one hand, and to use immunological parameters to monitor the effects of training programs on the health of athletes.
7. **SCOPE OF THIS THESIS**

The aims of this project were to assess:

(i) the acute and chronic effects of exercise (at moderate and maximal intensity) on various neutrophil microbicidal activities; and

(ii) the immunological consequences of exercise-induced changes in these activities, and the cellular and humoral mechanisms involved in these responses.

During the course of these studies, a neuroendocrine/cytokine hypothesis was developed to explain the intensity-dependent effects of exercise on neutrophil microbicidal activity. Thus, I also investigated the role of cytokines and neuroendocrine hormones on the differential responses of neutrophil microbicidal activities to moderate and intensive exercise.

The specific hypotheses tested in this part of the thesis are presented in the separate introductions to each experimental paper.
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CHAPTER 2

EXPERIMENTAL

(Neutrophil microbicidal activity)
CHAPTER 2.1

Exercise, training and neutrophil microbicidal activity
Exercise, Training and Neutrophil Microbicidal Activity

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Abstract


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The concentration in human plasma of putative neutrophil-"priming" cytokines like endogenous pyrogens is known to increase significantly in response to moderate exercise (11). This is characteristic of an acute-phase response. The ability of blood neutrophils isolated from both trained and untrained human subjects (n = 11, 9) to produce microbicidal reactive oxygen species was determined using luminol-enhanced chemiluminescence both before and after one hour of aerobic exercise at 60% VO2max. Irrespective of training and stimulus concentration, exercise nearly always caused significant "priming" of the capacity of neutrophils to produce H2O2 and HOCl upon stimulation with opsonized zymosan (P < 0.01); however, compared to their untrained counterparts, the activity of cells isolated from trained individuals was depressed about 50% at unit stimulus concentration, both before and after exercise (P < 0.075), whilst remaining unaltered at saturating concentrations.

Although neutrophil oxygenation activity is only one parameter that contributes to immunological status, regular episodes of moderate exercise may increase resistance to infection by priming the "killing capacity" of neutrophils. In contrast, prolonged periods of intensive training may lead to increased susceptibility to common infections by diminishing this activity.

Key words

Exertion, neutrophil, cytokines, priming, chemiluminescence, reactive oxygen species, training.

Introduction

The influence of exercise on immunity is an important community health issue that encompasses a wide range of activities from recreational jogging to the performance of elite athletes undertaking strenuous training programs. Reports suggest that, whilst moderate exercise may enhance resistance to common infections, the effects of excessive physical activity may be detrimental. The direct or indirect effects of exercise that have been documented include increased resistance of individual human subjects to infection (34), retardation of tumour growth in treadmill-trained experimental animals (26) and, in contrast, an apparent increase in susceptibility of some highly-trained athletes to common infections (37, 39). Acute physical exertion in human subjects is accompanied by physiological changes that are remarkably similar, in some respects, to those induced by infection (11): there is a substantial increase in circulating white blood cells (WBC) (specifically neutrophils and lymphocytes), an induction of mild fever and an increase in the concentration of serum factors that influence white blood cell function in vitro. These factors include fever-inducing "pyrogens" like interleukin-1 (13) and interferon-α (47) as well as a number of acute-phase proteins (17, 31) including complement factors (19). Their elevation, accompanied by a fall in the serum concentrations of iron and zinc, produces a distinctive acute-phase response to exercise. These responses are likely to be multi-faceted processes that vary widely from individual to individual.

Neutrophils - which represent 50–60% of the total circulating white blood cell population - constitute the "first-line-of-defence" against foreign organisms (53). Microbes are ingested (phagocytosed) upon contact with these cells, incorporated into vacuoles and subjected to intravacuolar contact with toxic reactive oxygen species and hydrolytic enzymes. Host tissues may be damaged in the process if oxidants and enzymes are released extracellularly. Since, ultimately, it is the phagocytic and cytotoxic capacity of these short-lived cells that determines whether there is a progression to clinical manifestation of infection, the influence of exercise on their microbicidal activity may be of crucial importance. Cytokines like endogenous pyrogens may contribute to increased resistance to infection throughout a period of moderate training (12) by augmenting the neutrophil oxidative burst. The present work questions whether a single episode of moderate exercise, sufficient to elevate endogenous pyrogens, alters the capacity of peripheral blood neutrophils to produce microbicidal reactive oxygen species upon stimulation in vitro under conditions that simulate a microbial challenge. We have also examined whether the acute effects of exercise are different in trained and untrained individuals and whether the al-
tered response is sustained for a significant period after exercise has ceased. The results suggest that neutrophil oxygenation activity is enhanced, acutely, by moderate exercise but depressed, chronically, by a prolonged period of intensive training. The mechanism may involve complex humoral and cellular alterations that are confined to the circulatory environment.

**Methods**

**Materials**

Hank's balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were prepared by conventional methods and sterilized. HBSS contained 5 mM D-glucose. Luminol was prepared as a chemiluminogenic indicator by suspending ten mg of luminol (5-amino-2,3-dihydro-1,4- phthalazine dione) (Boehringer, Mannheim, FRG) in five ml of PBS containing 8 µM triethylamine. The mixture was sonicated for one minute and then shaken by hand to give a clear solution. A single batch of opsonized zymosan (used for all experiments) was prepared by incubating ten mg of Zymosan-A particles (highly glycosylated fragments of yeast cell walls) (Sigma, St. Louis, MO) with one ml of fresh human plasma for 30 min at 37 °C. The suspension was centrifuged at 400 × g for two minutes and the pellet washed twice in PBS before re-suspension in PBS at a final concentration of ten mg/ml. Aliquots (1 ml) were stored frozen and used only once after thawing. Counting by haemocytometer revealed that this preparation contained approx. 10⁶ zymosan particles/microlitre.

**Human Subjects**

Nine healthy untrained subjects (average VO₂max 48.1 ml/kg×min, average age 22 years) and eleven elite cyclists (average VO₂max 71.4 ml/kg×min, average age 19 years) were recruited for the project. All of the subjects were male. No experimental subject had experienced symptoms of acute illness or had taken medication for six weeks prior to the test. The majority of the cyclists were competing at Australian national standard and maintained an intensive training regime of at least four hours/day. In contrast, all untrained subjects devoted less than three hours/week to exercise-related activities. The project was approved by the Ethics in Human Experimentation Committee of the Australian National University. All subjects signed a consent form that described the aims of the project and acknowledged its attendant risks.

**Determination of VO₂max**

On a day prior to the test, the maximum rate of oxygen uptake (i.e. VO₂max) was determined for each individual by subjecting them to a progressive increase in workload to voluntary exhaustion on a racing cycle ergometer (Exertech, Melbourne, Vic). A work monitor unit (Exertech, Melbourne, Vic) which incorporated a magnetic sensor located close to a 60-tooth cog mounted on the front wheel provided the subject with a continuous analogue display of power output in watts (W). The initial power output was 50 W, and increments of 25 W were introduced each min. The subjects breathed through a one-way valve (Hans Rudolph 2700, Kansas City, MO) and the volume of air inspired was measured with a Morgan ventilation meter (Medox, Melbourne, Vic). Samples of inspired air were drawn continuously through a desiccant (CaCl₂) into oxygen and carbon dioxide analysers (Applied Electrochemistry, Sunnyvale, CA) which were calibrated regularly against gravimetric standards. Electrocardiograms (ECG) were recorded during the final ten seconds of each minute and the readings were transferred to an ECG computer (Quinton Instruments, Seattle, WA). Outputs from the ventilation meter, gas analysers and ECG computer were monitored by a LSI 11/21 computer (Digital Electrical Corporation, Sydney, N. S. W.) programmed to give minute-by-minute readouts of pulmonary ventilation at standard temperature and pressure for dry gas (STPD). Oxygen uptake, carbon dioxide output, respiratory exchange ratio, heart rate and ventilatory equivalents for oxygen (VO₂) and carbon dioxide (VCO₂) were recorded for each subject.

**Exercise Protocol**

On the day of the test, a standardized exercise schedule was undertaken by each participant. Food intake before the test was restricted to a light “non-fatty” meal. The majority of the experiments were carried out in the morning (i.e. 9.30–10.30 am) to take advantage of the overnight rest; some additional experiments were performed in the afternoon (i.e. 3.30–4.30 pm) to test for circadian variation in neutrophil oxygenation activity. Apart from the exercise test, no other strenuous activity was undertaken throughout the 24 hour period. All subjects exercised on a racing cycle ergometer (Exertech, Melbourne, Vic) for one hour at a work output equivalent to 60% of their individual VO₂max values. This workload is sufficient to elevate the circulating endogenous pyrogen activity of human subjects (11). Heart rates were monitored continuously with a Sportstester P. E. 3000 heart rate meter (Polar Electro, Kempele, Finland) and the power output with a work monitor unit (Exertech, Melbourne, Vic). Blood samples were taken by venipuncture (arm antecubital vein) immediately before (i.e. at rest) and after exercise. A third sample was taken six hours later to check for the persistence of exercise-induced changes.

**Preparation of Neutrophils**

Ten ml of heparinized blood was decanted onto a three ml Ficol-paque cushion (Pharmacia, Uppsala, Sweden) in a 15 ml sterile tube and centrifuged for 15 minutes at 600 × g. Under these conditions the neutrophils sedimented directly on top of the red blood cell layer. The neutrophil layer was removed by aspiration and transferred to a second sterile tube. Contaminating red blood cells were removed by hypotonic shock with 0.83% NH₄Cl (which also prevents cellular aggregation (25)) and lysis was assumed to be complete when the opaque mixture became translucent. The mixture was then centrifuged at 400 × g for 5 minutes. The cell pellet was washed twice in 0.83% NH₄Cl to remove residual red blood cells and then resuspended in HBSS. The suspension was stored on ice before use. At the time of testing, no cells were “older” than three hours and the experimental schedule was organized so that the same time interval elapsed between blood sampling and testing whether the neutrophils were collected from resting subjects or after exercise. The purity of the cell preparation, checked by differential staining with Harclo “diff-quik” (Lab-Aids, Sydney, N. S. W.), was found consistently to be greater than 95% neutrophils. Viability checked by the ability of the cells to exclude trypan blue was found to be
always greater than 95%. The total neutrophil count was determined with a semi-automated Coulter Counter (Coulter Electronics, Hialeah, FL). Total white blood cells and differential counts of whole blood were determined using the same techniques.

Chemiluminescence

The production of reactive oxygen species by $10^5$ neutrophils in response to stimulation with opsonized zymosan particles in the concentration range 5–500 particles/cell was estimated by measuring the chemiluminescence generated by the co-oxidation of luminol by the hydrogen peroxide and hypochlorous acid produced by the cells; it is a measure of "true phagocytosis" since myeloperoxidase activity requires azurophilic degranulation (1). The production of reactive oxygen is an essential prerequisite for chemiluminescence to take place (2). This method exceeds the sensitivity of both spectrophotometric and fluorometric techniques by several orders of magnitude (10, 41). Chemiluminescence was measured in a 1251 Luminometer (LKB Wallac, Turku, Finland) at 37 °C with continuous stirring of the reaction mixture (25). Neutrophils suspended in HBSS were added to polystyrene luminometer tubes (Clinicon 2174-086, Turku, Finland) containing 900 µl of HBSS and luminol (113 µM). Before stimulation, the reaction mixture was preincubated for five min to determine baseline chemiluminescence. The stimulus (i.e. opsonized zymosan) was then added to give a total volume of 1.0 ml. The chemiluminescent signal (expressed in millivolts (mV)) was recorded continuously up to peak velocity on an LKB 2210 potentiometric chart recorder (LKB-produkter AB, Bromma, Sweden). Tests for the loss of neutrophil oxygenation activity over the time interval taken to complete each opsonized zymosan titration showed no significant change. To test for plasma "pyrogenic" activity, 10^6 neutrophils isolated from rested subjects (i.e. prior to exercise) were preincubated for 30 minutes at 37 °C with 40 µl of plasma isolated from either rested or exercised subjects before being stimulated with opsonized zymosan at a single concentration (i.e. 50 particles/cell). This procedure was adopted because some "pyrogens" (e.g. tumour necrosis factor) take 30 minutes to bind maximally to neutrophil receptors (43), and this concentration of plasma produced the largest response.

Statistical analysis

Wilcoxon’s sum rank test (30) and Student’s t-test were used to assess the significance of differences attributable to either training or acute exercise (i.e. untrained/trained; rested/exercised; rested/six hours post exercise; and exercised/six hours post exercise). Non-parametric methods were used to check the values arrived at using Student’s t-test because the values of k did not appear to be distributed normally. Significance levels, however, agreed quite closely in most cases. Where data are presented as means±SEM, the significance of the differences claimed are the larger of those obtained from the two tests.

Results

The exercise test

All of the trained individuals performed the one hour workload at 60% VO2max with relative ease, as reflected in a low stable heart rate that remained relatively unchanged throughout the test; the majority of the untrained individuals, however, reported some subjective difficulty in accomplishing the task, which was reflected in a steadily rising (and significantly higher) heart rate.

Exercise-induced changes in neutrophil oxygenation activity

The responsiveness of neutrophils isolated from the blood of exercised individuals (whether trained or untrained) to opsonized zymosan – as judged by comparing their luminol-amplified chemiluminescence with that of neutrophils obtained from the same subjects at rest – was nearly always enhanced. There were no changes in baseline chemiluminescence. Fig. 1 (a) shows the time course of a typical response of neutrophils isolated from an exercised subject, indicating that the enhancement induced by exercise (initially two-fold) was still evident six hours later, although somewhat reduced in magnitude. For some individuals the response at six hours was either slightly larger or similar to that measured.
immediately after the test (data not shown). Fig. 1 (a) also shows that the kinetics of the chemiluminescent response to opsonized zymosan consisted of a characteristic lag phase followed by a phase of acceleration that tapered off slowly before peak velocity was reached; this was followed by a slow phase of deceleration to zero (not shown). The time taken to reach the peak velocity is known as the nodal time as it separates the accelerative and decelerative phases (1).

Fig. 1 (b) shows that, at peak velocity, the enhanced activity of the cells from exercised subjects was sustained over the complete range of opsonized zymosan concentrations tested (i.e. 5–500 particles/cell). Transformation of the data according to the method of Allen (1) (Fig. 2) revealed that the values calculated for: (a) peak velocity, (c) maximum rate of acceleration and (d) nodal times for both resting and independently of the trained state and at each concentration of opsonized zymosan – a consistent increase in peak velocity (k1) to well above the resting values; and (ii) the trained group showed – independently of the effect of exercise – a significant depression of the neutrophil response (Fig. 3 b). Fig. 4 and Table 1 summarize the mean values of each descriptive parameter arrived at according to the transformation of the data described in Fig. 2. The following conclusions can be drawn from the values reported in both Fig. 4 and Table 1.

(i) One hour of moderate aerobic exercise was responsible for "priming" neutrophils so that, upon isolation and stimulation, they formed reactive oxygen species at an elevated rate (P < 0.01) quite independently of the trained or untrained state of the subject; this was true whether the stimulus (opsonized zymosan) was applied at a low concentration (at a single particle/cell) or at saturation (i.e. at 500 particles/cell). The increase in peak velocity (k1) (Fig. 4 a) was always accompanied by an increase in the maximum rate of acceleration (k2) (Table 1). In the majority of cases, neutrophils isolated from exercised subjects reached the peak velocity faster (i.e. with a decreased nodal time (k3) (Table 1)) than those from their resting counterparts. For all parameters measured in Fig. 2, the episode of exercise did not alter the order of the reaction as reflected in the parallel slopes of the lines in each data set (Fig. 4 d). No significant changes were observed after exercise in the number of particles/cell required to generate chemiluminescence at half of the maximum rate (i.e. Km) (Fig. 4 c).

(ii) Whilst training had no significant effect on the maximum rate of reactive oxygen production (Vmax) by stimulated neutrophils from rested or exercised subjects (Fig. 4 b), it did cause reproducible changes in most of the descriptive parameters represented in Fig. 4 and Table 1. In particular, when extrapolated to unit stimulus concentration, the peak velocity (k1) of the response of neutrophils isolated from trained subjects was strongly depressed (P < 0.075) compared with the responses of cells from untrained controls both at rest

As the non-linear response of peak velocity to particle concentration (Fig. 1 b) is formally analogous to an enzyme-substrate reaction conforming to Michaelis-Menten kinetics (1), these data were also analysed by Lineweaver-Burk analysis (Fig. 2 b). These plots generated values descriptive of: (i) the maximum rate of reactive oxygen formation at saturating particle concentration (Vmax); and (ii) the particle concentration required to reach half of this maximum rate (Km).

In both the trained and untrained groups, the absolute values obtained from the kinetic analysis showed considerable individual variation (Fig. 3 a, b). Despite this: (i) individual responses to exercise showed – independently of the trained state and at each concentration of opsonized zymosan – a consistent increase in peak velocity (k1) to well above the resting values; and (ii) the trained group showed – independently of the effect of exercise – a significant depression of the neutrophil response (Fig. 3 b). Fig. 4 and Table 1 summarize the mean values of each descriptive parameter arrived at according to the transformation of the data described in Fig. 2. The following conclusions can be drawn from the values reported in both Fig. 4 and Table 1.

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![Graph](image)

**Fig. 3** To illustrate the variability of individual responses to exercise within and between the (a) trained ( ) and (b) untrained ( ) groups, individual values of \( k_1 \) (designated A–K for the trained and 1–9 for the untrained subjects) are displayed for the resting, exercised and six hour post-exercised states. The 'box' by each data set contains the middle 50% of the values where half lie above the median (at the point of indentation) and half below it, and the dashed lines represent the upper and lower 25% extremities (23). Significant differences (Wilcoxon test): (P < 0.01) due to exercise ( ); and (P < 0.075) due to training ( ).

**Fig. 4** Effect of exercise and training on the mean kinetic constants of neutrophil peak chemiluminescence (Mean values ± SEM) determined from the summarized data for the untrained ( ) and trained ( ) groups. Mean values are shown for: (a) Peak velocity at unit particle concentration \( (k_1) \); (b) Peak velocity at saturating particle concentration \( (V_{max}) \); (c) Number of particles/cell required to produce \( V_{max}/2 \) \( (K_m) \); and (d) Order of the reaction with respect to stimulus concentration \( (S_1) \). Significant differences (Student's t test): (P < 0.05) due to exercise ( ) and training ( ).

and after exercise (Fig. 4). In contrast, the order of the reaction \( (S_1) \) with cells from trained individuals was higher, approaching first order, consistent with the fact that the depressed activity observed at unit stimulus concentration rose much more steeply as the particle concentration approached saturation. Whilst training did not alter, significantly, either the intercept \( (k_2) \) and slope \( (S_2) \) values for the maximum rate of acceleration, it did reduce the nodal time \( (k_3) \) and allowed cells from trained individuals to reach, irrespective of exercise, their lower peak velocities more quickly (Table 1).

In all of the experiments reported, the exercise test was performed in the morning (i.e. 9.30–10.30 am) to take advantage of the overnight rest. To eliminate the possible influence of circadian variation in neutrophil oxygenation activity, the experiment was repeated on some subjects in the after-
Table 1 Effect of exercise and training on the mean kinetic constants§ of neutrophil chemiluminescence (rate and nodal time) determined from the summarized data for each experimental group

<table>
<thead>
<tr>
<th></th>
<th>Untrained</th>
<th>Trained</th>
<th>Untrained</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_2$</td>
<td>9.8±1.8</td>
<td>0.73±0.014</td>
<td>8.5±1.9</td>
<td>0.79±1.8</td>
</tr>
<tr>
<td>$S_2$</td>
<td>14.5±1.20</td>
<td>0.010±0.023</td>
<td>12.2±0.56</td>
<td>0.008±0.012</td>
</tr>
<tr>
<td>Nodal Times</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_3$</td>
<td>15.5±2.3</td>
<td>0.75±0.02</td>
<td>14.7±4.3</td>
<td>0.81±0.04</td>
</tr>
<tr>
<td>$S_3$</td>
<td>13.9±1.10</td>
<td>0.010±0.023</td>
<td>10.8±0.42</td>
<td>0.007±0.009</td>
</tr>
</tbody>
</table>

§Mean values (±SEM) of the kinetic constants for both rates and nodal times determined for each individual subject were assessed for statistically significant differences, as described in the Methods section. Significant differences (Student’s t test) from rest (P < 0.05); and untrained (P < 0.05).

Table 2 Effect of exercise and training on the number of circulating white blood cells (WBC)§ before and after exercise

<table>
<thead>
<tr>
<th></th>
<th>Untrained</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>6.53±0.36</td>
<td>6.00±0.37</td>
</tr>
<tr>
<td>Exercise</td>
<td>9.60±0.61</td>
<td>7.70±0.41</td>
</tr>
<tr>
<td>P. E. (6 h)</td>
<td>9.50±0.46</td>
<td>8.62±0.54</td>
</tr>
</tbody>
</table>

§Mean values ([× 10⁶ WBCs/mL±SEM]) of individual white blood cell counts in whole blood were assessed for statistically significant differences, as described in the Methods section. Significant differences (Student’s t test) from rest (P < 0.05); and untrained (P < 0.05).

noon (i.e. 3.30–4.30 pm). The effect of exercise on all parameters measured occurred irrespective of the time of day.

White blood cell counts

Exercise of all individuals, whether trained or untrained, caused the expected increase in the total number of circulating white blood cells (WBC). This “demargination” was substantially greater in the untrained group (P < 0.05) although, quite independently of training, circulating white blood cell numbers (in whole blood) remained well above the resting values six hours after exercise (Table 2). The relative proportions of each white blood cell type did not change significantly in response to exercise in either the untrained or trained group (data not shown) nor was there any significant elevation of immature (i.e. non-segmented) neutrophils.

Plasma “pyrogen” activity

When neutrophils isolated from resting subjects (trained or untrained) were preincubated for 30 minutes with plasma obtained either before or after exercise, no significant difference in their zymosan-stimulated oxygenation activity was induced by the presence of “exercised” plasma (Fig. 5).

Discussion

The results demonstrate, firstly, that, irrespective of training, physical exertion sufficient to elevate circulating pyrogenic activity enhances or “primes”, for up to six hours, the capacity of circulating neutrophils to produce microbicidal reactive oxygen species upon stimulation. Secondly, they show that endurance training depresses neutrophil oxygenation activity (compared to untrained subjects) at low-stimulus concentration only – irrespective of exercise effects. The potential of the neutrophil population to kill foreign pathogens – particularly in the low, physiologically-significant concentration range – is increased substantially by acute aerobic exercise, yet depressed, chronically, by intensive training; this may increase the susceptibility of trained individuals to common infections. While the critical threshold of neutrophil oxidative capacity that limits cytotoxicity is not known, a correlation between the intensity of reactive oxygen formation and “killing capacity” is expected since patients with severe impairments in this pathway (e.g. chronic granulomatous disease) suffer from recurrent infections (14). Macrophage chemotactic and phagocytic activities also increase after strenuous exercise and they are attenuated by endurance training (21, 36).
Basal neutrophil bactericidal activity (measured as a percentage of viable intracellular bacteria), but not phagocytosis, has also been shown to be lower in trained than in untrained subjects before exercise (32). This difference was exaggerated following maximal physical exertion, which significantly depressed the bactericidal activity of cells from trained but not from untrained subjects. Whilst the authors suggested that a deficiency of hydrolytic enzymes might have lowered bactericidal activity, our results suggest that a reduced capacity to produce microbicidal oxidants may be a sufficient explanation. Myeloperoxidase activity appears to be important in preventing bacterial growth within the neutrophil; its release may be an important prerequisite that enables the efficient killing and degradation of ingested micro-organisms (33). Furthermore, the involvement of chlorinated oxidants in neutrophil-mediated tissue injury is related to their ability to sustain the activities of released proteases (52); it is not yet known whether the same interplay between oxidative and non-oxidative mechanisms is involved in neutrophil microbicidal activity.

There is a number of possible explanations for the observed alterations in neutrophil oxygenation activity. A reduction in the antioxidant status of blood cells after exercise might be anticipated as a result of the oxidative stress imposed by the large throughput of oxygen to meet the demands of exercising muscles. However, neutrophils have a considerable capacity to maintain antioxidants in the reduced state during phagocytosis (48) and those expressing a larger chemiluminescent signal purely because of reduced antioxidant protection would be, in fact, more vulnerable to irreversible oxidative damage during phagocytosis, perhaps leading to dysfunction or cell death (35) in spite of their apparently greater microbicidal activity.

Quite independent of antioxidant status, exercise-induced enhancement of neutrophil oxygenation activity is more likely to be due to cytokines released into the circulation during exercise (i.e. pyrogens or other neutrophil priming agents) or, more indirectly, to the selective release from isolated vascular pools ("demargination") of a subpopulation of neutrophils with intrinsically higher activity. Neutrophil heterogeneity has been identified by various parameters (22) and a subpopulation of "primed" neutrophils has been observed in the blood of patients throughout an acute bacterial infection (4). Our recent finding (J. A. Smith, B. Gray, R. D. Telford and M. J. Weidemann, unpublished observations) that one minute of cycling at maximal effort depresses the capacity of neutrophils to produce reactive oxygen in spite of significant "demargination" excludes the possibility that "priming" can be attributed to an increase in the percentage of "demarginated" neutrophils possessing higher oxidative capacity.

The endogenous pyrogens interleukin-1 (IL-1) (45), tumour necrosis factor (TNF) (28) and granulocyte-macrophage colony stimulating factor (GM-CSF) (51) all enhance neutrophil oxygenation activity and degranulation in vitro. TNF is the most potent of these and recent experiments in this laboratory (J. A. Smith, G. Chaudhri and M. J. Weidemann, unpublished observations) have shown, using a specific enzyme-linked immunosorbent assay, that its concentration in human blood increases two-to three-fold (n=5) after exercise for one hour at 60% \( VO_2 \text{max} \). In addition, IL-1, which also increases (13), stimulates T-cells to synthesize and release interleukin-2 (IL-2). Interleukin-2 also activates neutrophil oxygenation activity and degranulation in vitro (29), and the ability of mitogen-stimulated T-cells to synthesize and secrete this cytokine in vitro also increases after an identical workload (44). Synergism between these cytokines sufficient to overcome the effects of endogenous inhibitors of IL-1 activity (46) would provide a mechanism for amplifying IL-1-induced priming. Our failure to reproduce in vitro the "priming" effect of exercise (Fig. 5) is similar to the negative result obtained when plasma isolated from human febrile patients was co-incubated with control cells (3). Thus, neutrophil "priming" either involves a critical step restricted to the circulatory environment, perhaps requiring endothelial cell-neutrophil interaction (54), or its development in vitro is masked by pyrogen inhibitors or plasma antioxidants.

Neutrophil microbicidal activity can be influenced – both positively and negatively – by a diverse range of mediators including adenalin (7), neuropeptides like substance P (42) and \( \beta \)-endorphin (15) as well as various eicosanoids. Thus, the molecular basis for exercise-induced "priming" in vivo is likely to be complex, involving the modification of one or more cellular parameters (27):

(i) The number and/or affinity of neutrophil plasma membrane receptors for activating cytokines (e.g. IL-1) or opsonins (e.g. complement factor 3b (C3b)) may change. TNF, for example, increases C3b receptor expression on neutrophils (5) and leukocyte cytotoxic activity increases in parallel with enhanced C3b receptor expression after an exercise-induced asthma attack (38). Since the effect of intensive training manifests as a depression of neutrophil oxygenation activity at low stimulus concentration (Fig. 4 a), cells from trained individuals behave as if they have a reduced affinity for opsonins. This is reflected in their tendency to require a higher number of particles/cell to produce half-maximal stimulation (Fig. 4 c). The simultaneous increase in the reaction order ensures that this defect is eliminated when the stimulus concentration reaches saturation.

(ii) Signal transduction may be influenced negatively by endogenous glucocorticoids that inhibit both IL-1 (6) and TNF activity (49). Elevated corticosteroid levels associated with the insistent energy demands of intense training (9) may also inhibit, indirectly, phospholipase A2, thereby suppressing a primary source of eicosanoids (e.g. prostaglandins, leukotrienes) and platelet activating factor (50) that are known to "prime" neutrophil oxygenation activity in vitro (16).

(iii) The catalytic efficiency and/or content of the enzyme(s) involved in producing oxidants in the cell may change. The exercise-induced increase in \( V_{\text{max}} \) (Fig. 4 b) may reflect a parallel increase in the \( V_{\text{max}} \) of one of the components involved in reactive oxygen formation (e.g. NADPH oxidase, cytochrome \( \text{b}_245 \) or myeloperoxidase). The plasma concentration of lipopolysaccharide (endotoxin), itself a primary stimulus of pyrogen activity, increases significantly after strenuous exercise (8); endotoxin may also "prime" neutrophils directly by increasing the \( V_{\text{max}} \) of the NADPH oxidase in the absence of any alteration in its \( K_m \) (24). The individual variation in the magnitude of the neutrophil response...
to exercise and training (Fig. 3 a, b) may have its origin in the extent to which endotoxin "leakage" from the gut (itself likely to be highly variable) is neutralized by circulating anti-lipopolysaccharide immunoglobulin (8).

Whilst an increase in neutrophil oxygenation activity may be advantageous immunologically, it may also have a detrimental effect if tissues infiltrated with "primed" neutrophils are subjected to tissue-damaging oxidants and enzymes in response to circulating particles (e.g. cellular debris) or immune complexes that are elevated transiently after prolonged running (18, 19). The presence of elastase in plasma increases about 180% in runners after a ten-kilometre race (40), most likely due to neutrophil degranulation in vivo, although mechanical rupture of neutrophils or release of elastase from other sources cannot be excluded. No significant neutrophil damage was observed in blood smears examined during the present work, possibly because cycling is atraumatic compared with running. The partial suppression of neutrophil oxygenation activity at low stimulus concentration in trained subjects may reduce exercise-induced inflammatory damage by limiting neutrophil stimulation by "auto-antigens" present in cellular debris. This interpretation is supported by the work of Lewicki et al. (32), which showed a significant increase in the plasma concentration of \( \beta \)-glucuronidase (a "marker" of azurophilic degranulation) in untrained but not in trained human subjects. Stimulated, as opposed to "primed". neutrophils have been implicated in several pathological conditions including exercise-induced asthma (38) and local tissue inflammatory damage associated with muscle injury and other forms of trauma (20). Exercise and training-induced changes in "immune cell" activity may also contribute significantly to degenerative and regenerative processes in the skeletal muscular system (36).

The most obvious practical conclusion to be drawn from this work is that moderate aerobic exercise is more likely to have a beneficial than a deleterious effect on the phagocytic system. Because neutrophils are involved in both immunity and inflammation, changes in their activity may provide a sensitive "marker" for overtraining. In the case of highly-trained athletes, where there is already evidence for increased susceptibility to common infections during periods when training is "tapered" suddenly (e.g. just before a major competition), it would seem advisable to avoid sudden reduction or cessation of the training program in order to maintain optimum neutrophil activity. Depressed activity in the absence of the regular "priming" contributed by moderate daily exercise could lead to a net depression (below a critical threshold) of the potential microbicidal response. Further work may provide useful input into the design of exercise programs that optimize training regimes for elite athletes (to prevent over-training or excessive tapering) and provide a secure framework within which normally inactive people are able to exercise to improve their general health.

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Exercise, Training and Neutrophil Microbicidal Activity


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CHAPTER 2.2

Further characterization of the neutrophil oxidative burst by flow cytometry
Short communication

Further characterization of the neutrophil oxidative burst by flow cytometry

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The ability to generate reactive oxygen species is essential for neutrophils to kill infectious microorganisms. We have investigated the 'oxidative burst' in human neutrophils stimulated in vitro with either opsonized zymosan (OZ) or phorbol myristate acetate (PMA) at the single cell level by flow cytometry using dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR) as oxidative probes. These responses have been compared to results obtained with chemiluminescence and spectrophotometric assays. DHR was the most sensitive oxidative probe and PMA the more potent stimulus. The magnitude of the intracellular fluorescence generated with both probes was increased substantially by inhibiting the activities of H$_2$O$_2$-consuming enzymes with a relatively low concentration of azide, which also influenced the distribution of neutrophil subpopulations that expressed fluorescence differentially. Positive correlations between the DCFH-DA and DHR assays were found with both stimuli, and between these assays and ferricytochrome c reduction with OZ-stimulated cells only. We conclude that the DHR flow cytometric assay is the most sensitive technique available for investigating changes that may occur in the oxidative activities and distribution patterns of active neutrophil subpopulations in response to a variety of physiological and pathological conditions.

Key words: Azide; Dichlorofluorescein diacetate; Dihydrorhodamine 123; Flow cytometry; Granulocyte; Reactive oxygen species

Introduction

Stimulation of isolated neutrophils with either phagocytic or soluble stimuli in vitro activates the 'oxidative burst'. This pathway is a crucial arm of the neutrophils' microbicidal activity but it has also been implicated in inflammatory damage to host tissues. The oxidative burst begins with the production of superoxide by a reaction catalysed by NADPH oxidase. Processes distal to this reaction involve the formation of H$_2$O$_2$ (which is catalysed by superoxide dismutase) and the release of myeloperoxidase from the azurophilic granules (Morel et al., 1991). Myeloperoxidase catalyses a reaction between H$_2$O$_2$ and Cl$^-$ ions which generates highly microbicidal chlorinated oxidants (Morel et al., 1991).

The production of reactive oxygen species (ROS) by stimulated neutrophils can be detected...
using a variety of spectrophotometric, fluorimetric and chemiluminescent techniques which measure the collective oxidative response of the cell suspension (Weber, 1990). It can also be detected at the single cell level by flow cytometry using probes that become fluorescent upon oxidation by ROS such as superoxide (Rothe and Valet, 1990) or \( \mathrm{H}_2\mathrm{O}_2 \) (Bass et al., 1983). However, these probes must compete with enzymes and endogenous antioxidants that reduce the concentration of ROS so that the fluorescence intensity is not necessarily directly proportional to the production of ROS.

In this communication we compare directly two flow cytometric probes (2,7-dichlorofluorescein diacetate and dihydrorhodamine 123) which are used routinely to assess the production of intracellular ROS in neutrophils, and describe how the inhibition of myeloperoxidase and catalase activities with azide influences fluorescence intensity and the distribution of responsive neutrophil subpopulations in cells stimulated in vitro with either opsonized zymosan (OZ) or phorbol myristate acetate (PMA). Furthermore, we have compared the DHR and DCFH-DA responses with measurements of the oxidative burst made by luminol-amplified chemiluminescence and ferricytochrome c reduction.

Materials and methods

Materials

Ferricytochrome c, formyl-methionyl-leucyl-phenylalanine (fMLP), luminol, PMA, superoxide dismutase (SOD) and zymosan A were purchased from Sigma (St. Louis, MO, USA). Ficoll-Paque was obtained from ICN Pharmacia (Upsala, Sweden), Mono-poly resolving medium from ICN Biomedicals (Sydney, Australia), 2,7-dichlorofluorescein diacetate (DCFH-DA) from Serva (Heidelberg, Germany), dihydrorhodamine 123 (DHR) from Molecular Probes (Junction City, OR, USA) and sodium azide from Ajax Chemicals (Sydney, Australia). The stock solutions of DCFH-DA and DHR were prepared by the methods described by Bass et al. (1983) and Rothe et al. (1991). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were prepared by conventional techniques. Luminol and OZ were prepared as described previously (Smith et al., 1990). PMA was dissolved initially in dimethyl sulphoxide to a concentration of 1 mM and further dilutions were made with HBSS. Ferricytochrome c and SOD were dissolved in HBSS.

Neutrophil isolation procedure

Blood samples were taken from the antecubital veins of eight healthy male volunteers on six separate occasions. Neutrophils (granulocytes) were isolated from heparinized blood by the following procedure (Kalamar et al., 1988). Ficoll-Paque (1 ml) was layered on top of Mono-poly resolving medium (3 ml) in a sterile tube. Whole blood (5 ml) was then layered on top of this cushion. The tubes were centrifuged for 30 min (room temperature) at 600 \( \times \) g. The neutrophil layer was aspirated and transferred to another sterile tube. Contaminating erythrocytes were removed by hypotonic lysis (30 s in ice-cold distilled water). Isotonicity was restored immediately by adding double-strength PBS. This step was performed twice. The cells were pelleted by centrifugation for 5 min (4°C) at 500 \( \times \) g between each wash. The cells were finally washed with PBS and resuspended in HBSS containing 5 mM D-glucose. The cell count was determined with an automated JT haematology analyser (Coulter Electronics, Hialeah, FL, USA). Neutrophil viability and purity, which were assessed by the ability of the cells to exclude trypan blue and on the basis of flow cytometric forward and right angle light scatter dot-plot profiles, were always found to be greater than 90%. This purification procedure has been reported recently to induce minimal functional changes to neutrophils (Watson et al., 1992).

Flow cytometric assays

The intracellular production of ROS in stimulated neutrophils was quantified in individual cells by flow cytometry using the procedures reported by Bass and colleagues (1983) and Rothe et al. (1991). Neutrophils \( (5 \times 10^5) \) were suspended in a microfuge tube containing HBSS ( + 5 mM D-glucose) and either 10 \( \mu \)M DCFH-DA or 1 \( \mu \)M DHR. The suspension was incubated with gentle horizontal agitation for either 20 min (DCFH-DA)
or 5 min (DHR) at 37°C before addition of the stimulus (either 200 OZ particles/cell or 100 nM PMA) at time zero to give a total volume of 1.0 ml. Sodium azide (100 µM) was added to some of the tubes immediately before the stimulus. Control samples containing no stimulus were run in parallel; they were used to set the negative and positive regions for the analysis. The conversion of DCFH-DA and DHR to their fluorescent derivatives (i.e., 2',7'-dichlorofluorescein and rhodamine 123 (Bass et al., 1983; Rothe et al., 1991)) was assessed in aliquots which were taken 20 and 40 min after stimulation and stored on ice before analysis. Flow cytometric analysis was performed on 5000 cells using an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA). OZ particles, which were not autofluorescent, were excluded by setting a narrow gate around the neutrophil population.

Other assays
Luminol-amplified chemiluminescence was measured continuously as described previously (Smith et al., 1990), and ferricytochrome c reduction was used to measure the extracellular release of superoxide. Neutrophils (1 x 10⁶) were added to HBSS containing 5 mM D-glucose and 50 µM ferricytochrome c (±0.2 mg SOD). The tubes were preincubated for 5 min at 37°C and the stimulus (either 100 OZ particles/cell or 100 nM PMA) was added at time zero to give a total volume of 1.0 ml. The tubes were agitated horizontally for 10 min at 37°C and placed on ice. The supernatants were collected after centrifugation for 30 s at 8000 rpm in a microcentrifuge. The reduction of ferricytochrome c was measured spectrophotometrically at 550 nm using the supernatants containing SOD as blanks (Metcalf et al., 1986).

Fig. 1. Typical profiles of rhodamine 123 fluorescence in stimulated neutrophils. The cells were stimulated with either OZ (a, b) or PMA (c, d) in the absence (a, c) or presence (b, d) of azide under the conditions described in the materials and methods section. Intracellular fluorescence was measured by flow cytometry and is plotted on a log₁₀ scale from channel numbers 0.1 to 1023 on the abscissa. The count on the ordinate represents the number of cells in each channel. The lower analysis region in each plot represents the cells emitting fluorescence above that of the control (unstimulated) sample while the other analysis regions were used to calculate the mean fluorescence per cell and the percentage of cells within each neutrophil subpopulation.
Data analysis

Measurements from 48 preparations of neutrophils were pooled for data analysis. Correlations between different variables were calculated with an appropriate software package (Cricket Graph) on an Apple Macintosh computer.

Results

In agreement with the brief report of Rothe and colleagues (1991), we found that DHR was a much more sensitive ROS-detection probe than DCFH-DA, particularly when a relatively low concentration of azide (100 µM) was added to the incubation medium (see below). Furthermore, there was only negligible autofluorescence in control (non-stimulated) samples incubated with DHR compared to the high background levels found with DCFH-DA (Figs. 1 and 2). The mean fluorescence intensity per responding neutrophil (i.e., mean channel number) detected with PMA-stimulated cells was much greater than the values found when the cells were stimulated with OZ, particularly in the DHR assay (Tables I and II). In contrast, only weak responses were obtained with fMLP (1 µM), and no measurable responses were found with unopsonized zymosan (200 particles/cell) with either DHR or DCFH-DA (n = 5, data not shown). There was a strong positive correlation between the DHR and DCFH-DA assays with OZ-stimulated cells (r = 0.63) but only a weak positive correlation between the two probes when the cells were stimulated with PMA (r = 0.34).

Dihydrorhodamine

Fig. 1 shows, using typical green fluorescence histograms, that two distinct 'subpopulations' of responding neutrophils were detected when the cells were stimulated with OZ or PMA and DHR was used as the ROS-detection probe. There was some intra- (day-to-day) and considerable inter-individual variation in the magnitude of the mean fluorescent responses obtained with both stimuli, but significant positive correlations were found between the responses to PMA and OZ (r = 0.52). The mean channel numbers were substantially

![Fig. 2. Typical profiles of 2',7'-dichlorofluorescein fluorescence in stimulated neutrophils. The presentation of the profiles is identical to those described in the legend to Fig. 1. The lower analysis region represents the cells emitting fluorescence above the value of the control.](image)

larger 40 min after activation in cells stimulated with either OZ or PMA than they were after 20 min (Tables I and II).

Azide-mediated inhibition of MPO and catalase activities, which effectively blocks the consumption of H\textsubscript{2}O\textsubscript{2} by these enzymes (Wymann et al., 1987), increased the magnitude of the intracellular fluorescence by an average of five-fold in cells stimulated with OZ and three-fold in cells stimulated with PMA (Tables I and II). Furthermore, in some cases, three distinct subpopulations of responsive neutrophils were detected in OZ-stimulated cells (Fig. 1b) or, if only two subpopulations were present, they were separated clearly by one to two orders of magnitude. Azide treatment did not alter the pattern of the response in cells stimulated with PMA (Fig. 1d), but it did, occasionally, merge the two distinct subpopulations into a relatively homogeneous single population. Strong positive correlations were found between the responses of azide-treated and untreated cells to both stimuli ($r = 0.71$).

### TABLE I

**NEUTROPHIL MICROBICIDAL ACTIVITY ACTIVATED BY OPSONIZED ZYMOSAN**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DCFH-DA</th>
<th>DHR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(- azide)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% active neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>86.6±29.1</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>97.7±24.6</td>
<td></td>
</tr>
<tr>
<td>Mean channel number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>5.21±4.30</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>7.67±5.42</td>
<td></td>
</tr>
<tr>
<td><strong>(+ azide)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% active neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>88.7±26.9</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>98.6±10.2</td>
<td></td>
</tr>
<tr>
<td>Mean channel number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>26.4±28.8</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>36.3±30.7</td>
<td></td>
</tr>
</tbody>
</table>

* Results are mean±SD. The percentage of active cells are those producing fluorescence above the control value, which was always less than 2% of the positive cells. The mean channel number indicates the fluorescence intensity on a log\textsubscript{10} scale from 0.1 to 1023.

### TABLE II

**NEUTROPHIL MICROBICIDAL ACTIVITY ACTIVATED BY PHORBOL MYRISTATE ACETATE**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DCFH-DA</th>
<th>DHR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(- azide)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% active neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>100±0.00</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>100±0.00</td>
<td></td>
</tr>
<tr>
<td>Mean channel number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>30.9±22.3</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>68.1±56.0</td>
<td></td>
</tr>
<tr>
<td><strong>(+ azide)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% active neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>95.1±6.84</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>100±0.00</td>
<td></td>
</tr>
<tr>
<td>Mean channel number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 min)</td>
<td>130±83.0</td>
<td></td>
</tr>
<tr>
<td>(40 min)</td>
<td>180±113</td>
<td></td>
</tr>
</tbody>
</table>

* Results are mean±SD. The percentage of active cells are those producing fluorescence above the control value, which was always less than 2% of the positive cells. The mean channel number indicates the fluorescence intensity on a log\textsubscript{10} scale from 0.1 to 1023.

2,7-dichlorofluorescein diacetate

Distinct subpopulations of neutrophils were also found when cells were incubated with DCFH-DA but these usually consisted of an active (positive) and inactive (negative) group rather than the separate active subpopulations identified with DHR. Only a small percentage of the OZ-stimulated neutrophils generated intracellular fluorescence in excess of the background level measured in unstimulated cells (Table I). This was in contrast to the much higher percentage exhibiting enhanced fluorescence when PMA was the stimulus (Table II). Inclusion of azide in the incubation medium substantially increased the percentage of positive cells as well as the magnitude of ROS formation per individual cell, irrespective of whether the cells were stimulated with OZ or PMA (Fig. 2; Tables I and II). Strong positive correlations were found between the responses of the azide-treated and untreated cells to both stimuli ($r = 0.77$). As shown with DHR, there was considerable intra- and inter-individual variation between the magnitude of the mean channel numbers with both stimuli, but signifi-
cant positive correlations were found between the responses to PMA and OZ \((r = 0.56)\). While PMA-stimulated neutrophils were always more oxidatively active than the cells stimulated with OZ, the magnitude of this difference was much larger when DHR was used as the probe (Tables I and II).

**Comparison of flow cytometric and other oxidative assays**

We also examined the relationship between the results obtained with DHR and DCFH-DA and those found when conventional assays were used to monitor the oxidative burst. Whilst a positive correlation was found between the reduction of ferricytochrome c and both DHR and DCFH-DA fluorescence in OZ-stimulated cells \((r = 0.64\) and \(r = 0.47\) respectively), there was no correlation with the peak response detected by luminol-amplified chemiluminescence and, furthermore, no correlations were found between any of the assays with PMA-stimulated cells. We also confirmed that azide, at the relatively low concentration employed \((100 \mu M)\), was inhibiting myeloperoxidase activity because luminol-amplified chemiluminescence (which is peroxidase-dependent (Allen, 1986)) was virtually abolished in its presence (data not shown). The levels of intra- and inter-individual variation observed in these assays were similar to those found with the flow cytometric methods (data not shown).

**Discussion**

These results confirm that DHR is a more sensitive flow cytometric probe for intracellular ROS detection than DCFH-DA (Rothe et al., 1991) and that azide-mediated inhibition of myeloperoxidase and catalase activities increases intracellular fluorescence in both PMA- and OZ-stimulated cells by 3–5-fold. The difference in the responses to OZ and PMA is likely to be due to the different mechanisms used by these stimuli to activate the oxidative burst: OZ is a phagocytic stimulus which binds to complement and immunoglobulin receptors on the cell surface which, in turn, activates NADPH oxidase and myeloperoxidase activities independently of protein kinase-C activity (Allen, 1986); in contrast, PMA stimulates NADPH oxidase indirectly by activating protein kinase C (Takahashi et al., 1991; Morel et al., 1991), but it is only a weak trigger of myeloperoxidase release (Allen, 1986). Therefore, the large increase in intracellular fluorescence detected in PMA-stimulated cells in the presence of azide may mainly reflect the inhibition of catalase activity. Our failure to detect positive correlations between the flow cytometric assays and luminol-amplified chemiluminescence is likely to be due to the dependence of the latter assay on peroxidase activity and the likelihood that it detects extracellular as well as intracellular ROS (Allen, 1986).

Whilst flow cytometric assays have been used to identify deficiencies in the oxidative burst pathway in cells isolated from individuals with chronic granulomatous disease (Rothe and Valet, 1990), they have further clinical and physiological applications. For example, the distribution of stimulus-responsive neutrophil subpopulations may be altered by infectious (Bass et al., 1986) and/or inflammatory diseases which cause a proportion of the circulating cells to shift to a primed (potentially more active) or more subdued state. Neutrophil microbicidal activity may be primed or suppressed by a variety of humoral mediators including cytokines (Steinbeck and Roth, 1989) and neuroendocrine hormones (Berczi, 1986) or, alternatively, it may become temporarily elevated if neutrophils become oxidatively active in vivo following stimulation with immune complexes (Robinson et al., 1992). Priming may have beneficial effects, if on the one hand, it enhances the killing of infectious agents but may become detrimental if it increases the potential of neutrophils to damage host tissues on the other. If neutrophils become fully activated in vivo, they may not respond to a second stimulus until a substantial refractory period has elapsed (Prasad et al., 1991). During this recovery period, a 'window of opportunity' may exist which allows infections to become established.

The sensitivity of the DHR assay may permit a reference range to be established which defines the limits of normal and abnormal neutrophil oxidative responses so that the clinical consequences of responses that are above or below this
range can be assessed. For example, our group has shown that neutrophils isolated from elite swimmers produced responses to OZ and PMA that were 2- and 4-fold lower, respectively, in the DHR assay, compared to the values detected in cells from untrained controls (D.B. Pyne, unpublished results); this result confirms our earlier work, using luminol-amplified chemiluminescence, which showed that the specific oxidative activities of neutrophils from trained cyclists were significantly lower than in cells from untrained controls (Smith et al., 1990). Neutrophil dysfunction, which may occur when oxidative activity falls below a critical threshold, may contribute to the susceptibility of athletes to respiratory infections inter alia during strenuous phases of their training programs (Heath et al., 1992).

Both DCFH-DA (Bass et al., 1986) and DHR (Rothe et al., 1991) have been reported to detect the generation of intracellular \( \text{H}_2\text{O}_2 \). Because azide substantially increased the fluorescence of cells incubated with either DCFH-DA and DHR, it is unlikely that peroxidase activity, as suggested by Rothe and Valet (1990), is involved in the oxidation of either probe. The enhancing effect of azide suggests that myeloperoxidase- and/or catalase-deficient cells may be identified as more active in these assays. The individual potentiating effects of selectively inhibiting myeloperoxidase and catalase activities were not investigated because specific inhibitors for each of these haem enzymes are not available. Furthermore, other reactive species may oxidise these probes. DCFH-DA, for example, has been reported to be oxidized by reactive nitrogen species (Rao et al., 1992). Thus, it is not surprising that the correlations between the fluorescence intensities of the two probes were not stronger, particularly in PMA-stimulated cells. In relation to the apparent non-specificity of these ROS-detecting probes, there has also been considerable debate as to the specificity of luminol, which is used widely in chemiluminescence assays; superoxide, \( \text{H}_2\text{O}_2 \), chlorinated oxidants (Allen, 1986; Takahashi et al., 1991) and reactive nitrogen species (Wang et al., 1991) have all been claimed to excite luminol. It is likely that DHR and DCFH-DA may be oxidized differentially by \( \text{H}_2\text{O}_2 \) when it is present in combination with other reactive species and, therefore, it is not surprising that no strong correlations were found between the relative intensities of the DHR assay and other assays used to measure the oxidative burst. The specificities of these oxidative probes may be investigated further by using specific inhibitors that can be trapped intracellularly or by using a chemical or cell-free system in combination with specific ROS-trapping reagents.

In conclusion, we have confirmed that DHR is a more sensitive probe than DCFH-DA for quantitating intracellular ROS generation and the distribution of stimulus-responsive neutrophil sub-populations in preparations isolated from peripheral blood. Furthermore, the inhibition of myeloperoxidase and catalase activities by a relatively low concentration of the haem inhibitor azide increases the magnitude of the fluorescence 3–5-fold. The combination of high sensitivity of the DHR assay and analysis of the responses at the single cell level by flow cytometry offers considerable advantages over conventional techniques for analysing neutrophil oxidative responses to a variety of acute and chronic physiological (e.g., exercise, psychological stress) and pathological conditions.

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References


CHAPTER 2.3

Mechanisms involved in exercise-induced priming of neutrophil microbicidal activity.

2. Cellular aspects
MECHANISMS INVOLVED IN EXERCISE-INDUCED PRIMING OF NEUTROPHIL MICROBICIDAL ACTIVITY.

1. CELLULAR ASPECTS

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ABSTRACT

Neutrophils constitute the "first-line-of defence" against infectious agents but have also been implicated in inflammatory tissue damage. We investigated the mechanisms involved in exercise-induced priming of \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) production by neutrophils \textit{in vitro} and whether this led to enhanced killing of bacteria. We also determined whether this pathway was affected by exercise at maximum activity acutely and by endurance training chronically. These studies were followed up by investigating the stimulus-specificity of priming using either receptor-dependent or soluble stimuli and by measuring the production of different reactive oxygen species using several independent assays including flow cytometry. Preliminary work showed that moderate exercise primed - but exercise at maximal effort attenuated - the specific activity of \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) production (\( k \)) in response to the phagocytic stimulus, opsonized zymosan (\( P < 0.05 \)). The depressive effect of athletic training was confirmed by both cross-sectional and longitudinal studies (\( P < 0.05 \)). Moderate exercise also amplified the phagocytic and bacterial killing capacities of the cells as determined by following a green (alive) to red (dead) transition of acridine orange-stained bacteria (\( P < 0.01 \)). Mechanistic studies showed that, despite some time-dependent variability in some neutrophil responses, moderate exercise increased both the expression of complement receptors by 20% (\( P = 0.045 \)) and the capacity of neutrophils to generate \( \text{H}_2\text{O}_2 \) intracellularly in response to stimulation with phorbol myristate acetate by three-fold (\( P = 0.025 \)). A 10% increase in the rate of \( \text{O}_2^- \) release extracellularly after stimulation with OZ (\( P = 0.012 \)), as well as a two-fold increase in the plasma concentration of complexed elastase (\( P = 0.003 \)), were also found after exercise. Overall, the results suggest that the intensity-dependent effects of exercise on neutrophils may involve a shift in the proportion of low- to high-activity cells, which may due to priming of some cells and activation of others, the net response reflecting a balance between the two states. This may explain the variable but "real" intensity-dependent responses of neutrophils to exercise and offers insights as to why the effects of exercise on immunity has been a highly-controversial field. This work indicates that the controversy may now be resolved.
INTRODUCTION

Epidemiological evidence and studies on specific cellular and humoral immune responses indicate that, while moderate exercise has beneficial effects on the immune system, the vigorous training programs undertaken by endurance athletes are potentially immunosuppressive [reviewed by Smith and Weidemann, (1990)]. Neutrophils - which represent 50-60% of the total circulating white blood cell population - constitute the "first-line-of-defence" against infectious agents. Paradoxically, these phagocytes have also been implicated in the pathology of various inflammatory diseases such as rheumatoid arthritis and the adult respiratory distress syndrome (Martin et al., 1991). The importance of neutrophils in host defence is demonstrated clearly in patients with inherited defects in their microbicidal armoury, as these people suffer from recurrent infections (Gallin et al., 1991). Our initial work showed that moderate exercise may enhance the microbicidal activity of neutrophils because the oxidative burst of human neutrophils (stimulated with opsonized zymosan particles in vitro) was primed significantly, for up to six hours, following one hour of cycling at 60% of maximal aerobic capacity (VO_{2 max}) (Smith et al., 1990). Priming is a state of pre-activation in dormant cells that enables a more powerful response to be generated upon activation. It may involve transformation from a non-responsive to a responsive state.

The biochemical mechanisms that regulate neutrophil microbicidal responses are extremely complex. Phagocytosis begins with attachment of the target to the cell surface via opsonins (antibody or complement components) which interact with complimentary receptors on the neutrophil plasma membrane. Neutrophil microbicidal mechanisms, which are activated simultaneously with phagocytosis, consist of both oxygen-dependent and oxygen-independent processes. Activation of the neutrophil "oxidative burst" is accomplished by transmembrane signalling events involving phospholipid hydrolysis which mobilize endogenous Ca^{2+} (triggered by inositol triphosphate) and lead to the phosphorylation of transmembrane protein(s) catalysed by protein kinase-C. This process culminates in the delivery of single electrons originating from NADPH to the cell surface where they reduce O_{2} to the superoxide anion (O_{2}^{-}) in a reaction catalysed by the NADPH oxidase complex (reviewed by Morel et al., 1991). Processes distal to this reaction involve the formation of H_{2}O_{2}, which is catalysed by superoxide dismutase (SOD), and the release of myeloperoxidase (MPO) from the azurophilic granules. This enzyme catalyses a reaction between H_{2}O_{2} and Cl^{-} ions which generates highly
microbicidal chlorinated oxidants (Weiss, 1989). Various components of the oxidative burst pathway can be identified by using physiological or soluble chemical stimuli which activate individual steps in the reaction sequence.

The major aim was to identify biochemical parameters in the neutrophil oxidative burst pathway which respond positively to moderate exercise (i.e. mechanisms of priming). Furthermore, we investigated whether priming of these oxygen-dependent processes increased the bacterial killing capacity of the cells. The second aim was to determine whether the depressive effect of endurance training on the oxidative burst (Smith et al., 1990) could be shown longitudinally and whether this pathway was also affected acutely by exercise at maximum intensity. Whilst this paper reports changes at the cellular level, we have determined, simultaneously, changes in neuroendocrine responses to exercise which may relate to the acute priming effect of moderate exercise and the immunosuppression caused by endurance training. These results are reported in the accompanying paper (Smith et al., 1993).
MATERIALS AND METHODS

Materials
Acridine orange, ferricytochrome-c (purified from horse heart), formyl-methionyl-leucyl-phenylalanine (fMLP), luminol (5-amino-2,3-dihydro-1,4-phthazinedione), paraformaldehyde, phorbol myristate acetate (PMA), superoxide dismutase and zymosan-A were all purchased from Sigma (St. Louis, MO). 2,7-Dichlorofluorescin diacetate was obtained from Serva (Heidelberg, Germany). The fluorochrome-conjugated monoclonal antibodies against complement receptor CR 3 (CD 11b) and Fc (Type III) receptor (CD 16), non-specific fluorochrome-conjugated IgG antibodies, and FACSlyse® were all purchased from Becton Dickinson (San Jose, CA, USA). The IMAC® kit for measuring human neutrophil elastase was obtained from Merck (Darmstadt, Germany).

Hank's balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were prepared by conventional techniques. Opsonized zymosan (OZ) and luminol were prepared as described previously (Smith et al., 1990). The stock solution of 2,7-dichlorofluorescin diacetate was prepared by the method of Bass et al. (1983). PMA was dissolved in dimethyl sulphoxide to a concentration of 1 mM. Further dilutions were made with HBSS. Ferricytochrome-c and SOD were dissolved in HBSS.

Preliminary Studies

Human subjects and exercise protocol
Six untrained and eight trained male subjects were recruited for the maximal exercise study. The maximum aerobic capacity (VO₂ max) of each individual was predetermined as described previously (Smith et al., 1990). Their profiles were similar to those of the subjects used in our pilot study (Smith et al., 1990). Eleven untrained male subjects participated in the phagocytic and bacterial killing study. Their profiles have been reported previously (Smith et al., 1992). The project was approved by the University's Ethics in Human Experimentation Committee; all subjects gave informed consent.

Eight untrained male subjects were recruited for the longitudinal study. These subjects (16 yr old males) were selected by the Australian Institute of Sport, using a talent-identification test (Hahn et al., 1991), to undertake a training program aimed at producing rowers of national standard. No individual had rowed previously or was training regularly. While initial training was technical, the intensity was increased progressively from the initial 12 hours/week at the first sampling point to
25 hours/week at the end when they then competed at the Australian National Rowing Championships with distinction. Five subjects completed the entire program. Blood samples were taken from these subjects on a rest day when they had not exercised for the previous 24 hours. Blood was drawn from the anticubital vein into sterile syringes and transferred immediately to sterile heparinized tubes. Neutrophils were isolated from whole blood using the procedure described previously (Smith et al., 1993).

Extracellular H$_2$O$_2$ was measured by a catalase-sensitive Clark-type oxidase electrode (Yellow Springs Instruments, OH, USA) (Test and Weiss, 1984). For each experiment, the electrode was calibrated with a fresh solution of standard H$_2$O$_2$. The neutrophil suspension (1 x 10$^6$ cells) in HBSS containing 0.2 µM azide (final volume 2 ml) was stirred continuously in a 37°C water-jacketed incubation chamber. Baseline H$_2$O$_2$ release was measured for five min before the addition of OZ (250 particles/cell). Total H$_2$O$_2$ release was then recorded until the baseline rate was re-established.

**Neutrophil phagocytic and bacterial killing capacity**

The number and viability of individual *Staphylococcus aureus* ANS46 cells phagocytosed by neutrophils was determined by the staining procedure described by Smith and Rommel (1977) with modifications by Pantazis and Kincker (1979) and Hed (1977). Neutrophils (5 x 10$^5$) were suspended in HBSS (400 µl) on a glass coverslip (18 mm diameter) mounted on foam platforms in Petri dishes (containing moistened filter paper) during the incubations at 37°C. The coverslips were washed gently with warm HBSS to leave a monolayer of adherent neutrophils. HBSS was left on the coverslips to prevent drying. The monolayers were incubated at 37°C with serum (10%) and bacteria (50 per cell) in HBSS (400 µl). The monolayers were then stained with acridine orange (0.01%). A wet mount was prepared by placing the coverslip, monolayer-side down, on a microscope slide and sealing the edges of the coverslip with nail polish. Phagocytosis and killing of the bacteria were determined (100 neutrophils) by following a green (living) to red (dead) transition of acridine orange-stained bacteria by fluorescence microscopy. The slides were examined (in a double-blind design) using a oil immersion objective (100x).

**Mechanistic studies**

The mechanistic studies were conducted in two phases. While the techniques used in both phases are described in phase-1, some methods and the statistical analyses are described separately.
PHASE-1

Human Subjects and exercise protocol
Fifteen untrained male subjects recruited for the first phase cycled on a
geread bicycle ergometer at a heart rate of ~ 140 beats/min for one hour; this
working heart rate was calculated from previous studies to approximate
60 % VO$_2$ max (Smith et al., 1992).

Measurement of neutrophil opsonin receptor expression and flow
cytometric light scatter
Non-specific antibodies (5 µl), and specific anti-CR-3 (C$_3$b$_1$-receptor) & anti-
FcyRIII (CD 16) antibodies (15 µl), were added in excess (as determined by
saturation experiments). EDTA-Anticoagulated whole blood (50 µl) was
added to separate tubes containing either the non-specific (control)
antibodies or the specific antibodies and mixed gently. The tubes were
incubated in the dark for 30 minutes at room temperature. FACSLYSE$^\circledR$
(1 ml) was added (to remove erythrocytes) and, after vortexing, all tubes
were allowed to stand for 10 minutes before being centrifuged at 2200 rpm
for 5 min. The supernatants were removed and the cells were washed with
PBS before resuspension in PBS (1 ml) containing paraformaldehyde
(1% w/v). The expression of FcyRIII (green fluorescence) and CR 3 (red
fluorescence) were measured simultaneously by flow cytometry (5000 cells)
using an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL,
USA) equipped with an argon laser emitting light at 488 nm. Flow
cytometric forward and right angle light scatter were measured
simultaneously in each sample.

Assessment of the neutrophil oxidative burst
The oxidative burst was assessed by several independent techniques:
(i) luminol-amplified chemiluminescence, which detects ROS generated by
myeloperoxidase activity, was carried out as described previously (Smith
et al., 1990); (ii) superoxide release was measured by ferricytochrome-c
reduction as described previously (Smith and Weidemann, 1993a); (iii) the
production of H$_2$O$_2$ was quantified in individual cells by flow cytometry
using the probes 2,7-dichlorofluorescein diacetate and dihydorhodamine-
123 as described previously (Smith and Weidemann, 1993a). The
concentrations of stimuli were: OZ (200 particles/cell); and PMA (100 nM).

Determination of neutrophil elastase
Elastase released into the circulation was measured by determining the
plasma concentration of free elastase and elastase/$\alpha_1$-protease inhibitor
complexes together using the IMAC® procedure (Hafner et al. 1991). Plasma or standard elastase (50 µl) was mixed with antibody against human elastase conjugated to horseradish peroxidase (200 µl). Control assays (to account for interference) were run by mixing a sample blank (50 µl) with a non-specific antibody conjugate solution (200 µl). All tubes were incubated at 37°C for 10 minutes before colour reagent containing H₂O₂ (0.5 ml) was added. After a further 10 min incubation at 37°C, the reaction was terminated by adding a stopping reagent supplied with the kit (2 ml). The optical density of each tube was determined spectrophotometrically at 500 nm.

Statistical analysis
The results were analysed by Student's t test for paired data.

PHASE-2

Human subjects and exercise protocol
Eight physically-active male subjects were recruited (age range 25 to 35 y). Individuals cycled on a geared bicycle ergometer at a heart rate of approximately 140 beats/min for one hour on two separate days. On one day each subject completed the test without drinking while, during the second test, they consumed 250 ml of a glucose-enriched solution (5% w/v) at 15 min intervals. Glucose was administered to prevent the large increase in growth hormone concentration (which may prime neutrophils) in the circulation caused by exercise (see accompanying paper; Smith et al., 1993) Variability in all of the parameters measured was determined by taking two resting blood samples one hour apart on another day when no exercise was performed and no glucose consumed. All subjects completed the three phases.

Statistical analysis
The results were analysed by a multilevel analysis of variance which incorporated the non-exercise time-controls and the exercise responses in the presence and absence of glucose. Where there was evidence that the underlying assumptions for the validity of tests were not satisfied, the data were transformed to logarithms.
RESULTS

PRELIMINARY STUDIES

Effect of maximal exercise
Because exercise at maximum intensity may dampen the activities of some immune cell subpopulations (reviewed by Smith and Weidemann, 1990), we investigated whether this was also the case with neutrophils. In contrast to the priming effect of moderate aerobic exercise (Smith et al., 1990), one minute of cycling at maximum capacity depressed the specific capacity per cell \( k \) of OZ-activated neutrophils to produce \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) \( (P < 0.05) \). Depressed activity was detected immediately following exercise and was even greater one hour later in both trained and untrained subjects (Fig. 1). The negative effect of regular intensive endurance training on the capacity of neutrophils to generate \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) both before and after exercise (Smith et al., 1990) was confirmed \( (P < 0.01; \text{Fig. 1}) \). This workload also doubled the circulating white blood cell count, which returned to pre-exercise levels within one hour after exercise (data not shown).

Longitudinal study
Because our two independent “cross-sectional” studies had shown that endurance training depressed the specific activity of \( \text{H}_2\text{O}_2 \) and \( \text{HOCl} \) production \( (k) \) from OZ-activated neutrophils chronically, we investigated whether this negative effect of regular intensive training could be demonstrated “longitudinally” in a group of initially untrained subjects undertaking a 13 month intensive training program. The major decline in neutrophil activity \( (k) \) occurred during the final two sampling periods when the intensity and duration of training both increased substantially (Fig. 2; \( P = 0.03 \)). A mean 15% increase in the maximum aerobic power \( (\text{VO}_2 \text{ max}) \) of the subjects occurred as a result of the overall training program \( (P = 0.03; \text{data not shown}) \).

The phagocytic and microbicidal responses of neutrophils before and after moderate exercise.
To determine whether exercise-induced priming of the neutrophil oxidative burst was physiologically significant, the adherence and bacterial killing capacities of the cells were measured \textit{in vitro}. Adherence of neutrophils to the glass coverslips was not altered significantly by exercise. The number of bacteria ingested by adherent cells increased progressively over the 45 min time course. Neutrophils isolated immediately after exercise showed increased phagocytic capacity at all time points measured
The effect of single episodes of maximal exercise, and endurance training, on H$_2$O$_2$ and HOCl production in neutrophils.

The results are presented as means ± SEM of kinetic constants (k) determined immediately before and after one minute of exercise at maximum capacity, and one hour post exercise (PE), in neutrophils isolated from trained and untrained men (n = 8,6). The experimental conditions are described in the Materials and Methods section. The kinetic constants were derived using the analysis described previously (Smith et al., 1990).
The progressive effects of endurance training on $\text{H}_2\text{O}_2$ and HOCl production in neutrophils.

The results are presented as means ± SEM of kinetic constants (k) determined in non-exercised men (n = 5) at defined periods over a 13 month period.
(Fig. 3a ;P < 0.01) compared with pre-exercise controls. While there was a consistent mean increase (5-10%) in the percentage of phagocytically-active neutrophils (detected at four out of the five time points measured) immediately after exercise, this was not significant (Fig. 3b). The phagocytic index (number of bacteria phagocytosed/active neutrophil) increased significantly immediately following exercise at all time points (P < 0.01) (Fig. 3c). The killing of phagocytosed bacteria (based on a green to red colour transition) was greater than 90% during the first ten min of the time course both before and after exercise. While exercise did not alter the bacterial killing capacity of the cells during the initial 20 min of the assay, it appeared to mitigate against the rapid decline in bactericidal activity observed in neutrophils isolated before exercise. Maximal killing capacity was sustained for at least 45 min (P < 0.01) in cells isolated after exercise while a large decline in bacterial killing was observed in neutrophils isolated from the same subjects before exercise (Fig. 3d).

We also found that the specific activity of H$_2$O$_2$ and HOCl generation (k), as detected by luminol-amplified chemiluminescence (pre-exercise: 2873 ± 418 mV; to exercise: 3763 ± 528 mV; P = 0.028), and the release of H$_2$O$_2$ into the extracellular medium (pre-exercise: 6.25 ± 0.283 μM; to exercise: 6.94 ± 0.316 μM; P = 0.02) by OZ-activated neutrophils increased immediately after moderate exercise in this group (*data are means ± SEM).

MECHANISTIC STUDIES

These studies were confined to the moderate exercise protocol only. Although priming and activation of neutrophils at the cellular level may be regulated by a multistep mechanism, we have studied these processes at the receptor and post-receptor levels by using stimuli (i.e. phorbol myristate acetate & opsonized zymosan) that activate the oxidative burst via separate signal transduction mechanisms (Takahashi et al., 1991). The first aim was to investigate, using these different stimuli, defined steps in the oxidative burst pathway which may be primed by exercise of moderate intensity. The second aim was to determine whether intermittent glucose consumption during exercise, which substantially diminishes the secretion of growth hormone into the circulation during exercise (see accompanying paper, Smith et al., 1993), prevented exercise-induced priming of various neutrophil functions. The cellular results are described in terms of the exercise-induced changes and the extent to which they are affected by the consumption of glucose. The variability in the time-control measurements in non-exercised subjects is also presented. The associated neuroendocrine
Fig. 3. The effect of moderate exercise on the phagocytic and bacterial killing capacities of neutrophils.

(a) Phagocytosis of *S. aureus* as a function of time.
(b) The percentage of phagocytically-active neutrophils.
(c) The number of bacteria phagocytosed per active neutrophil.
(d) The percentage of dead bacteria per active neutrophil.

All these parameters were measured both before (rest) and immediately after exercise. Data are means ± SEM.
Figure 3

(c) Bacterial/active neutrophil

(d) % dead bacteria

Time (min)
responses to exercise and their correlations to the neutrophil responses are presented and discussed in the accompanying paper (Smith et al., 1993).

**Effect of moderate exercise on flow cytometric light scatter and opsonin receptor expression**

Changes in flow cytometric light scatter were determined as measures of cell size (forward scatter) and granularity (side scatter) (Fletcher and Seligmann, 1985). In phase-1, the exercise-induced changes in the expression of cell surface opsonin receptors (see below) were accompanied by small reductions in flow cytometric side-scatter ($P = 0.014$) and forward scatter ($P = 0.005$) (Table 1). In the second phase, a decrease in forward scatter of similar magnitude was detected after exercise ($P = 0.061$) which was not affected by glucose consumption. The decrease in side scatter found after exercise was not significantly different to that found over the same period in non-exercised subjects; glucose consumption during exercise had no additional effect (Table 2).

Microbicidal activity is activated *in vivo* when opsonized microbes bind to complement and/or immunoglobulin Fc-receptors on the surface of the neutrophil plasma membrane. Moderate exercise increased the expression of complement (C$_3$b$_1$) receptors by 15% ($P = 0.049$) but triggered a concomitant down-regulation of FcγRIII ($P = 0.041$; Table 1) in the first phase of the study. Exercise did not alter the percentage of neutrophils expressing these receptors, which was always greater than 90%. Binding of the control antibodies was always less than 2% of the value found with the receptor-specific antibodies. In phase-2, the expression of C$_3$b$_1$ receptors increased by 20% after exercise (Table 2; $P = 0.045$) and this was not modulated by glucose consumption. Exercise did not alter the expression of FcγRIII significantly when changes in neutrophils isolated from non-exercised subjects studied over the same time period were taken into account.

**Effect of moderate exercise on the neutrophil oxidative burst**

In this series of experiments, we investigated steps in the oxidative burst pathway distal to receptor expression. In these experiments, each stimulus was tested at a single concentration only because of limitations imposed by the maximum volume of blood that could be taken from each subject to obtain a sufficient number of cells for each assay. As reported previously (Smith et al., 1990), there was a small increase in the number of circulating leucocytes immediately after exercise but the percentages of neutrophils and lymphocytes did not change; these responses were not affected by glucose
<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>PRE-EXERCISE</th>
<th>EXERCISE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side scatter</td>
<td>119.2 ± 2.32</td>
<td>114.7 ± 2.11*</td>
</tr>
<tr>
<td>Forward scatter</td>
<td>114.7 ± 2.13</td>
<td>111.2 ± 2.37*</td>
</tr>
<tr>
<td>Complement receptors (C3bi)</td>
<td>12.0 ± 0.99</td>
<td>13.7 ± 0.93*</td>
</tr>
<tr>
<td>Antibody receptors (FcγR III)</td>
<td>14.0 ± 1.55</td>
<td>13.2 ± 1.45*</td>
</tr>
</tbody>
</table>

All units are mean channel number ± SEM. The mean channel number indicates the fluorescence intensity (measured on a log scale from 0.1 to 1023) caused by binding of the fluorochrome-conjugated monoclonal antibodies to specific receptors on neutrophils. Flow cytometric forward and side scatter was also measured by channel number on a linear scale (0-255).

* Significantly different from pre-exercise, $P < 0.05$. 

†
Table-2  The effects of time and exercise on flow cytometric light scatter and neutrophil opsonin receptor expression

<table>
<thead>
<tr>
<th>TIME (min)</th>
<th>Non-exercise</th>
<th>Exercise (+ glucose)</th>
<th>Exercise (- glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Side scatter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>108.14</td>
<td>101.88</td>
<td>111.68</td>
</tr>
<tr>
<td>60</td>
<td>105.64</td>
<td>97.29</td>
<td>107.03</td>
</tr>
<tr>
<td></td>
<td>Forward scatter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>148.85</td>
<td>135.80</td>
<td>148.31</td>
</tr>
<tr>
<td>60</td>
<td>147.15</td>
<td>132.28</td>
<td>143.93</td>
</tr>
<tr>
<td></td>
<td>C₃b₁ receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.07</td>
<td>6.71</td>
<td>5.43</td>
</tr>
<tr>
<td>60</td>
<td>4.72</td>
<td>8.85</td>
<td>6.57</td>
</tr>
<tr>
<td></td>
<td>FcγIII receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.910</td>
<td>2.404</td>
<td>3.404</td>
</tr>
<tr>
<td>60</td>
<td>1.814</td>
<td>2.324</td>
<td>3.175</td>
</tr>
</tbody>
</table>

*The results are presented as mean channel number only (see Table 1). Statistical analysis was carried out as described in the Materials and Methods section. The pre-exercise (rest) sample was taken at time = 0 min; the exercise or non-exercise control samples were taken at time = 60 min. Each subject completed the three phases of testing.
consumption (data not shown). The leucocyte count was stable in non-exercised subjects.

Chemiluminescence. In phase-1, we confirmed the results of the published study (Smith et al., 1990) and the preliminary studies reported here by showing that the OZ-activated, (MPO dependent) luminol-amplified chemiluminescence was primed by moderate exercise ($P < 0.05$) at a single subsaturating concentration of OZ (100 particles/cell). Exercise-induced priming was confined to the phagocytic stimulus (OZ) (Table 3) because the peak velocity of $H_2O_2$ and HOCl production did not change significantly after exercise in either PMA (Table 4) or fMLP-activated neutrophils [pre-exercise: $120 \pm 20.4$ mV$^+$; to exercise: $124 \pm 24$ mV ($^*$data are means $\pm$ SEM)].

Only OZ was used in the second phase. The ability of OZ-stimulated neutrophils to produce $H_2O_2$ and HOCl tended to increase after exercise but this was not significant when compared to non-exercise time controls (Table 5; $P = 0.20$). Glucose consumption during exercise had no significant effects on chemiluminescence. As all but one of the non-exercised subjects showed a decrease in chemiluminescence in the 60 min control sample, the non-significance of the exercise effect is likely to be due to the fact that only four of the eight subjects showed an exercise-induced priming response.

Ferricytochrome-c reduction. We also investigated the direct effect of moderate exercise on superoxide production by superoxide dismutase inhabitable ferricytochrome-c reduction. In phase-1, superoxide ($O_2^-$) was produced at a higher rate immediately after exercise, irrespective of whether OZ (Table 3; $P = 0.04$) or PMA (Table 4; $P = 0.047$) was used to stimulate the cells. In the second phase, the rate of $O_2^-$ release into the extracellular medium in OZ-stimulated neutrophils was also enhanced after exercise ($P = 0.012$) and glucose consumption had no effect (Table 5). There was also no time-dependent variability in non-exercised subjects in the rate of $O_2^-$ production in neutrophils stimulated with PMA but, in contrast to OZ-stimulated cells, the exercise-induced increase was not significant (Table 6; $P = 0.12$). In contrast, there was a small decrease in the rate of $O_2^-$ production in PMA-stimulated cells when exercising subjects consumed glucose (Table 6; $P = 0.12$).

Flow cytometry. Whilst the chemiluminescence and spectrophotometric assays reported above detect the aggregate oxidative response of all the cells present in a suspension, the production of intracellular $H_2O_2$ is detectable
Table-3  Neutrophil oxidative burst activity activated by opsonized zymosan†

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>REST</th>
<th>EXERCISE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence (mV)</td>
<td>444 ± 73.7</td>
<td>580 ± 95.5*</td>
</tr>
<tr>
<td>Superoxide release (nmol/10⁶ neutrophils)</td>
<td>1.47 ± 0.21</td>
<td>1.67 ± 0.18*</td>
</tr>
</tbody>
</table>

**Flow cytometry**

| Active neutrophils (%)                  | 38.4 ± 4.6 | 48.1 ± 4.9* |
| Mean channel number                     | 1.45 ± 0.11 | 1.43 ± 0.11 |

† Results are mean ± SEM. The percentage of active cells are those producing H₂O₂ above the control value of 0.6. The mean channel number indicates the fluorescence intensity (i.e. H₂O₂ production) on a log scale (0.1 to 1023).

* significantly different from pre-exercise, P < 0.05.
Table 4: Neutrophil oxidative burst activity activated by phorbol myristate acetate

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>REST</th>
<th>EXERCISE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence (mV)</td>
<td>571 ± 131</td>
<td>567 ± 101</td>
</tr>
<tr>
<td>Superoxide release (nmol/10^6 neutrophils)</td>
<td>1.84 ± 0.23</td>
<td>2.09 ± 0.28*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flow cytometry</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Active neutrophils (%)</td>
<td>65.5 ± 6.0</td>
<td>75.0 ± 5.6*</td>
</tr>
<tr>
<td>Mean channel number</td>
<td>1.72 ± 0.18</td>
<td>1.96 ± 0.21*</td>
</tr>
</tbody>
</table>

Results are mean ± SEM. The percentage of active cells are those producing H_2O_2 above the control channel number of 0.6 (see below). The mean channel number indicates the fluorescence intensity (i.e. H_2O_2 production) on a log scale (0.1 to 10^23).

* Significantly different from pre-exercise; P < 0.05.
Table 5  The effects of time and exercise on the neutrophil oxidative burst*  
(opsonized zymosan)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Non-exercise Luminol-amplified chemiluminescence</th>
<th>Exercise (+ glucose) Luminol-amplified chemiluminescence</th>
<th>Exercise (- glucose) Luminol-amplified chemiluminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3805</td>
<td>788</td>
<td>751</td>
</tr>
<tr>
<td>60</td>
<td>3128</td>
<td>754</td>
<td>909</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ferricytochrome-c reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flow cytometry (DCFH-DA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active neutrophils (%)</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>Mean channel number</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>60</td>
</tr>
</tbody>
</table>

* The results are presented as means only. Statistical analysis was carried out as described in the Materials and Methods section. The sampling and testing procedures are described in the footnotes to Table 2.
Table 6  The effects of time and exercise on the neutrophil oxidative burst* (phorbol myristate acetate)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Non-exercise</th>
<th>Exercise (+ glucose)</th>
<th>Exercise (- glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ferricytochrome-c reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.164</td>
<td>1.046</td>
<td>0.991</td>
</tr>
<tr>
<td>60</td>
<td>1.120</td>
<td>1.035</td>
<td>1.135</td>
</tr>
<tr>
<td></td>
<td>Flow cytometry (DCFH-DA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active neutrophils (%)</td>
<td>0</td>
<td>71.9</td>
<td>72.4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>73.7</td>
<td>80.7</td>
</tr>
<tr>
<td>Mean channel number</td>
<td>0</td>
<td>1.686</td>
<td>1.879</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.818</td>
<td>2.175</td>
</tr>
</tbody>
</table>

* The results are presented as means only. Statistical analysis was carried out as described in the Materials and Methods section. The sampling and testing procedures are described in the footnotes to Table 2.
in individual neutrophils by flow cytometry. Our previous work has shown that distinct subpopulations of neutrophils can be identified using flow cytometry (Smith and Weidemann, 1993a) and the microscopic studies reported here earlier showed that about 20% of the neutrophil population were phagocytically inactive. The effect of exercise on the percentage of cells within these subpopulations, and their mean intensity of H$_2$O$_2$ production in response to stimulation with either OZ or PMA, was examined.

Only DCFH-DA was used as a H$_2$O$_2$-detection probe in phase-1. With this probe, neutrophils could be separated into non-responsive and responsive subpopulations. An exercise-induced increase in the percentage of OZ-responsive neutrophils was found (P = 0.03) but there was no significant change in the mean oxidative activity of the neutrophil population (Table 3). The percentage of PMA-responsive neutrophils also increased after exercise (Table 4; P = 0.03). and, in contrast to OZ, there was a substantial increase in the oxidative capacity of individual cells (Table 4; P = 0.04).

In the second phase, the intracellular production of H$_2$O$_2$ was measured using both DCFH-DA and dihydrorhodamine-123 (DHR) as oxidative probes in separate assays 40 min after stimulation with either OZ or PMA. As reported previously, DHR was a much more sensitive oxidative probe than DCFH-DA (Smith and Weidemann, 1993a). As found in phase-1 with DCFH-DA, an increase in the percentage of OZ-responsive cells occurred after exercise, even after consumption of glucose, but this was not significantly different to increases found over the same period in non-exercised controls (Table 5; P = 0.20). In contrast to the low percentage of responsive (positive) cells detected with DCFH-DA, all of the cells (100%) showed a positive response to stimulation with both OZ and PMA when DHR was used as the H$_2$O$_2$-detection probe. With DHR, there were again no significant exercise-induced changes in H$_2$O$_2$ production in OZ-stimulated cells when the response of the entire cell population were analysed statistically (Fig. 4a). This was also the case when the distribution of the two neutrophil subpopulations was assessed (data not shown).

The most salient exercise effect was found in the DHR assay after neutrophils were stimulated with PMA. There was a three-fold increase in the magnitude of intracellular H$_2$O$_2$ production after exercise (P = 0.025) which was attenuated by glucose consumption (P = 0.06; Fig 4b). When the effect of exercise on the distribution of subpopulations was analysed, the percentage of cells in the high-activity subpopulation (population-2) increased substantially after exercise (Fig 5a; P = 0.034) and this correlated
Fig. 4. The effect of exercise on the mean intensity of intracellular 
H₂O₂ production in neutrophils.

Isolated neutrophils were stimulated in vitro with either: (a) opsonized 
zymosan (200 particles/cell); or (b) phorbol myristate acetate (100 nM) under 
conditions described in the Materials and Methods section. The results 
obtained both before and immediately after exercise (± glucose 
consumption), and in non-exercised controls, are presented as means. Each 
subject completed the three phases of testing. Mean fluorescence intensity is 
proportional to H₂O₂ production measured on a log scale (0.1 to 1023).
The percentage of neutrophils in the two distinct neutrophil subpopulations identified both before and immediately after moderate exercise.

Isolated neutrophils were stimulated with phorbol myristate acetate (100 nM). Subpopulations were characterized using the procedure reported previously (Smith and Weidemann, 1993). The testing procedures and the presentation of data are described in the legend to Figure 4.

(a) High-activity subpopulation.
(b) Low-activity subpopulation.
Data are means.
with a decreased percentage in the low-activity subpopulation (population-1) (Fig. 5b; \( P = 0.033 \)). Despite this clear difference, there were no significant changes in the mean intensity of \( \text{H}_2\text{O}_2 \) produced per cell in these subpopulations (Fig. 6). With DCFH-DA, the percentage of PMA-responsive cells tended to increase after exercise (Table 6; \( P = 0.21 \)) and this was not affected by glucose consumption (Table 6). These responses were accompanied by an increase in the mean fluorescence intensity (Table 6; \( P = 0.08 \)).

**Effect of moderate exercise on plasma elastase.**

The release of elastase into the circulation during exercise was measured as an index of neutrophil activation (i.e. azurophilic degranulation) *in vivo*. In phase-1, the plasma concentration of total elastase increased two-fold immediately after exercise (\( P = 0.003 \)) in the untrained subjects but no significant changes were found in the trained subjects or unexercised controls (Fig. 7a). There were no significant differences in the mean pre-exercise values between the trained and untrained subjects (Fig. 7a). In phase-2, the plasma concentration of elastase increased about two-fold immediately after exercise (\( P = 0.003 \)) but this was attenuated to some extent by glucose consumption (Fig 7b; \( P = 0.10 \)).
Fig. 6. The effect of exercise on the mean intensity of neutrophil H₂O₂ production in the two neutrophil subpopulations.

The mean intensities of intracellular H₂O₂ production in the two subpopulations identified in Figure 5 are presented. The testing procedures and the presentation of data are described in the legend to Figure 4.

(a) High-activity subpopulation.
(b) Low-activity subpopulation.
Fig. 7. The effect of moderate exercise on the plasma concentration of complexed elastase.

(a) The effect of endurance training on the release of complexed elastase into the circulation in response to moderate exercise. Data are means ± SEM.

(b) The effect of glucose consumption on the exercise-induced release of elastase into the bloodstream. Data are means.
DISCUSSION

Previous studies by our group (Smith et al., 1990) and this work show that the capacity of circulating neutrophils to produce reactive oxygen species (ROS) was primed significantly immediately following one hour of moderate exercise. Although some of these putative exercise effects were not significantly different in samples taken over the same one hour period in non-exercised subjects, the increased expression of neutrophil C3b1 receptors, the primed capacity of neutrophils to generate H2O2 intracellularly in response to stimulation with PMA, and to release O2- extracellularly after stimulation with O2, as well as the increase in the plasma concentration of complexed elastase were all clearly exercise-dependent.

We focussed on the responses detectable in the immediate post-exercise period because initial work had shown that the primed capacity of neutrophils to generate H2O2 and HOCl over a wide concentration range was much larger immediately than at six hours after exercise (Smith et al., 1990). The major physiological consequence of exercise-induced priming of the oxidative burst was a significant increase in the phagocytic and bacterial killing capacities of the isolated cells. The accompanying release of elastase into the circulation after exercise is indicative that a subpopulation of neutrophils may have undergone complete activation in vivo as a consequence of moderate exercise. In contrast to this protocol, single episodes of exercise at maximal intensity and/or intensive periods of endurance training both had detrimental effects on the capacity of neutrophils to produce H2O2 and HOCl which may, in turn, have reduced their ability to kill infectious agents once the intensity of these cytotoxic reactions fell below a critical threshold.

The combined results of our mechanistic studies suggest that, despite some discrepancies, exercise-induced priming was evident at both the receptor and post-receptor levels. The discrepancies may be due to differences in the proportions of various ROS generated intracellularly that diffuse into the extracellular medium, and the specificity and reactivity of the oxidative probes or reagents used to detect different ROS. This is discussed in more detail later.

Exercised-induced priming appears to involve a shift towards a increased percentage of high-activity cells in a population that contains two distinct "subpopulations", especially when PMA is used as a stimulus. The low-activity cells may include neutrophils activated during exercise which have subsequently returned to a dormant, but non-responsive, state after
exercise. This discussion will focus on the cellular aspects only. Neuroendocrine parameters and their associations with the neutrophil responses are discussed in the accompanying paper (Smith et al., 1993).

Contrasting responses of neutrophils to moderate exercise
While some of the cellular responses to exercise that failed to reach significance were clearly due to time-dependent variation and not to exercise *per se*, the chemiluminescence response to OZ was not. Because all but one of the non-exercised subjects in phase-2 showed a time-dependent decrease in H$_2$O$_2$ and HOCl production in the 60 min control sample, the non-significance ($P = 0.20$) of the exercise effects detected in phase-2 may have been due to the restriction of the primed response to four subjects immediately after exercise while a sharply contrasting decrease was found in the other four. The decreases may have been due to the priming threshold being exceeded in some individuals, leading to the full activation of a subpopulation of their cells during exercise. The increase in the plasma concentration of complexed elastase after exercise is an indication of neutrophil activation *in vivo* and this observation is supported by recent work showing that moderate exercise activates translocation of p$47^{phox}$ from the cytosol to the membrane (A.B. Gray et al., unpublished results). This normally cytosolic protein was not detectable in neutrophil membranes prepared from cells isolated just before exercise, and its translocation to the membrane is an essential step in the activation of NADPH oxidase (Dusi et al., 1993). The refractory period expected during the post-exercise recovery period would leave activated neutrophils in a non-responsive state for some hours (Prasad et al., 1991). Because the production of H$_2$O$_2$ intracellularly in response to stimulation by PMA showed exercise-induced priming, this postulated mechanism may only apply to receptor-dependent stimuli such as OZ and it may involve uncoupling of the link between activation of NADPH oxidase and the release of MPO from the azurophilic granules (see next section).

It is not surprising that variability in the majority of neutrophil functions to time and exercise were found considering the extremely diverse mitogenic responses of lymphocytes to acute exercise that have been reported, reflecting, to some degree, the current training load of each subject (Verde et al., 1992). Variability in immune cell activities may also be related to diet and other lifestyle factors.
Stimulus dependence and assay specificities

These results emphasize the stimulus-specificity of the oxidative burst in terms of NADPH oxidase and MPO-dependent activities, and the independence of the techniques used to measure the production of ROS. Stimuli such as PMA, OZ and fMLP activate the oxidase through distinct proximal signal transduction pathways (Takahashi et al., 1991). OZ is a phagocytic stimulus that binds to C3b/Fc receptors on the neutrophil plasma membrane and activates, in turn, the oxidative burst via Ca2+ and bioactive lipids released as a result of the stimulation of phospholipases A2, C & D (Della-Bianca et al., 1990). These steps occur independently of the activity of PK-C (Maridonneau-Parini et al., 1986) which may only be required to extend or sustain the duration of the oxidative burst (Watson et al., 1991; Koenderman et al., 1989). The bacterial peptide, fMLP, binds to a specific receptor, linked to a G-protein, on the neutrophil plasma membrane which also activates Ca2+ release via phospholipase-C activation independently of PK-C (reviewed by Tauber, 1987; Babior, 1992). In contrast, PMA bypasses the receptor-mediated steps (including Ca2+ release and azurophilic degranulation) by activating PK-C activity directly (Segal and Abo, 1993). Investigation of the effects of exercise on other receptors linked to various stimuli (e.g. fMLP-receptor, FcyRII) and the intracellular concentrations of Ca2+, cyclic nucleotides and bioactive lipids such as platelet activating factor may indicate the kind of changes that occur in receptors and signalling molecules linked to NADPH oxidase in response to single episodes of exercise at various intensities.

Compared to OZ, PMA and fMLP are both weak activators of MPO release from the azurophilic granules into the phagosome (Allen, 1986), so it is not surprising that exercise-induced priming was not detected in PMA- or fMLP-stimulated cells in the chemiluminescence assay. Recent reports suggest that reactive nitrogen species (RNS) are also involved in luminol (Wang et al., 1991; Radi et al., 1993) and DCFH (Rao et al., 1992) excitation generated during the phagocytic oxidative burst. The oxidative killing of micro-organisms may be mediated by a combination of ROS and RNS, and, perhaps, by other unknown species that may be "detected" differentially by these assays. Thus it is not surprising that other workers have reported that there is no correlation between superoxide release (detected extracellularly only by ferricytochrome-c) and luminol-amplified chemiluminescence, which appears to be primarily intracellular (Takahashi et al., 1991). We also found no consistent relationship between ferricytochrome-c reduction, luminol-amplified chemiluminescence and the flow cytometric assays in neutrophils stimulated with either OZ or PMA (Smith and Weidemann,
1993a). The assay differences could also be related to a number of parameters: reactivity of the various cytotoxic species with different oxidative probes or detection molecules, and their competition for substrate with cellular antioxidants and detoxification enzymes; secondary reactions between various reactive species; and, with some molecules, their distribution between the intracellular and very large extracellular compartments.

Mechanism(s) of exercise-induced priming of neutrophils.
There are several explanations which may account for the significant priming/activation phenomena detected immediately after moderate exercise.

Marginated versus circulating pools. Neutrophils which demarginate from capillary beds in the lungs and spleen during exercise (Peters et al., 1992) may have intrinsically higher activities than those in the general circulation. Because both moderate and maximal exercise workloads trigger the release of neutrophils from marginated vascular pools into the circulation - and because neutrophil oxidative burst activity is diminished significantly after vigorous exercise - it is unlikely that priming would be due solely to the release of a neutrophil “subpopulation” with greater oxidative capacity into the bloodstream.

Opsonin receptor expression. Whilst C$_3$b$_1$ receptors enhance the phagocytosis of OZ particles, they do not mediate ROS production directly (Wright and Silverstein, 1983). Therefore, the increase in neutrophil C$_3$b$_1$ receptor expression detected after exercise is unlikely to fully account for the increased responsiveness of the cells to OZ in the ferricytochrome-c and luminol-amplified chemiluminescence assays after exercise; nor it is indicative of activation of the entire neutrophil population because C$_3$b$_1$ receptor expression increases at least 10-fold in activated cells (Berger et al., 1988). Because these receptors are bypassed by PMA, the priming response to PMA occurs independently of changes in opsonin receptor expression at the level of PK-C. However, the exercised-induced increases in the expression of C$_3$b$_1$ receptors and ROS generation may be coupled to some extent and may be triggered by the same humoral mediator(s) (Smith et al., 1993). The decrease in flow cytometric forward light scatter is indicative of a increase in the cell surface area to volume ratio which may occur during priming (Fletcher and Seligmann, 1985). Although not statistically significant in phase 2, the reduction in the expression of neutrophil Fc$_y$RIII
found in phase-1 is a further indication (together with elastase release and light scatter changes) that a subpopulation of neutrophils has been activated during exercise. Shedding of FcγRIII into the circulation is usually associated with inflammation and neutrophil activation (Huizinga et al., 1990; Fleit et al., 1992). The physiological role of soluble FcγRIII may be to bind circulating immune complexes, thus ensuring their destruction by neutrophils which take up the complex via FcγRII (Huizinga et al., 1990). Immune complexes are possible triggering agents for the exercise-induced activation of a small percentage of circulating neutrophils. Measurement of soluble FcγRIII in plasma samples taken before and after exercise may resolve whether the decrease in neutrophil FcγRIII expression is an authentic exercise effect.

*Priming or activation?*

The biochemical mechanisms involved in neutrophil priming and activation are coupled to some extent. Thus, it is not surprising that substimulating concentrations of some activating agents (e.g. PMA) may prime but not activate the oxidative burst (Lipschitz et al., 1991). Whilst moderate exercise tends to prime the oxidative burst, a subpopulation of cells appears to have become fully activated during exercise. In fact, ligation of FcγRIII activates exocytosis of granule proteins (Huizinga et al., 1990) and an increased plasma concentration of complexed elastase has been reported after intensive running (Kokot et al., 1988; Dufaux et al., 1989). This suggests that the percentage of neutrophils that become fully activated by exercise may be intensity-dependent, with a threshold that may vary considerably from individual to individual. Our results show this distinction clearly because the exercise-induced release of elastase into the circulation was significant only in the untrained subjects. Activated cells have a decreased capacity to produce ROS upon secondary stimulation with OZ, but it appears that this recovers with time (Prasad et al., 1991). This is suggestive that the cells either enter a lengthy refractory period or are replaced gradually with low-activity immature cells from the bone marrow.

Exercise-induced activation of neutrophils may have some benefits. Free elastase may deactivate soluble mediators and/or toxins which are released into the circulation during exercise; elastase degrades humoral mediators such as TNF-α *in vitro* but rapid complexing of the enzyme with α1-protease inhibitor prevents this (Scuderi et al., 1991). Elastase release within inflammatory sites may play an autocrine/paracrine role in enhancing the oxidative burst but this is also blocked by complexing the enzyme (Bucurenci et al., 1992). In addition, free elastase may be involved
in the proteolytic cleavage of FcγRIII from the neutrophil plasma membrane (Fleit et al., 1992) and the degradation of fibrin deposits at injury sites (Kazura et al., 1989). Complexed elastase is a neutrophil chemoattractant (Banda et al., 1988) and elastase release during exercise may be responsible for the activation of proteolytic cascades, including those of the complement and fibrinolytic systems (Dufaux and Order, 1991).

To discriminate between priming and activation, an extracellular marker that is expressed exclusively on primed but not dormant or activated neutrophils needs to be identified. Several novel neutrophil activation markers have been described recently, including CD 66 and CD 67, which are stored in the specific granules, and CD 63, a marker of azurophilic degranulation; the functions of these proteins are not known (Ducker and Skubitz, 1992; Niessen and Verhoeven, 1992). CD 43 and LECAM-1 are also shed rapidly from the plasma membranes of activated neutrophils (Rieu et al., 1992). Whilst no marker that is exclusive to primed neutrophils has been described yet, the expression of CD10 and binding of the monoclonal antibody 7D5 have been reported to be early activation markers in circulating neutrophils (Kuijpers et al., 1991).

Other groups have found that various neutrophil functions respond differentially to submaximal exercise. The chemotactic and phagocytic activities of neutrophils were enhanced significantly immediately after one hour of cycling at 50% VO₂ max but their Candididal capacity was not altered from pre-exercise values (Ortega et al., 1993a). Thus, the ability of neutrophils to kill different types of micro-organisms may be primed differentially by moderate exercise. In contrast to the results reported here, Macha and colleagues (1990) reported that the responsiveness to phorbol esters of neutrophils isolated immediately after a similar exercise workload was decreased significantly. The latter finding may reflect differences in intra- and extra-cellular assays for H₂O₂ and, perhaps, the fact that only a small amount of the H₂O₂ generated may diffuse into the extracellular medium when PMA is used as the activating stimulus.

Recent studies by our group have confirmed that the type and intensity of exercise affect the oxidative burst of neutrophils differentially. Intensive uphill treadmill running has a suppressive effect on the oxidative burst but moderate downhill running produces a delayed two-three fold increase in the O₂-activated burst which is sustained for six to 24 hours post exercise (D.B. Pyne et al., unpublished results). This may explain why moderate exercise has a priming effect but intensive exercise is detrimental because of “over-activation”. Furthermore, activation may be
initiated once the plasma concentration of growth hormone reaches a critical threshold, and immunosuppressive hormones such as adrenalin and cortisol may sustain the resulting refractory period during the recovery phase (Smith and Weidemann, 1993).

Training also has intensity-dependent long-term affects on neutrophil microbicidal activities. The basal phagocytic and chemotactic activities of neutrophils, and their capacity to kill in vitro the fungus Candida albicans, were significantly higher in cells isolated from female basketball players compared to cells from untrained controls (Ortega et al., 1993b). In contrast, Hack and colleagues (1992) reported that the phagocytic and chemotactic activities, and superoxide production, of neutrophils were not different in cells isolated from either highly-trained runners and triathletes (during a moderate training period) or untrained controls, suggesting that tapering of the training program may have normalised these parameters. Lewicki and colleagues (1987) reported that the bactericidal activity of neutrophils was significantly lower in trained athletes before exercise and this training difference was even greater after a session of maximal exercise. Our group has also shown that neutrophils isolated from elite swimmers produce responses to OZ and PMA in the DHR assay that are two- and four-fold lower, respectively, than the values detected in cells from untrained controls (D.B. Pyne, unpublished results); this result confirms our earlier work, using luminol-amplified chemiluminescence, which showed that the specific oxidative activities of neutrophils from trained cyclists were significantly lower than in cells from untrained controls (Smith et al., 1990).

Conclusions
In summary, our results show that exercise has intensity-dependent effects on some neutrophil microbicidal functions that are significantly greater than those found in non-exercised subjects over the same period. While the mechanisms responsible for the priming effects of moderate exercise have not been characterized unequivocally, it is possible to conclude, tentatively, that moderate exercise brings about at least three changes to the circulating neutrophil pool: (i) the recruitment of low-activity cells into a pool that responds more strongly to both phagocytic and soluble stimuli; (ii) a relatively small increase in intracellular H₂O₂ production per responding cell that can be evoked, independently of opsonin receptors or degranulation, at the level of protein kinase-C by PMA; and (iii) direct activation of a distinct subpopulation of neutrophils.
At present, the clinical implications of exercise-induced changes in immune function detected *in vitro* are unknown. However, because the presence of a high percentage of neutrophils that are non-responsive to activating agents such as PMA correlates with an increased incidence of bacterial infection (Bass *et al.*, 1986) and is a poor prognosis in patients with advanced carcinomas of the head and neck (Kaffenberger *et al.*, 1992), priming of neutrophil microbicidal activity may partially explain why moderate exercise reduces the susceptibility of humans to infectious disease and some cancers and overtraining is a high-risk factor for infectious disease (reviewed by Smith and Weidemann, 1990; Shephard, 1993). This work shows that investigations of whether the ability of neutrophils to kill various types of micro-organisms (e.g. gram-positive and-negative bacteria, fungi, protozoa, etc) is modified by exercise in an intensity-dependent manner should be undertaken. The variable but "real" intensity-dependent responses of neutrophils to exercise may explain why exercise and its effects on the immune system has remained a controversial subject for decades (Cannon, 1993). These results suggest ways in which this controversy may be resolved.
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REFERENCES


CHAPTER 3

EXPERIMENTAL

(Immunoregulation)
CHAPTER 3.1

The exercise and immunity paradox: a neuroendocrine/cytokine hypothesis
Forum

The exercise and immunity paradox: a neuro-endocrine/cytokine hypothesis

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Exertion, training, stress, hormone, neurohumor, neutrophil, lymphocyte, neuropeptide, cellular and humoral immunity.

Introduction

Whilst the health benefits of regular exercise (e.g. reduced susceptibility to cardiovascular disease) are well known, the relationship between exercise and immunity has only recently become a focus of intense research activity. This is surprising considering that anecdotal evidence exists dating back to 1920 linking these two phenomena [1]. At present, some reports suggest that moderate exercise increases resistance to infection; beyond this point of moderation, however, the daily exercise undertaken by athletes during intensive training periods appears to depress immunity (see reviews [1-3]). The psychological stress associated with intensive training and competition may exacerbate this tendency. The mechanisms by which these paradoxical responses to exercise are produced are poorly understood.

Despite the dearth of epidemiological studies, several investigations have shown that, compared to untrained individuals, athletes show increased incidence of upper respiratory tract infections [4, 5], infectious hepatitis and aseptic meningitis [2]. Highly-stressed individuals are also more susceptible to respiratory infections [6] and some cancers [7]. In contrast, moderate training programs (26-29 miles/week of running) reduce the occurrence of infectious episodes compared with those experienced by individuals running less than 15 miles/week [8]. Moderate exercise programs and/or physically active jobs may also reduce susceptibility to colon and breast cancers [9] and the severity and duration of depression-related illness [10].

The human immune system consists of a complex network of interacting cells and humoral factors. The mechanisms mediating immunity can be divided functionally into natural (non-specific) and adaptive elements. In this forum, we discuss the available evidence (confined mostly to human studies) and propose a mechanism that may explain why low to moderate exercise may boost immune responses while intensive training is potentially immunosuppressive.

Exercise and the immune response

Submaximal (aerobic) and maximal (anaerobic) exercise cause substantial but transient increases in circulating leucocyte (immune cell) numbers due to their release from marginated vascular pools and the bone marrow [11]. Blood leucocyte counts can remain significantly elevated for up to eight hours after moderate exercise but they return to pre-exercise (resting) levels within one hour after a brief episode of exercise at maximal capacity. The magnitude and period of the leucocytosis depends upon the intensity and duration of the workload; it is mediated by elevations in circulating catecholamines, cortisol and increased blood flow. Training reduces the magnitude of these responses. Lymphocytes and neutrophils contribute jointly to the leucocytosis induced by submaximal and maximal exercise but increased neutrophil numbers become predominant once cortisol release is activated [11]. In general, exercise of all intensities lowers both the T-cell to B-cell and the helper T-cell to suppressor/cytotoxic T-cell ratios among the circulating cells [3, 11]. The potential immunological consequences of these changes are not clear.

The majority of studies that have attempted to assess the effects of exercise on cell-mediated immune responses have focused on lymphocytes and natural killer (NK) cells but there are some recent reports where neutrophil and macrophage functions have been examined. Many independent investigations have reported significant reductions in the responsiveness of isolated lymphocytes to T-cell and B-cell mitogens after exercise at maximum intensity, while variable results have been documented for submaximal exercise [1-3]. This variability may be due to differences in the intensity and duration of the submaximal exercise protocols. For example, exercise at maximum intensity and marathon running both cause significant reductions in NK-cell cytotoxicity while moderate exercise is stimulatory [1, 12]. Prolonged submaximal exercise and brief maximal exercise both lower serum and salivary immunoglobulin levels, complement components and neutrophil microbicidal activity [1, 13-15]. Surprisingly, macrophage phagocytic capacity has been reported to increase immediately after a bout of exhaustive exercise [16]. In contrast to intensive exercise, moderate exercise potentiates antibody-dependent cytotoxicity, NK-cell cytotoxicity and the phagocytic and microbicidal activities of neutrophils [1-3, 15].

Several striking immunodeficiencies have been reported in "cross-sectional" studies of athletes undergoing intensive training programs compared to untrained controls. These include reductions in serum complement factors, salivary IgA, NK-cell cytotoxicity, lymphocyte mitogenesis and neutrophil microbicidal activity [2, 3, 13, 15]. "Longitudinal" studies have not been undertaken, however, to determine at what stage of the training program these responses begin to diminish. In a 15 month longitudinal study of five untrained males selected to participate in an intensive rowing training program, we observed a substantial fall in neutrophil microbicidal activity shortly after the training intensity increased dramatically (Smith et al., unpublished). This confirmed the results of our original cross-sectional study on male cyclists [15].
Trauma causes similar defects in T-cell, macrophage and neutrophil functions that may explain the high infection rates observed in these individuals [17]. Transient perturbations of immune responses after intense exercise or trauma may be a protective response that prevents severe inflammatory damage to body tissues, particularly at injury sites. For instance, elevated levels of neutrophil elastase (which may damage body tissues) have been detected in plasma after prolonged running [18]. Impaired mitogen responsive responsiveness of lymphocytes and reductions in NK-cell cytotoxicity have also been reported following bereavement and in response to the psychological stress of college examinations [19].

These observations show that the intensity and duration of exercise may exert, via humoral mechanisms, a behavioural influence on cellular immunity. Interestingly, the depression of some immune cell functions in response to psychological and traumatic stress shows a remarkable similarity to that induced by intensive training. The variability of the exercise effects may be due to the failure of some investigators to express their results in terms of a defined cell number (to take account of any leucocytosis) or to the tendency of others to restrict sampling to the periods just before and immediately after exercise. NK-cell cytotoxicity, for example, may increase immediately after moderate to intense exercise, only to fall substantially below resting values two hours later, with recovery to normal levels occurring over the next 21 hours [12, 20]. Some immune responses may also show endogenous diurnal fluctuations in their activities.

Exercise and immunoregulation

Various humoral messengers including hormones, neurotransmitters and cytokines regulate cellular and humoral immunity. Immune cells express plasma membrane receptors for many classical neuro-endocrine messengers [e.g. catecholamines, some pro-opiomelanocortin (POMC)-derived peptides (i.e. adrenocorticotropic hormone (ACTH) and β-endorphin), thyrotropin, gonadotropin, growth hormone and prolactin]; they are also able to synthesise and release small amounts of most of these factors in vitro [21].

Cytokines were first reported as interleucocyte hormones (interleukins) but they are now known to be produced by, and to act on, virtually all nucleated cells [22]. Thus, it is not surprising that communication between the neuro-endocrine and immune systems mediated by hormones and cytokines is one of the most active areas of current biological research. In this section, we will consider the range of interactions between hormones, cytokines and immune cells that may be induced by exercise and the potential immunological consequences that may flow from such wide ranging interactions.

The intensity and duration of exercise also influences the extent to which many neuro-endocrine hormones are released into the circulation [23]. The magnitude of the response is substantially lower in trained subjects exercising at the same absolute workload as untrained counterparts [23]. Stress hormones like catecholamines and cortisol are thought to be secreted in response to the direct metabolic and physiological demands of exercise. Circulating and marginated immune cells with receptors for these hormones are highly susceptible targets. With some hormones, the plasma concentrations peak immediately after a critical threshold is reached in workload intensity but with others this may not occur until several hours after the episode of exercise has ceased. There may also be a substantial lag time between the attainment of peak plasma concentration of a particular hormone and the appearance of a functional cellular response. The release of cytokines from circulating monocytes has been observed following submaximal exercise [24, 18, Smith et al., unpublished].

The circulating concentration of growth hormone (GH) is elevated significantly after 30 minutes of exercise of very low intensity (10–15% VO₂max) and it continues to rise with increasing workload [25]. Prolactin, which shares considerable structural and functional homology with GH [26], does not increase in plasma until exercise intensity exceeds 50% VO₂max and the magnitude of the increase is much smaller than that of GH [23, 27]. The rapidly accumulating evidence that these pituitary hormones are potent activators of immune mechanisms has been reviewed recently [26]. GH is essential for the development of the immune system and maintenance of immunocompetence [28]. GH-deficiency, which reduces the responsiveness of virtually all immune mechanisms, leads to increased vulnerability to infection.

Specifically, GH is essential for thymus development but it also enhances lymphocyte proliferation, T-cell cytotoxicity, NK-cell cytotoxicity and is as potent as γ-interferon in priming macrophage microbicidal activity [26]. GH also primes the basal rate of superoxide production by neutrophils but, paradoxically, has been reported to inhibit the oxidative burst when opsonised zymosan is the stimulus [26]. Superoxide may, however, be utilised by the myeloperoxidase network (which is absent in macrophages) to produce chlorinated oxidants (like hypochlorous acid) with greater microbicidal potency.

Prolactin augments macrophage tumouricidal activity and interferon synthesis and appears to be essential for maximum T-cell responsiveness to antigenic stimulation [29, 30]. The immunosuppressive action of cyclosporin-A, which is regularly prescribed for transplant patients, may be due in part to its ability to displace prolactin from its lymphocyte receptor [30]. Prolactin also primes the oxidative burst of macrophages to the same intensity as that induced by GH [31]. Despite their close functional and structural similarities, GH and prolactin are not necessarily released from the pituitary gland in a coordinated manner, endotoxin and interleukin-1 both stimulate GH release, for example, but inhibit that of prolactin [26].

The neuropetide substance P also potentiates many cellular and humoral immune responses [32]. These include the enhancement of neutrophil microbicidal activity and phagocytosis, B-cell differentiation and T-cell proliferation. Substance P also blocks the secretion of ACTH [33] and can inhibit many functional disorders caused by chronic stress [34]. The plasma concentration of substance P may also be increased by moderate exercise [25] but, in general, its response to exercise has not been well characterised. Thus, further studies are warranted. Exercise does not produce significant changes in plasma gonadotropins or thyrotropin [23].

While POMC-derived peptides and glucocorticoids may enhance some immune responses at very low concentrations, they are generally considered to be immunosuppressive [19]. Adrenalin is also immunosuppressive [28]. The immunostimulatory properties of GH and prolactin are antagonised by ACTH and glucocorticoids [28]. ACTH – which stimulates cortisol release from the adrenal gland and is secreted concomitantly with β-endorphin – does not rise in the plasma until exercise intensity exceeds 60% VO₂max [35]. This threshold must be exceeded before cortisol (the major human glucocorticoid) and opioid release are triggered [35, 36]. These increases are not effected significantly by prolongation of the workload because maximum-intensity exercise causes...
plasma ACTH and β-endorphin concentrations to rise within 30-60 seconds [37, 38]. The rise in the plasma concentration of catecholamines is five-fold larger after maximal exercise than the small increase induced by moderate exercise [39]. Noradrenaline secretion is triggered at much lower workloads than those required to raise adrenalin [25] and noradrenalin, in turn, initiates GH release in the hypothalamus [40].

Correlation with increased circulating cortisol

Nearly all immunosuppressive responses induced by intense exercise correlate with increases in circulating cortisol, the plasma half-life of which is 60-90 minutes at rest. Cortisol release is slower than that of catecholamines and ACTH, and may not peak until 30 minutes after exercise has ceased. Sustained cortisol secretion, like the 200% increase observed after a marathon run [11], may represent failure of this glucocorticoid to trigger, under these conditions, the feedback signal that shuts down ACTH release [41]. While the plasma concentrations of opioids and adrenalin usually return to resting values 10-20 minutes after exercise, these hormones may also contribute to suppressed immune responses after intense exercise [12, 42]. The return of their plasma concentrations to resting values may not coincide with the disappearance of their immunosuppressiv effects. Some of these changes may be prolonged and may require the recruitment of recently matured cells from bone marrow to reverse them. The effects of training on the rate of leucocyte production and differentiation has not been investigated.

Although adrenalin and, possibly, opioids may initiate
transient perturbations in immune responses after intense exercise, cortisol appears to be the major mediator of sustained immunosuppression. Cortisol inhibits virtually all functions of cells of the monocyte/macrophage lineage and severely impairs neutrophil microbicidal activity [28]. The ability of cortisol to suppress every major T-cell function, including helper and killer activities, is quite well documented, but B-cell responses are variable [28]. The cytotoxic activity of NK-cells is also inhibited by glucocorticoids [28]. Adrenalin treatment of isolated cells in vitro inhibits mitogenesis of lymphocytes [3], the oxidative burst of macrophages and neutrophils [28] and the tumouricidal and antiviral activities of macrophages [43].

Opioid addiction increases susceptibility to a variety of infections. This may be due to suppressed T-cell proliferation and neutrophil macrobicidal activity [44]. While the transient 15 minute increase in NK-cell cytotoxicity induced by prolonged submaximal exercise may be mediated by low concentrations of endorphins, the post exercise decrease—sustained for 24 h—may be due to excessive release or slow catabolism of the same agents [12, 45]. A small proportion of the major immune cell populations (discrete subpopulations) may escape these suppressive effects by “down regulating” the appropriate receptors and/or uncoupling signal transduction mechanisms.

Psychological stress is also associated with elevated cortisol activity and impaired immune responses [46]. Stress caused by trauma, infection, pain and fear also stimulates the secretion of cortisol [47]. Because the neuro-endocrine responses to intensive physical exertion and psychological stress bear striking similarities, it is not surprising that similar changes in immune responses also occur. In contrast, moderate training reduces the basal circulating concentration of β-endorphin and this correlates with greater emotional stability and reduced depression [48].

Do cytokines have a role?

Members of the cytokine family include monokines, lymphokines, interferons and haemopoietic growth factors [22]. They are present in plasma in minute concentrations, except under pathological conditions, and may act locally at picomolar concentrations [22, 49]. Cytokines act both alone and/or synergistically to regulate the proliferation and activation of all immune cells in vitro [22], but their exact functional roles and their interactions with neuro-endocrine hormones in vivo are not known. The signal that causes a significant rise in corticotropin release hormone (CRF) to stimulate ACTH release from the anterior pituitary is not known with certainty but the rapidity with which it responds to intense exercise suggests that local (hypothalamic/pituitary), rather than systemic, factors are responsible. In this context, the monokines (interleukin-1 and tumour necrosis factor) are potent activators of CRF and ACTH release in vitro [50]. Monokines may thus play a pivotal role as mediators between the immune and neuro-endocrine systems.

Cannon and Kluger [51] were the first investigators to report changes in cytokine activity after exercise. They showed that plasma and supernates from monocytes isolated from human subjects after one hour of cycling at 60% V̇O₂max contained a substance (not endotoxin) capable of eliciting a significant rise in rat body temperature upon injection. Further work using a thymocyte proliferation assay suggested that this agent was interleukin-1 (IL-1) [24]. The plasma concentration of interleukin-1 has been shown, additionally, to increase after a similar workload [52].

We originally proposed that the priming of neutrophils induced by exercise may be due to IL-1 release into the circulation following moderate exercise; in contrast, the depressed neutrophil activity observed after one minute of exercise at maximal capacity may be mediated through cortisol, adrenalin and opioids [15]. However, other cytokines like tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are more potent neutrophil-priming agents in vitro [53]. IL-1 and TNF secretion from monocytes may be stimulated by endotoxin released from indigenous gut microflora into the portal circulation as a result of the reduced splanchnic blood flow and mild hypoxia that accompanies exercise of moderate intensity [54].

Endotoxin, itself, may also contribute to priming of neutrophils [53]. The endotoxia that has been reported after ultramarathon running [54] could initiate a cascade that triggers the sustained release of cortisol observed [11]. Low concentrations of circulating endotoxin may be responsible for the small increases in plasma monokines we have observed after moderate exercise.

We demonstrated that the plasma concentrations of the monokines IL-1β and TNF-α (detected by immunoradiometric assays) increased significantly 3–6 h after moderate exercise, but the kinetics of the response did not coincide with the priming of neutrophil oxidative burst activity (Smith et al., unpublished), which always appeared immediately after exercise. Monokines may be active, instead, in maintaining the primed state for at least six h [15]. Prolonged running causes a similar elevation in plasma TNF-α [18]. Conversely, very large increases in plasma monokine concentration may be detrimental. While local hypothalamic/pituitary factors may trigger CRF to induce immediate ACTH release in response to maximal exercise, monokines—which can be released into the portal circulation of the pituitary gland [55]—may activate a sustained release of ACTH during prolonged submaximal exercise, perhaps leading to the chronic immunosuppression observed in some (perhaps, overtrained) athletes [2].

While moderate exercise did not alter the plasma concentrations of the lymphokines interferon-γ and GM-CSF, we detected a significant acute-phase response (i.e., presence of C-reactive protein in plasma) in untrained subjects 24 h post exercise (Smith et al., unpublished). The occurrence of an acute-phase response after intense exercise is well known [56]. It is also indicative of the presence of IL-6 (IL-6 release is triggered by IL-1 and TNF) because this cytokine is known to induce the synthesis of all major acute-phase proteins in the liver [57].

Cytokine responses to maximal exercise have not been studied in vivo. After progressive exercise to maximum capacity, the ability of endotoxin-stimulated human monocytes to release IL-1 increased two-fold but IL-2 release from PHA-stimulated lymphocytes decreased 30% [58]. These responses were sustained for two hours after exercise. However, in vitro assays of this type measure only the potential for cytokine release. The influence of exercise intensity and duration on plasma cytokine concentrations in vivo requires further investigation.

While the ability of cytokines (IL-1 and TNF) to stimulate neuro-endocrine hormone release and glucocorticoids to inhibit cytokine release from immune cells in vitro is well established, the influence of hormones on cytokine production and secretion has received limited attention.

Substance P stimulates the production and secretion of IL-1 and other monokines preferentially from pre-activated mono-
cytes in vitro [59]. Arginine vasopressin and oxytocin trigger γ-interferon release from mouse lymphocytes in vitro [60]. The diverse range of immunomodulatory effects documented for GH and prolactin are likely to extend to cytokine production and release. Preliminary reports suggest that prolactin is required for the normal secretion of γ-interferon by antigen-stimulated mouse lymphocytes in vitro [61] and GH treatment in vivo augments the capacity of endotoxin to induce greater necrosis of Meth A tumours in mice. These results suggest that GH, and possibly prolactin, may prime macrophages for greater TNF release in vivo [62] and they correlate with the previous findings of this group showing that GH and prolactin also prime superoxide release from opsonised zymosan-stimulated macrophages [31].

Hypothesis

The observations showing that GH and prolactin are potent macrophage priming agents suggest that changes in the circulating concentrations of these putative hormones may trigger the exercise-induced priming of phagocytic cells. Additionally, the leakage of small amounts of endotoxin into the circulation during moderate exercise may trigger monokine release and sustain the exercise-induced priming of phagocytic activity. Because the plasma concentration of GH is elevated at lower workloads than those that increase prolactin, it is the most likely candidate responsible for enhancing immunity in response to moderate exercise. The release of substance P during mild exercise may also contribute. The plasma concentration of GH increases 10-fold after 30 minutes of exercise at an intensity of 60% VO2,max, but this is not sufficient to induce adrenocorticotrophic hormone (ACTH) release or the secretion of glucocorticoids and opioids.

We propose that low to moderate levels of exercise may boost immunity through the action of GH (and, possibly, substance P) on cellular and humoral immune mechanisms. These beneficial effects may be negated once exercise intensity reaches a critical threshold beyond which, through feedback mechanisms initiated by high concentrations of monokines (interleukin-1 and tumour necrosis factor which act as the molecular switch), ACTH release is activated. ACTH, in turn, induces the release of immunosuppressive glucocorticoids and opioids into the circulation. Immunosuppression may be manifested as a consequence of sustained ACTH release and cortisol elevation (Figure 1).
CHAPTER 3.2

Cytokine immunoreactivity in plasma does not change after moderate endurance exercise
Cytokine immunoreactivity in plasma does not change after moderate endurance exercise

JOHN A. SMITH, RICHARD D. TELFORD, MARK S. BAKER, ANDREW J. HAPEL, AND MAURICE J. WEIDEMANN

Division of Biochemistry and Molecular Biology, School of Life Sciences, Faculty of Science; and Division of Clinical Sciences, John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory 2600; and Department of Physiology and Applied Nutrition, Australian Institute of Sport, Belconnen, Australian Capital Territory 2616, Australia

SMITH, JOHN A., RICHARD D. TELFORD, MARK S. BAKER, ANDREW J. HAPEL, AND MAURICE J. WEIDEMANN. Cytokine immunoreactivity in plasma does not change after moderate endurance exercise. J. Appl. Physiol. 73(4): 1396-1401, 1992.—We investigated whether increased concentrations of circulating cytokines may be responsible for exercise-induced priming of blood neutrophils (J. A. Smith et al. Int. J. Sports Med. 11: 179-187, 1990). The plasma concentrations of tumor necrosis factor-α, interleukin-1α, IL-6, granulocyte-macrophage colony-stimulating factor, and neopterin in trained and untrained human subjects were measured by immunoassay before and after 1 h of cycling at 60% of maximal oxygen uptake. C-reactive protein and creatine kinase (CK) were also measured before and 24 h after exercise as markers of the “acute-phase response” and muscle damage (C. Taylor et al. J. Appl. Physiol. 62: 464-469, 1987), respectively. The small changes in the plasma concentrations of cytokines or neopterin observed after exercise in both trained and untrained subjects were not significantly different to those found in a control group of nonexercised subjects. However, untrained subjects did exhibit an acute-phase response (P = 0.04) 24 h after exercise without additional release of CK into plasma. Baseline training differences were confined to a twofold elevation in CK activity (P = 0.04). The results show that circulating cytokines are unlikely to be responsible for the priming of neutrophil microbicidal activity observed after moderate endurance exercise (J. A. Smith et al. Int. J. Sports Med. 11: 179-187, 1990).

acute-phase response; exertion; granulocyte-macrophage colony-stimulating factor; immunity; neutrophils; neopterin; neurohumor; stress

ACUTE EXERCISE by human subjects is capable of inducing some immune or inflammatory responses similar to those that accompany infection and mild trauma (5). Although many reports suggest that moderate exercise may enhance resistance to infection, the effects of prolonged or intense physical activity are thought to be detrimental (11, 18, 25). The mechanisms by which these paradoxical physiological responses to exercise occur are poorly understood.

We reported recently that the capacity of human peripheral blood neutrophils to produce microbicidal reactive oxygen species on stimulation in vitro was “primed” significantly after 1 h of cycling at 60% of maximal oxygen uptake (VO_{2 \text{ max}}) (24). While the mechanism(s) responsible was not elucidated, “priming” might have occurred in response to putative neutrophil-priming cytokines like interleukin-1 (IL-1) released into the circulation (3). In fact, endogenous pyrogen and thrombocyte-stimulating activities have been detected in plasma collected up to 6 h after the completion of exercise and in the supernatants of monocytes isolated from the same subjects (3, 5, 9). This heat-labile activity (designated IL-1) was also found before exercise but at higher levels in trained subjects than in untrained controls (9). Data obtained from bioassays (3, 5, 9), however, are rarely wholly specific, and the results may be influenced by the presence of inhibitory or stimulatory agents in plasma (26). For instance, tumour necrosis factor-α (TNF-α) has also been shown to act as an endogenous pyrogen and to stimulate thymocyte proliferation (16). In addition, IL-1 and TNF both exist in two distinct molecular forms (i.e., α and β), and one of these (IL-1β) has been detected immunochemically in human skeletal muscle after vigorous eccentric exercise (4).

The neutrophil “oxidative burst” is known to be primed in vitro by various cytokines that may act individually or synergistically (27). Because IL-1α and IL-1β are only weak neutrophil-priming agents (27), we hypothesized that other cytokines, particularly the more potent priming agents like TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (27) might be responsible for the exercise-induced priming of neutrophil microbicidal activity (24). The aim of this study was to determine whether 1) an acute episode of moderate exercise and 2) regular intensive training caused detectable changes in the plasma concentrations of several relevant cytokines, in particular, immunoreactive forms of IL-1α, IL-6, TNF-α, and GM-CSF. Because interferon-γ (IFN-γ) is degraded rapidly after extracellular release, the concentration of the more stable monocyte metabolite neopterin was measured in plasma as an indirect indicator of the presence of IFN-γ (15, 29). To monitor whether exercise or training induced a detectable acute-phase response and/or significant muscle damage, plasma C-reactive protein (CRP) and total creatine kinase (CK) activities were measured before exercise in all subjects and at 24 h after exercise in untrained subjects only (28).
TABLE 1. Physical and physiological characteristics of the untrained and trained human subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Age, yr</th>
<th>Weight, kg</th>
<th>Height, cm</th>
<th>Work load, W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untrained</td>
<td>8</td>
<td>25.8±4.66</td>
<td>69.7±6.75</td>
<td>175.4±4.93</td>
<td>175.0±25.0</td>
</tr>
<tr>
<td>Trained</td>
<td>8</td>
<td>26.1±3.53</td>
<td>71.6±8.58</td>
<td>179.5±6.46</td>
<td>250.0±25.0</td>
</tr>
<tr>
<td>Runners</td>
<td>6</td>
<td>26.9±4.10</td>
<td>67.2±5.01</td>
<td>174.6±3.50</td>
<td></td>
</tr>
<tr>
<td>Swimmers</td>
<td>7</td>
<td>20.0±1.83</td>
<td>78.1±7.69</td>
<td>183.9±7.17</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of subjects.

MATERIALS AND METHODS

Exercise protocol and human subjects. Eight untrained and eight trained male subjects (6 triathletes, 2 endurance runners) cycled on a geared bicycle ergometer at a heart rate of ~140 beats/min for 1 h; this working heart rate was calculated from previous studies to approximate 60% \( V_{O_2\text{max}} \) (1, 24). The working heart rate remained relatively constant throughout the test (140 ± 10 beats/min). Physical and physiological characteristics of the subjects are presented in Table 1. To compare the effects of very intensive training on baseline plasma concentrations of cytokines, six elite endurance runners and seven elite swimmers were also recruited. The project was approved by the Ethics in Human Experimentation Committee of the Australian National University; all subjects gave informed consent.

The untrained subjects devoted <5 h/wk to recreational physical activity. The six triathletes and the two runners who participated in the exercise experiments were training at a much lower intensity than the two internationally ranked groups (see below). The triathletes were training up to 20 h/wk and competing at national level; their weekly training program consisted of 50–60 km running, 10–12 km swimming, and 200–250 km of cycling and included no resistance or weight work. At the time of the test, they were in the precompetition phase of their training program. The two runners were running about 60 km/wk. Of the internationally ranked groups, the seven swimmers, who all ranked in the top Australian three in their respective events (100–400 m), were training 80–100 km/wk in the pool. This represented an endurance-development phase of their training program, which also included three sessions of gymnasium work (including weight training) per week. The six elite runners were all ranked in the top Australian five in their respective events, which involved regular competitions over distances varying between 800 m and 10,000 m. Training involved 12–14 running sessions/wk, covering a total distance that varied between 100 and 180 km. At the time of sampling, the runners were completing an endurance phase of their training program that was focused on the development of aerobic power. Training also included two sessions of weights per week. Plasma samples analyzed for GM-CSF were taken from the trained and untrained groups used in our previous study (24).

Blood samples. Blood samples were taken before exercise (after subjects had rested in the sitting position for 10 min), immediately after exercise, and 3 and 6 h postexercise in all subjects. An additional sample was taken at 24 h postexercise from the untrained subjects. Blood samples were taken from five untrained subjects (who did not exercise) at each time point to assess the influence of circadian variations in situations where evidence of exercise effects was found. Blood was drawn from the antecubital vein into sterile syringes and transferred immediately to sterile EDTA tubes. No subject had exercised in the 24-h period before the test, nor did they exercise again during the blood sampling period. All exercise testing was carried out between 9:00 and 11:00 a.m. Apart from advising subjects to eat light meals on the day of testing, there were no dietary restrictions. Red blood cell and white blood cell counts, hematomers, and hemoglobin concentrations were determined by an automated Coulter Counter (Coulter Electronics, Hialeah, FL). The changes in plasma volume after exercise were calculated by the equation of Schmidt et al. (22) and found to be negligible (0–6%) in all subjects. Plasma was separated from whole blood at 4°C by centrifugation at 500 g for 10 min and prepared as "platelet-poor plasma" by subjecting it to an additional centrifugation step (3,000 g for 20 min). Plasma was stored immediately at −196°C in liquid nitrogen until assayed.

Assays. All assays were performed in duplicate, and intra-assay variation was <10%. IL-1β, IL-6, and TNF-α plasma concentrations were determined by immunoradiometric assays (Medgenix, Brussels, Belgium). The assays are based on coated-tube separation in which a mixture of several monoclonal antibodies against distinct epitopes of the relevant cytokine were fixed to the lower and inner surfaces of the plastic tube. After addition of the standard, control, or sample (200 µl), [125I]-labeled signal antibody (50 µl) was dispensed into each tube to trigger the immunological reaction and the mixture was incubated for 18 h at room temperature (RT). After washing, the remaining radioactivity bound to each tube was counted in a gamma counter for 3 min; the counts reflected the specific cytokine concentration. "Spiked" serum controls were supplied with the immunoradiometric assay (IRMA) kits, and their values were found to be in the expected range. The detection limit of the assays were TNF-α (1 pg/ml) (13), IL-1β (5 pg/ml), and IL-6 (6 pg/ml).

GM-CSF was measured by enzyme-linked immunosorbent assay (ELISA) (6) (Insight GM-CSF ELISA, kindly provided by Medical Resources, Sydney, Australia). Washing buffer (200 µl) was added to each well of a microtitre plate coated with a monoclonal antibody raised against recombinant human GM-CSF, and the plate was incubated for 30 min at RT. After removal of the buffer, samples and standards (150 µl) were then added to each well and the plate was incubated at 4°C for a further 1 h. On rewarming to RT, the standards and samples were discarded and the plate was washed three times with washing buffer before a rabbit anti-GM-CSF polyclonal antibody (40 µl) was added to each well. After a 2-h incubation at RT, any unbound second antibodies were removed by three washes. After the addition of an anti-rabbit antibody conjugated to horseradish peroxidase (HRP; 40 µl), the plate was subjected to another 1-h incubation at RT. The unbound conjugate was removed by six washes, and HRP substrate solution (100 µl) was added to each well. The reaction was terminated by addi-
TABLE 2. Baseline (preexercise) plasma concentrations of representative parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untrained (n = 8)</th>
<th>Trained (n = 8)</th>
<th>Runners (n = 6)</th>
<th>Swimmers (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK, units/I</td>
<td>112±21</td>
<td>165±20</td>
<td>325±55*</td>
<td>249±30*</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>2.40±0.30</td>
<td>1.96±0.40</td>
<td>2.93±0.26</td>
<td>6.7±3.4</td>
</tr>
<tr>
<td>IL-1β, pg/ml</td>
<td>12.9±2.7</td>
<td>10.6±0.6</td>
<td>10.5±1.9</td>
<td>15.9±9.0</td>
</tr>
<tr>
<td>Neopterin, pmol/ml</td>
<td>7.67±2.7</td>
<td>7.0±0.38</td>
<td>3.66±1.0</td>
<td>6.62±3.2</td>
</tr>
<tr>
<td>GM-CSF, pg/ml</td>
<td>19.4±3.0*</td>
<td>18.9±4.9*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. CK, creatine kinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; GM-CSF, granulocyte-macrophage colony-stimulating factor. * Subjects from Ref. 24 (n = 8 untrained, 8 trained). " Significantly different from trained and untrained subjects at 5% level.

tion of a stop solution (20 µl) after a 15-min incubation at RT. The absorbance was measured on a Dynatech plate reader at 410 nm using a reference filter at 450 nm. The detection limit of the assay was 10 pg/ml.

Neopterin was measured by radioimmunoassay (Henning, Berlin, FRG) (29). In separate tubes, sample, control or standard (20 µl) was mixed with [125I]-labeled neopterin tracer (100 µl) and preprecipitated antiserum (100 µl). The tubes were incubated in the dark for 1 h at room temperature, and, after addition of washing solution (1 ml), they were centrifuged for 10 min at 2,500 g. The supernatant was aspirated, and the radioactivity of each tube was counted in a gamma counter for 3 min. Control values were within the expected range, and the detection limit of the assay was 1 pmol/ml.

Total plasma CK activity was assessed spectrophotometrically using the Kodak Ektachem system (Eastman-Kodak). The plasma sample (10 µl) was placed on a Ektachem DT slide, and, in a series of coupled reaction sequences that began with the conversion of creatine phosphate and ADP to creatine and ATP by the CK in each sample, a leukocyte precursor was finally oxidized by H2O2 to produce a chromophore. Reflection density was monitored continuously at 680 nm throughout the 5-min incubation period, which was carried out at 37°C. The analyzer was calibrated with standards supplied by the manufacturer. The detection limit of the assay was 20 units/l.

CRP was measured by radial immunodiffusion. The standard, control, or sample (20 µl) was added to wells of low concentration- (LC) Partigen plates (Behring, Marburg, FRG) and incubated for 3 days at RT. The diameters of the precipitates were measured and sample and control values were compared with standards. Control values were within the expected range. The detection limit of the assay was 0.06 mg/dl.

Statistical analysis. Where single comparisons only were required, the results were analyzed by the appropriate Student’s t-test. A method for comparing profiles of repeated measures was performed to analyze exercise and circadian effects where more than one postexercise or control measurement was made (19).

RESULTS

With the exception of CK, endurance training did not significantly influence the baseline (resting) plasma concentrations of any of the parameters measured and there was considerable variability between each subject (Table 2). This was also the case after exercise. Therefore, the pooled observations from both groups were analyzed statistically for exercise effects.

The plasma concentration of IL-1β ranged from 0 to 20 pg/ml. Small increases in plasma IL-1β were detected in the 6-h period after exercise, but these differences were not significant compared with control measurements taken over the same time period with five unexercised subjects (Fig. 1). In addition, plasma IL-1β concentration did not change significantly 24 h after exercise when measured in the eight untrained subjects.

TNF-α was detectable over the range 0–8 pg/ml in plasma. Like IL-1β, the plasma concentration of TNF-α increased during the 6-h period after exercise, but these changes were not significant compared with control measurements on unexercised subjects (Fig. 2). In eight untrained subjects, there were no significant changes 24 h after exercise. A positive correlation was observed between the baseline plasma concentrations of TNF-α and IL-1β (r = 0.83) with IL-1β being about twofold higher than TNF-α.
The plasma concentrations of neopterin, GM-CSF and IL-6 did not change significantly during the 6-h period after exercise in both the trained and untrained groups or at 24 h after exercise in the eight untrained subjects. In the absence of significant change, no control measurements in unexercised subjects were required. Plasma neopterin was detected over a range of 0–21 pmol/ml (Fig. 3). Plasma GM-CSF concentration was detected in the range of 10–100 pg/ml, but, in contrast to the small changes in the other cytokines detected after exercise in individual subjects, nearly all individual postexercise values were equal to the resting values. IL-6 was detectable in plasma from only four of the eight untrained subjects both before and after exercise (range 0–20 pg/ml). No significant exercise effects were found. Samples from trained subjects were not assayed for IL-6.

There were no subjective reports of delayed muscle soreness after the exercise test, nor did CK activity increase in the plasma of untrained subjects 24 h after exercise. Trained subjects were not tested for plasma CK activity 24 h after exercise because the test work load was considerably lighter than that undertaken daily. Mean baseline plasma CK activities in both elite runners and swimmers were not different from each other but were significantly higher than those measured in the trained and untrained subjects (Table 2; P = 0.04). There were no correlations between the baseline plasma concentrations of CK and any cytokine.

Along with CK, CRP was assessed, first, as a marker of exercise and training-induced tissue injury and, second, as a product of the acute-phase response (28). CRP was not detected in plasma from trained subjects before exercise but did, unexpectedly, register detectable levels in two of the eight untrained subjects at rest. Twenty-four hours after exercise, however, there was detectable CRP in plasma from six of the eight untrained subjects (P = 0.04).

DISCUSSION

In contrast to previous reports (3, 5), the results show that 1 h of cycling at 60% $V_{O_2 max}$ did not significantly alter the plasma concentration of IL-1β or any of the other cytokines measured compared with circadian variations over the same time period. Furthermore, with the exception of CK, endurance training did not have an effect on the resting (baseline) or postexercise plasma concentrations of any of these parameters (although IL-6 was not measured in trained subjects). Therefore, elevation in the circulating concentrations of these cytokines appears unlikely to be responsible for the priming of neutrophil microbicidal activity observed after the same exercise work load (24).

Cannon and Kluger (5) originally showed that plasma isolated from human subjects immediately after 1 h of exercise at 60% $V_{O_2 max}$ contained detectable endogenous pyrogen activity that was even higher 3 h later. Further work showed that, when plasma samples were assayed for IL-1 by a thymocyte-proliferation assay, this cytokine could only be detected at 3–6 h after the same work load (3). The presence of IL-1 was confirmed with an IL-1-neutralizing antibody, and no circadian variations were reported over this time period (3). Thus it is likely that small increases in the plasma concentrations of a combination of cytokines (e.g., TNF-α, IL-1β, and IFN-γ) may have contributed to the exercise-induced increases in plasma endogenous pyrogen and thymocyte-proliferation activity reported previously (3, 5, 30), perhaps through synergistic amplification. The appearance of CRP in the plasma of the majority of the untrained subjects 24 h after the exercise test is indicative of the local action of cytokines (28) and shows that the acute-phase response occurred in the absence of significant muscle damage.

As a result of cellular uptake, the net release of these pleiotropic cytokines into the circulation may only approximate the activity found in localized tissue sites and cytokine-responsive cells (20). High circulating cytokine activity is associated with pathological conditions where the concentrations are 10–50 times higher than those found in healthy subjects (13, 20). In addition, cytokine immunoreactivity or bioactivity is likely to be masked, to some extent, by circulating carrier proteins or endogenous inhibitors (17, 26). These proteins may include soluble receptors shed into the circulation under normal conditions (26) by cytokine-responsive cells like neutrophils (21). Despite these concerns, we are confident that the IRMAs used in this study are detecting biologically active cytokines because a significant positive correlation has been demonstrated between the TNF-α IRMA assay used here, a radioimmunoassay and the L929 bioassay for TNF-α (13).

While the trained and untrained groups worked at an intensity of 60% $V_{O_2 max}$ for 1 h, cytokine secretion may be influenced by other factors like anaerobic threshold, power output, and associated heat production. In fact, several reports suggest that increasing the intensity and duration of exercise triggers cytokine release into the circulation. For example, strenuous running for 2.5 h has been shown to raise the plasma concentrations of TNF-α, neopterin, soluble IL-2 receptor, and complexed neutrophil elastase; the presence of elastase in plasma is indicative of neutrophil degranulation (7). After a 5-km race, the median plasma concentration of TNF-α in 11
highly trained runners showed a small but significant increase 2 h after the race and returned to normal 24 h later; in contrast, plasma IL-2 concentration, which decreased immediately after the race, was followed by a significant increase 24 h later (8). Endotoxin, a potent stimulus of cytokine secretion, has also been detected in plasma after an ultratriathlon (2). Measurements of cytokine release from isolated mononuclear cells stimulated with mitogens in vitro have shown that exercise for 1 h at 80% \( \text{VO}_{\text{max}} \) significantly increased the secretion of both IL-6 and IL-1 without enhancing the secretion of TNF-\( \alpha \), IFN-\( \gamma \), or IL-2 (14), whereas, in contrast, IL-2 secretion from mitogen-stimulated mononuclear cells in vitro has been reported to increase 44% after 60 min of exercise at 60% \( \text{VO}_{\text{max}} \) (23). Assays of this type measure only the potential capacity of cells to release cytokines in vivo. None of these studies controlled, however, for the influence of circadian variations.

Because the plasma concentrations of neopterin did not change significantly in response to exercise or endurance training, these results suggest that T-cell capacity to secrete IFN-\( \gamma \) was not affected by acute or chronic endurance exercise. It is not known whether other circulating agents like hormones and neuropeptides affect neopterin production directly, but neopterin release from macrophages is not triggered directly by TNF (\( \alpha \) and \( \beta \), IL-1 (\( \alpha \) and \( \beta \)), IL-2, IL-6, or GM-CSF (15, 29). Although neopterin has no known immunological function itself, the plasma and urine concentrations of neopterin correlate with activation of the cell-mediated immune response and high values (which are found in patients with infectious or inflammatory diseases) may indicate a refractory response of IFN-\( \gamma \) release to antigenic stimulation (15, 29). Thus neopterin measurements may be useful in the assessment of suspected cases of overtraining or chronic fatigue where there is concomitant immune dysfunction.

The higher baseline activity of CK detected in plasma from the internationally ranked swimmers and runners is likely to be due to mild muscle damage caused by eccentric contractions during resistance and weight training and their greater training intensity (4, 9). While no correlations were found between plasma cytokines and centric contractions during resistance and weight training, these results suggest that T-cell capacity to secrete cytokines may be confined to local sites like muscle where they may ameliorate damage and promote wound healing and tissue remodeling by activating the removal of damaged cells by phagocytes.

In conclusion, our results show that the plasma concentrations of IL-1\( \beta \), IL-6, TNF-\( \alpha \), GM-CSF, and neopterin were not affected by 1 h of cycling at 60% \( \text{VO}_{\text{max}} \) in both endurance-trained and untrained human subjects. Furthermore, endurance training did not influence the baseline plasma concentrations of these factors. Thus the evidence suggests that these cytokines are unlikely to be responsible for exercise-induced priming of neutrophil microbicidal activity (24). A recent report that indicates that growth hormone is a potent neutrophil-priming agent (12) suggests that this factor may be the responsible mediator because its concentration in plasma increases at least 10-fold after the same work load (10; J. A. Smith and M. J. Weidemann, unpublished data). This hypothesis (25) is currently under investigation.

NOTE ADDED IN PROOF

Sprenger et al. (26a) have reported that cytokine concentrations do not change significantly in the plasma of well-trained runners after a 20-km road race. They were able to demonstrate, however, quite large increases in IFN-\( \gamma \), TNF-\( \alpha \), IL-1\( \beta \), IL-6, and soluble IL-2 receptor in urine samples taken after the race, which is indicative of enhanced production and/or release of these factors during the run (26a). Thus failure to detect changes in plasma cytokine concentrations may be due to their rapid clearance from the circulation.

We are grateful to the athletes and untrained subjects for their time and cooperation. We thank Ross Cunningham (Department of Statistics, Australian National University) for performing the statistical analyses of the repeated-measures data. We also thank Australian Laboratory Services (Sydney, NSW) for assistance in obtaining the Medgenix assay kits promptly.

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CHAPTER 3.3

Mechanisms involved in exercise-induced priming of neutrophil microbicidal activity.
2. Neuroendocrine aspects
MECHANISMS INVOLVED IN EXERCISE-INDUCED PRIMING OF NEUTROPHIL MICROBICIDAL ACTIVITY.

2. NEUROENDOCRINE ASPECTS

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ABSTRACT

Exercise appears to modulate the immune system in an intensity-dependent manner in which moderate exercise is potentiating but strenuous effort results in suppression. We have developed a neuroendocrine/cytokine hypothesis to explain this paradox. The first phase of this work indicated that cytokines were not involved (Smith et al., 1992). The neuroendocrine arm is tested here by determining whether increases in the plasma concentrations of various immunoregulatory hormones (measured by immunoassays) in response to moderate exercise was associated with priming of the oxidative burst of stimulated neutrophils in vitro. One hour of moderate exercise increased the plasma concentration of growth hormone by 10- to 20-fold in both trained and untrained subjects but it did not alter the concentrations of prolactin, substance-P or endotoxin. In contrast to previous reports, we did find increases in the plasma concentrations of adrenocorticotropic hormone (50%) and cortisol (20%) in the untrained subjects only (P < 0.05). While consumption of a glucose solution (5% w/v) every 15 min during exercise substantially attenuated growth hormone secretion, a three-fold increase was still found; glucose consumption did not influence the responses of any other hormone to exercise. However, while this modest rise in growth hormone was associated with priming of intracellular H₂O₂ production in response to stimulation with phorbol myristate acetate, increased complement receptor expression, and the release of elastase into the circulation, the magnitude of all these responses declined progressively once plasma growth hormone concentration reached and increased beyond a critical threshold. These results are consistent with the hypothesis that growth hormone may differentially modulate the intensity-dependent responses of neutrophils, and perhaps other immune cells, to exercise.
INTRODUCTION

In the preceding paper we reported that, while single episodes of moderate aerobic exercise apparently boost some aspects of neutrophil microbicidal activity, an acute bout of maximal exercise or regular intensive endurance training both tend to depress the cytotoxic reactions associated with the oxidative burst (Smith et al., 1993). The mechanisms that underlie these paradoxical responses to exercise are not known; however, cytokines and neuroendocrine hormones may be involved (Smith and Weidemann, 1990).

We originally suggested (Smith et al., 1990) that exercise-induced priming of neutrophils may be mediated through pyrogenic cytokines that others had reported to be released into the circulation in response to one hour of moderate cycling (Cannon et al., 1986). This hypothesis was rejected because our own work showed that moderate exercise did not increase, in either trained or untrained human subjects, the immunoreactive plasma concentrations of the putative neutrophil-priming cytokines IL-1β, TNF-α, GM-CSF or IL-6 above those caused by time-dependent fluctuations in unexercised controls (Smith et al., 1992). Simpson and Hoffman-Goetz (1991) also reported that the concentration of IL-1β in the plasma was not altered by moderate exercise or endurance training to any greater extent than that caused by normal circadian variation. These results suggest that circulating cytokines are unlikely to be the major factors responsible for exercise-induced priming of neutrophils unless the process occurs extravascularly in localised marginated pools from which these cells are subsequently expelled into the general circulation during exercise. Neutrophils may also be primed directly by bacterial endotoxin (i.e. lipopolysaccharide) which is a potent stimulus of cytokine release from monocytes (Michie et al., 1988). Traces of free endotoxin have been detected in the circulation of triathletes at the end of a 70 km ultra-triathlon of six hours duration, perhaps in response to mild hypoxia in the splanchnic circulation and/or heat-stress, both of which lead to the release of endotoxin (derived from gut bacteria) into the portal circulation (Bosenberg et al., 1988).

In addition to cytokines and endotoxin, neuroendocrine hormones such as growth hormone (GH), prolactin (Fu et al., 1991), and substance-P (Perianin et al., 1989) have been reported to be potent neutrophil-priming agents. Three independent groups have now described priming effects of GH that show, unequivocally, its capacity to reproduce in vitro a priming phenomenon (Fu et al., 1991; Wiedermann et al., 1991; Spadoni et al., 1991) similar to the one we have observed immediately following moderate
exercise (Smith et al., 1990; 1993). The secretion of GH into the circulation is triggered by exercise of mild to moderate intensity but consumption of glucose during exercise antagonizes this response (Galbo et al., 1985). In contrast to moderate exercise, maximal exercise may have immuno-suppressive effects that may be mediated by “stress hormones” such as adrenalin, adrenocorticotropic hormone (ACTH) and cortisol which increase in the circulation only after exercise intensity exceeds 60% of maximal aerobic capacity (VO₂ max) (Smith and Weidemann, 1990).

The aims of this project were to investigate: (i) whether exercise-induced changes in the neutrophil oxidative burst are consistent with differential modulating roles for bacterial endotoxin and/or hormones that are released into the circulation; (ii) whether endurance-training influences these responses; and (iii) whether consumption of glucose during moderate exercise prevents the priming of neutrophil microbicidal activities through its action in limiting the exercise-induced rise in plasma GH.
MATERIALS AND METHODS

Human Subjects
The plasma samples were obtained from subjects used in exercise studies described previously: Group-1 (Smith et al., 1990); Group-2 (Smith et al., 1992); a longitudinal study; and from the phase-1 and phase-2 mechanistic studies reported in the accompanying paper on cellular parameters (Smith et al., 1993). The project was approved by the Ethics in Human Experimentation Committee of the Australian National University; all subjects gave informed consent.

Assays
The procedures common to all assays are described, followed by specific information on each assay. All assay kits were obtained commercially and carried out according to the manufacturers' instructions. The radioactivity bound to antibody-coated tubes or in pelleted immune complexes was determined for one min on a gamma counter. Non-antibody-coated tubes were used to measure the total counts of radioactive tracers and non-specific binding. All measurements were performed in duplicate and intra-assay variation was less than 10%. The activity of standards added to serum or plasma samples from non-exercised controls (i.e. “spiked” samples) was always greater than 90% of the expected value, with the exception of the endotoxin assay, which was 80%.

The plasma concentrations of growth hormone and prolactin were measured separately by radioimmunoassay in antibody-coated tubes (Farmos Diagnostica, Turku, Finland). The standard, “spiked” serum controls and samples (100 µl) were pipetted into separate tubes coated with a monoclonal antibody against either human GH or prolactin. Human GH-[125I]- or prolactin [125I]- tracer (200 µl) and antisera against these hormones (200 µl) were added to all tubes which, after mixing, were incubated for 21 hours at room temperature. The liquid contents of each tube were decanted and washing solution (1 ml) was added. The tubes were shaken gently and the washing solution removed by decantation before the bound radioactivity was counted. Detection limits: GH (0.4 ng/ml); prolactin (1.0 ng/ml).

ACTH concentration in plasma was measured by the Dynotest® immunoradiometric assay (Henning, Berlin, Germany). Saline buffer (100 µl) was added to each tube coated with mouse anti-ACTH (15-21) antibody. Standards, “spiked” serum controls and plasma samples (200 µl) were pipetted into separate tubes. All tubes were covered with aluminium foil and incubated on an orbital shaker (200 rpm) at room temperature for
two hours. Washing buffer (2ml) was added to each tube and the contents removed by decantation. The washing procedure was performed twice and the tubes were inverted and drained on blotting paper to absorb the remaining liquid. $^{125}$I-Anti-ACTH-[34-39] tracer (300 µl) was added to each tube. After a 20 hour incubation at $4^\circ$C, the liquid contents were removed by aspiration and the tubes washed three times with washing buffer (2 ml) before the bound radioactivity was counted. Detection limit: (1.0 pg/ml).

The plasma concentration of cortisol was measured by a specific radioimmunoassay (Farmos Diagnostica, Turku, Finland). Each standard, control and plasma sample (25 µl) was pipetted into the appropriate tube prior to the addition of cortisol-$^{125}$I reagent (100 µl) and cortisol antiserum (100 µl). All tubes were vortexed briefly and incubated for one hour at $37^\circ$C, and cooled to room temperature for 10 minutes prior to the addition of polyethylene glycol (1 ml). The tubes were vortexed and centrifuged for 15 mins at 2000 x g. The supernatants were removed by aspiration before counting of the radioactivity in the pellets. Detection limit: (2.0 ng/ml).

The testosterone concentration in plasma samples was measured by radioimmunoassay (Farmos Diagnostica, Turku, Finland). Standards, "spiked" serum controls and plasma samples (50 µl) were pipetted into separate tubes coated with anti-testosterone antibody. Testosterone-$^{125}$I tracer (200 µl) and antiserum to testosterone (200 µl) were added to all tubes, and they were incubated for two hours at $37^\circ$C with gentle shaking. The liquid contents were removed by decantation prior to the addition of washing solution (1 ml). The tubes were shaken gently and decanted before measurement of the bound radioactivity. Detection limit: (0.1 ng/ml).

The plasma concentration of Substance-P was measured by radioimmunoassay (Peninsula, Belmont, CA, USA). Following its extraction from plasma (1 ml) with the acetone/petroleum ether technique described by Vinik and colleagues (1990), the air-dried extracts were reconstituted with assay buffer (125 µl). The standards, "spiked" serum controls and samples (100 µl) were pipetted into separate tubes. Rehydrated rabbit anti-substance-P antiserum (100 µl) was added to each tube and they were vortexed and incubated for 16 hours at $4^\circ$C. The addition of rehydrated substance-P-$^{125}$I tracer (100 µl) was followed by incubation for a further 16 hours at $4^\circ$C. Rehydrated goat anti-rabbit IgG serum (100 µl) and normal rabbit serum (100 µl) were then added to each tube, and they were vortexed and incubated for 120 minutes at room temperature prior to the addition of RIA buffer (0.5 ml). The tubes were vortexed and centrifuged for 20 minutes at 1700 x g. The supernatants were removed by aspiration and the radioactivity of the pellets in each tube was measured. Detection limit: (1.0 pg/ml).
The plasma concentration of endotoxin was measured using the chromogenic limulus amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MA, USA). The assay was performed as described in the manufacturers' instructions with the following modifications (Parsons et al., 1989). The plasma samples were diluted with pyrogen-free water (1:10) and heated to 70°C for 5 mins to prevent inhibition of the enzyme activity of limulus amoebocyte lysate by plasma factors. The first incubation with limulus amoebocyte lysate was extended from 10 to 30 mins at 37°C as recommended by the manufacturer to achieve maximum sensitivity. The detection limit of the assay under these conditions was 0.1 endotoxin units (EU)/ml.

Glucose consumption
The release of GH into the circulation during exercise has been reported to be blunted if subjects consume glucose. To test whether prevention of GH release affected the exercise-induced priming of neutrophils, each subject consumed 250 ml of a glucose-enriched solution (5% w/v) every 15 min (Galbo et al., 1985) during the one hour submaximal exercise test as described in the accompanying paper (Smith et al., 1993).

Statistical analysis
The results were analysed by Student's t test for paired (exercise) and unpaired (training) data. Correlations were calculated with the software package, Cricket Graph™ (Cricket Software, PA, USA).
RESULTS

The major result of this study is that the exercise-induced increase in plasma growth hormone (GH) is consistent with the hypothesis that this hormone has the capacity to modulate some aspects of neutrophil microbicidal activity in a concentration-dependent manner. Increases in various neutrophils activities were detected when the exercise-induced increase in GH was modest, but once its plasma concentration reached a critical threshold, activation of a neutrophil subpopulation \textit{in vivo} and, possibly, the immunosuppressive arm of the pituitary/adrenal axis, may have been triggered.

EXERCISE, GROWTH HORMONE AND NEUTROPHIL MICROBICIDAL ACTIVITY

\textit{Plasma Growth hormone}

\textbf{Group-1} Irrespective of training status, one hour of moderate cycling induced an increase in the plasma concentration of GH of at least 10-fold (Fig. 1a; \( P = 0.005 \)). Whilst this coincided with priming of the neutrophil oxidative burst, as detected by luminol-amplified chemiluminescence (Smith \textit{et al.}, 1990), there was only a weak positive correlation between the magnitude of the two responses (\( r = 0.27 \)). The pre-exercise (rest) values tended to be higher, but were not significantly different, in trained subjects, but untrained subjects showed the largest increase in plasma GH in response to moderate exercise.

\textbf{Phase-2} Because several laboratories have reported that glucose consumption during exercise can virtually abolish GH secretion (Hansen, 1971; Galbo \textit{et al.} 1985), we investigated whether the ingestion of glucose at regular intervals during exercise would prevent the secretion of GH and the associated priming of neutrophil microbicidal activity. As reported above, all subjects showed, following exercise, at least a 10-fold increase in the plasma concentration of GH (Fig 1b). The exercise-induced increase was reduced substantially (\( P < 0.001 \)), but not abolished, by intermittent glucose consumption during exercise (Fig. 1b).

The kinetics of GH secretion during exercise consisted of a distinct lag phase (\( \sim 10 \) mins) followed by a rapid linear increase in the plasma concentration which peaked after 45 mins and was maintained until exercise ceased (Fig 2). While glucose consumption substantially diminished the magnitude of the large exercise-induced rise in plasma GH, its kinetics remained similar to those found with the unsupplemented controls. No significant intra-individual variation in the plasma concentration of GH was detected over a one hour period in non-exercised control subjects.
Fig. 1. Moderate exercise and plasma growth hormone concentration.

(a) Plasma growth hormone concentrations immediately before and after exercise in both trained and untrained subjects (n = 11,9). Data are means ± SEM.

(b) The responses of the phase-2 group to exercise and the effect of glucose consumption (n = 8,8). Data are means ± SEM.
Fig. 2. The kinetics of growth hormone secretion during exercise.

Mean ± SEM of plasma growth hormone concentration during the moderate exercise test. The data are from the same subjects (n = 4) who on one day consumed 250 ml of a solution that contained D-glucose (5% w/v) every 15 min during the test, or who did not consume fluids during test on the other day.
Neutrophil responses to exercise and growth hormone

As reported in the accompanying paper, moderate exercise increased several aspects of neutrophil microbicidal activity including the expression of C<sub>3b</sub> receptors, intracellular H<sub>2</sub>O<sub>2</sub> generation in cells stimulated with phorbol ester, and elastase release (Smith et al., 1993). As shown in Figure 3a, modest increases in the plasma concentration of GH (up to 10 ng/ml) in exercising subjects - irrespective of glucose consumption - were associated with the largest increases in the intensity of intracellular H<sub>2</sub>O<sub>2</sub> generation while, in contrast, priming appeared to be progressively ameliorated once the plasma GH increased beyond a critical threshold concentration (~20 ng/ml). To a lesser extent, similar relationships between moderate increases in GH and in the expression of C<sub>3b</sub> receptors (Fig. 3b) and the release of elastase into the circulation (Fig. 3c) were also observed.

OTHER IMMUNOPOTENTIATING MEDIATORS

In addition to GH, the plasma concentrations of prolactin, substance-P and endotoxin were measured because of their potential roles in mediating the exercise-induced increases in neutrophil function. The plasma concentration of PRL, which is structurally and functionally related to GH (Gala, 1991), did not change significantly after moderate exercise in the trained and untrained subjects (Fig. 4a). There were also no significant differences attributable to training in the resting (pre-exercise) values (Fig. 4a). Glucose consumption during exercise had no influence on PRL release into the circulation (Fig. 4b). Substance-P was not detectable in the plasma under any of the conditions tested. As reported previously with cytokines (Smith et al., 1992), the plasma concentration of free endotoxin, a potent stimulus of cytokine secretion, did not change significantly immediately after exercise in either the trained or untrained groups (Fig. 5). In addition, there were no significant differences in the plasma concentrations of endotoxin in the trained and untrained groups either before or after exercise.

IMMUNOSUPPRESSIVE MEDIATORS

The plasma concentrations of the potentially immunosuppressive hormones ACTH and cortisol were measured to determine whether exercise at moderate intensity triggered their release acutely, and whether training had any chronic effects on their circulating concentrations.

Group-2 Trained subjects displayed a higher baseline (pre-exercise) plasma concentration of ACTH than their untrained counterparts (Fig. 6a; P = 0.008). Figure 6a shows that, while a two-fold increase in the plasma
Fig. 3. The relationship between exercised-induced changes in plasma growth hormone concentration and various neutrophil activities.

(a) Intracellular H₂O₂ generation activated \textit{in vitro} by phorbol myristate acetate.
(b) The release of complexed elastase into plasma.
(c) Expression of cell surface complement (C₃b) receptors.

The experimental conditions and the methods used to measure these cellular parameters are described in the accompanying paper (Smith \textit{et al.}, 1993). Data (a-c) are individual values.
Fig. 4. Moderate exercise and plasma prolactin concentration.

(a) Plasma prolactin concentrations immediately before and after exercise in both trained and untrained subjects (n = 11,9). Data are means ± SEM.

(b) The responses of the phase-2 group to exercise and the effect of glucose consumption (n = 8,8). Data are means ± SEM.
Fig. 5. The effect of moderate exercise on the plasma concentration of endotoxin.

Mean ± SEM of plasma endotoxin concentrations immediately before and after exercise in both trained and untrained subjects (n = 7, 9).
Figure 6

The effect of moderate exercise on the plasma concentrations of ACTH and cortisol.

Mean ± SEM of plasma (a) ACTH; and (b) cortisol concentrations immediately before and after exercise in both trained and untrained subjects (n = 9,20).
concentration of ACTH was detected in the untrained subjects immediately after moderate exercise (P = 0.006), no significant change occurred in the trained group. There were no significant time-dependent changes in plasma ACTH concentration over one hour in non-exercised subjects. As with Group 2, the plasma concentration of ACTH in subjects used in the phase-2 experiments (Smith et al., 1993) increased to a similar extent after moderate exercise. This increase correlated positively with the exercise-induced increase in plasma GH (r = 0.52).

**Group 1** With cortisol, the pre-exercise plasma concentrations were similar in both the trained and untrained subjects (Fig. 6b). Like ACTH, however, the plasma concentration of cortisol only increased after moderate exercise in untrained subjects (P = 0.045). Plasma cortisol concentration fell over the same time period of the exercise test in non-exercised subjects (from 131 ± 21 ng/ml* to 83 ± 19 ng/ml; n = 5; P = 0.03). Maximal exercise for one minute appeared to give rise to an increase in plasma cortisol that was delayed for one hour after the cessation of exercise (from 117 ± 20 ng/ml* to 196 ± 38 ng/ml; n = 5) but this was not statistically significant. In both trained and untrained subjects, there were no direct correlations between the plasma concentrations of ACTH and cortisol either before or after exercise. Furthermore, there was no correlation between the plasma concentration of cortisol and the specific oxidative burst activity (k) of neutrophils isolated before or after exercise. (*data are means ± SEM).

**LONGITUDINAL STUDY**

The plasma concentrations of testosterone and cortisol are assessed routinely in male athletes as a measure of anabolic and catabolic activities respectively (Urhausen et al., 1987). A low testosterone/cortisol ratio has been proposed as a diagnostic marker of overtraining (Urhausen et al., 1987). In the five subjects followed longitudinally over a 13 month training program (Smith et al., 1993), there were no consistent changes in the plasma concentrations of either cortisol or testosterone. This was also the case with the testosterone/cortisol ratio (Table 1). In fact, cortisol values tended to be lower at the end of the study when training intensity was increased substantially. Thus, the significant fall in neutrophil oxidative burst activity found in these subjects (Smith et al., 1993) did not correlate positively with plasma cortisol concentrations, which is consistent with the results of our cross-sectional study described above. However, the results presented above suggest that cortisol concentrations in plasma can vary over intervals as brief as one to two hours.
Table 1. Profile of anabolic and catabolic hormones during a 13 month training program.

<table>
<thead>
<tr>
<th>Time</th>
<th>Testosterone (ng/ml)</th>
<th>Cortisol (ng/ml)</th>
<th>Testosterone/Cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>4.31 ± 0.399</td>
<td>110 ± 14.1</td>
<td>0.0439 ± 0.0090</td>
</tr>
<tr>
<td>April</td>
<td>4.39 ± 0.223</td>
<td>136 ± 42.6</td>
<td>0.0454 ± 0.0130</td>
</tr>
<tr>
<td>July</td>
<td>4.55 ± 0.369</td>
<td>165 ± 50.5</td>
<td>0.0347 ± 0.0675</td>
</tr>
<tr>
<td>October</td>
<td>4.22 ± 0.248</td>
<td>95.0 ± 29.1</td>
<td>0.0546 ± 0.0091</td>
</tr>
<tr>
<td>February</td>
<td>3.95 ± 0.691</td>
<td>99.4 ± 21.3</td>
<td>0.0486 ± 0.0117</td>
</tr>
</tbody>
</table>

† The results are presented as means ± SEM.
DISCUSSION

The results will be discussed in terms of their implications for the regulation of the intensity-dependent responses of neutrophils to exercise, and how they add to previous work on exercise and endocrine responses.

Implications for neuroendocrine regulation of neutrophil microbicidal activity during exercise

These results are consistent with the hypothesis that the exercise-induced increase in plasma growth hormone (GH) modulates some aspects of neutrophil microbicidal activity in a concentration-dependent manner (Smith and Weidemann, 1990). While a modest rise in plasma GH was associated with priming of the ability of neutrophils to generate H$_2$O$_2$ intracellularly in response to stimulation with PMA in vitro, the magnitude of the primed response declined progressively once the plasma concentration of GH reached a critical threshold. To a lesser extent, similar relationships between moderate increases in GH and in the expression of C$_3$b$_1$ receptors and the release of elastase into the circulation were also observed. The decline in these neutrophil activities may be due to a direct or potentiating effect of GH on a subpopulation of neutrophils leading to their full activation in vivo by stimuli such as immune complexes. Furthermore, the subsequent non-responsiveness of the activated cells to secondary stimulation (Prasad et al., 1991) may be sustained by hormones such as ACTH, adrenalin, and cortisol which are secreted into the bloodstream in response to activation of the immunosuppressive arm of the pituitary/adrenal axis via negative feedback by GH (Lanzi and Tannenbaum, 1992). Apart from the immunological implications, this work also shows that the secretion of neuroendocrine hormones into the circulation was modulated significantly by training and the duration of exercise.

None of the other humoral mediators measured in plasma appear to undergo changes consistent with their involvement in the priming process triggered by moderate exercise. In contrast to GH, the plasma concentration of prolactin did not change significantly after moderate exercise in both the trained and untrained subjects. This confirms previous reports suggesting that the anaerobic threshold must be exceeded, even when GH secretion is diminished substantially, before prolactin is secreted into the circulation (De Meirlier et al., 1985). The plasma concentrations of endotoxin and substance-P also did not change immediately after moderate exercise.

Apart from GH, other humoral mediators, including cytokines - either singly or in combination - may be involved in mediating exercise-induced priming and/or activation of neutrophils. While cytokine
concentrations do not increase significantly in the circulation after exercise, substantial increases were detected in urine samples taken from runners after a 20 km road race (Sprenger et al., 1992). This is consistent with a greater rate of production and secretion of some cytokines in response to exercise even though they fail to accumulate in the circulation. Therefore, a role for cytokines in exercise-induced priming of neutrophils cannot be ruled out completely.

These present results do not support the hypothesis that cortisol may be involved in the suppression of neutrophil microbicidal activity induced acutely by maximal exercise or chronically by regular endurance training. This is not surprising considering that nearly all reports of glucocorticoid-mediated suppression of phagocyte microbicidal activities have involved pharmacological rather than physiological concentrations of glucocorticoids and/or prolonged exposure to these agents (Fuenfer et al., 1985; Schleimer et al., 1989). Much larger and less transient increases in plasma cortisol concentration than the small changes reported here may be required for immunosuppression to be manifested either acutely or chronically. Also, because of the substantial lag time before the cortisol concentration in plasma increases after maximal exercise, other humoral factors that respond rapidly to exercise - such as adrenalin (Deuster et al., 1989) - are more likely to be involved in acute immunosuppression. Adrenalin can inhibit a variety of immune cell functions including the neutrophil oxidative burst (Fantozzi et al., 1985; Berczi, 1986). The rise in plasma catecholamines immediately after maximal exercise is five-fold larger than the small increase triggered by aerobic exercise of moderate intensity (Deuster et al., 1989) and noradrenalin secretion is triggered at much lower workloads than those required to raise adrenalin (Galbo, 1986). However, the effect of noradrenalin on neutrophil function is not known. This suggests that - if humoral agents are involved - adrenalin rather than cortisol may be responsible for decreasing the oxidative activity of neutrophils after single episodes of intense exercise. The role of ACTH and cortisol in exercise-induced suppression of various immune functions could be investigated by administering phosphatidylserine to exercising subjects as this compound has been reported to block the ACTH and cortisol responses to intense exercise in humans (Monteleone et al., 1990).

In contrast to previous reports (Urhausen et al., 1987), long-term endurance training had no significant chronic effects on the basal plasma concentrations of cortisol and testosterone. While blood samples were taken at the same time of day to avoid time-dependent variation, seasonal alterations in the secretion of these hormones cannot be ruled out. The integration of several measurements of plasma cortisol and testosterone
concentrations taken several hours apart should have alleviated variability due to pulsatile secretion of these hormones to some extent. It is clear that, however, that changes in plasma cortisol did not correlate positively with the suppression of the neutrophil oxidative burst activity detected acutely after single episodes of maximal exercise or chronically in response to intensive endurance training. It could be premature to rule out a role for cortisol in inhibiting neutrophil activity, however, because the dynamics of cortisol action on these cells and its plasma concentration may be unrelated. Cellular markers of cortisol action on neutrophils must be identified and examined to test this hypothesis.

The number of neuroendocrine factors known to influence neutrophil microbicidal activity - both positively and negatively - is expanding rapidly. This emphasizes the redundancy of this homeostatic system. Weidemann and colleagues (1992) reported, for example, that atrial natriuretic peptide (ANP) is a potent priming agent of superoxide release in vitro. This hormone is secreted into the circulation very rapidly in response to low-intensity exercise and it remains elevated until work ceases (Follenius and Brandenberger, 1988; Freund et al., 1991). Furthermore, the secretion of ANP into the circulation may be enhanced by glucose supplementation during exercise because hyperglycaemia is a potent trigger of ANP secretion (Clark et al., 1993). ANP has also been reported to inhibit ACTH release in humans (Kelner et al., 1992). GH, ANP and other immunopotentiating agents may be involved, either alone or synergistically, in initiating or modulating the priming/activation cascade of neutrophil microbicidal activity during exercise.

Because exercise-induced release of neuroendocrine factors into the circulation is intensity-dependent, priming/activation or suppression of neutrophil microbicidal activity in response to exercise are unlikely to be mediated by just one or two factors. While GH and ANP secretion into the circulation is triggered by exercise of low intensity and short duration, the plasma concentrations of ACTH, cortisol, renin, vasopressin and prolactin do not change until the intensity exceeds 60% VO₂ max (Freund et al., 1991). The net effect of exercise on neutrophil microbicidal activity may therefore represent the interplay between a variety of positive and negative mediators that are released in response to exercise. Furthermore, some mediators such as β-endorphin (Fiatarone et al., 1988) and prostaglandin E₁ (Farmer et al., 1991) exert positive effects on immune cells at low concentrations and become inhibitory only after a threshold concentration is exceeded which over-rides the stimulatory effect. A similar mechanism may explain the apparent concentration-dependent effects of GH on various neutrophil functions found in this work. Such contrasting effects may be
due to the presence of heterogeneous receptors for individual mediators in single cells which exert stimulatory and inhibitory effects at low and high mediator concentration respectively (Van den Bergh et al., 1991). In fact, the priming effect of GH on neutrophils in vitro is mediated by the binding of GH to the prolactin receptor in a zinc-dependent process (Fu et al., 1992). In contrast to the priming agents discussed above, histamine, adenosine, prostaglandin E\textsubscript{1} (Fantozzi et al., 1985), lipoxins A\textsubscript{4} & B\textsubscript{4} (Conti et al., 1990), some adenine nucleotides (McGarrity et al., 1988) and vasointestinal peptide are all potent inhibitors of neutrophil microbicidal activity. The responsiveness of the majority of these mediators to exercise has not been examined in human subjects. However, it is possible that a plethora of immunoregulatory mediators may be secreted into the circulation in an intensity-dependent manner during exercise and/or during the recovery period after exercise. Thus, cellular markers (e.g. receptors, intracellular signalling molecules) associated with a specific mediator need to be examined in parallel with the humoral responses to exercise in order to prove a direct cause-and-effect relationship.

Previous neuroendocrine studies

This work extends previous work on neuroendocrine responses to moderate exercise by showing that they are influenced by both training (fitness) and the duration of exercise. While Farrell and colleagues (1983) reported that 20 minutes of treadmill-running at 60% VO\textsubscript{2} max increased the plasma concentration of GH (7-fold), with no matching change in ACTH and cortisol, we found a much greater increase in plasma GH concentration and a significant rise in the plasma concentrations of ACTH and cortisol in untrained subjects only. Thus, duration also exerts a significant influence on neuroendocrine responses to exercise.

With moderate exercise protocols of short (< 30 min) duration, much higher exercise intensities that exceed the threshold of 60% VO\textsubscript{2} max are necessary to elevate circulating ACTH and, with it, an increase in circulating cortisol (Farrell et al., 1983) which follows after a lag period (Schwartz and Kindermann, 1990). Like ACTH, \(\beta\)-endorphin, which also has inhibitory effects on the neutrophil oxidative burst (Diamont et al., 1989), is derived from the same precursor as ACTH. \(\beta\)-Endorphin is not elevated in plasma after one hour of either cycling or running at 60% VO\textsubscript{2} max (Langenfeld et al., 1987; Goldfarb et al., 1991), but its concentration in plasma increases substantially after vigorous exercise of 30 to 60 sec duration (Rahkila et al., 1988). Because the plasma concentration of GH has been reported to be much higher after maximal (than submaximal) exercise (Farrell et al., 1983), negative feedback by GH (Lanzi and Tannenbaum, 1992)
may induce the secretion of ACTH, ß-endorphin and cortisol into the circulation. These hormones may be responsible, either singly or collectively, for the decrease in neutrophil activity observed after exercise at maximum effort (Smith et al., 1993). Buono and colleagues (1986) reported that one minute of maximal exercise increased the plasma concentration of ACTH immediately by three-fold while cortisol increased by 20% 15 minutes later. A similar result was reported by Schwartz and Kindermann (1990).

Conclusions

The results provide circumstantial evidence consistent with GH differentially modulating, in a concentration-dependent manner, some aspects of neutrophil microbial activity in response to moderate exercise. However, because of the potentially large number of humoral factors that may be involved in mediating the priming, activation or suppression of neutrophil microbial activity during exercise, a direct cause/effect relationship between a rise in the plasma concentration of a specific humoral mediator(s) and changes in immune cell activities will be difficult to prove. However, the putative immunomodulatory roles of some mediators can be ruled out on the basis of their failure to respond to exercise. Thus, in general, the intensity-dependent modulation of immunity by exercise is likely to involve complex interactions between several factors that are released into the circulation as the agents of a highly-redundant regulatory network.

To obtain further evidence that GH may participate in exercise-induced priming/activation of neutrophils in a concentration-dependent manner, we need to examine whether injection of this hormone into human subjects can raise its plasma concentration to the same levels as those found after moderate exercise in the presence and absence of glucose. This procedure may invoke changes in the microbicidal activities of neutrophils that are in similar to those found following moderate exercise. An understanding of how exercise regulates the immune system could have important benefits in the prevention and treatment of overtraining, immunodeficiency (Smith and Weidemann, 1990) and, perhaps, some cancers (Shephard, 1993).
REFERENCES


CHAPTER 4

GENERAL DISCUSSION
4.1 Introduction

As stated in the introduction (Chapter 1), the aims of this project were to assess the acute and chronic effects of exercise, at both moderate and maximal intensities, on: (i) various aspects of neutrophil microbicidal activity; and (ii) the cellular and humoral mechanisms involved in mediating exercise-induced changes in neutrophil function. The major conclusion of my study is that exercise has intensity-dependent effects on the neutrophil oxidative burst and the secretion of immunomodulatory neuroendocrine factors that are independent of normal intra-individual variation over an identical period in non-exercised subjects. The variable responses of neutrophils appear to be related to some extent to the magnitude of the exercise-induced increase in growth hormone (GH) secretion into the circulation. While modest increases in plasma GH concentration (up to 10 ng/ml) were associated with proportional increases in intracellular H₂O₂ production in response to stimulation with phorbol myristate acetate *in vitro*, complement (C₃b) receptor expression and the release of elastase into the circulation, the magnitude of all these neutrophil responses declined progressively once plasma growth hormone concentration reached and increased beyond a critical threshold (> 20 ng/ml). Overall, the results suggest that the intensity-dependent effects of exercise on neutrophils may involve a shift in the proportion of low- to high-activity cells, which may be due to priming of some cells and activation of others, the net response reflecting a balance between the two states. While the results do not allow inferences to be made directly about susceptibility to infection, they support the emerging epidemiological evidence that exercise has intensity-dependent effects on resistance to infection. I will also discuss a number of implications of the work and present a revised hypothesis which attempts to explain the experimental results.

I consider that neutrophils are an appropriate model to study modulation of immune cell function in human subjects by exercise. As discussed in the introduction (Chapter 1), neutrophils, as the major circulating WBC population, are essential to host defence. They interact with other immune cells through either direct cell-to-cell contact or via humoral factors. In particular, the microbicidal activities of neutrophils have been shown, *in vitro*, to be modulated by various humoral factors such as cytokines and neuroendocrine hormones (see Section 3, Chapter 1). Thus, the discussion will focus on the experimental results in terms of
exercise effects on neutrophil microbicidal activity and its possible implications for the immune system and, thus, resistance to infectious disease.

4.2 Overview of results

4.2.1 Exercise and neutrophil microbicidal activities
The work began with a pilot study which showed that a single episode of moderate exercise (in human subjects) primed, for up to six hours, the specific activity of $\text{H}_2\text{O}_2$ and HOCl production ($k$) in neutrophils stimulated with opsonized zymosan (OZ) (Chapter 2.1). This result was confirmed in a second study which also showed that moderate exercise primed the phagocytic and killing capacities of neutrophils in both trained and untrained men (Chapter 2.3). In contrast to moderate exercise, exercise at maximum effort substantially diminished the specific activity of $\text{H}_2\text{O}_2$ and HOCl production in OZ-stimulated neutrophils (Chapter 2.3). The intensity of this activity was considerably lower in neutrophils isolated from trained subjects, both before and after exercise, than in cells from their untrained counterparts in both of these cross-sectional studies; the endurance training effect was confirmed in a longitudinal study (Chapter 2.3).

The initial work using the moderate exercise protocol was extended by employing a number of independent assays of the neutrophil oxidative burst, including a flow cytometric technique that measured the intracellular production of $\text{H}_2\text{O}_2$ at the single cell level, and by using stimuli that activated the production of reactive oxygen species (ROS) at defined steps in the signal transduction pathway of NADPH oxidase activation. Flow cytometry showed conclusively that neutrophils consisted of one or more “subpopulations” whose distribution changed in response to exercise (Chapter 2.3). The choice of stimuli known to act at well-characterised steps of the priming process and the use of assays that were dependent on NADPH oxidase and/or MPO activities were made in an attempt to characterize mechanisms involved in the exercise-induced amplification (priming) of neutrophil microbicidal activity.

The final cellular experiments reported in this part of the thesis have examined the inherent variability, over one hour, and have attempted to elucidate whether the effects of moderate exercise are authentic or due merely to intra-individual time-dependent variation of the humoral and neutrophil activities studied. This type of experiment has not been conducted before in this field to my knowledge but is essential in view of the variable responses obtained when lymphocytes isolated after acute
exercise are exposed to mitogenic stimulation \textit{in vitro} (Verde \textit{et al.}, 1992). Immune cell responses to exercise may differ substantially between individuals because of dietary differences and other lifestyle factors that cannot be controlled for in the experimental design.

The mechanistic experiments (Chapters 2.3; 3.3) showed conclusively that the capacity of neutrophils to generate $\text{H}_2\text{O}_2$ intracellularly in response to stimulation with PMA, but not OZ, increased three-fold after one hour of moderate exercise. There was, however, a significant increase (10\%) in the rate of superoxide generation in OZ-stimulated cells after exercise. These responses were accompanied by a significant increase (20\%) in $\text{C}_3\text{b}1$ receptor expression. While these primed responses were not affected substantially by glucose consumption, the modest increase (up to 3-fold) in the plasma concentration of GH was associated with a progressive increase in these primed responses. The magnitude of priming declined once the GH concentration reached and increased more than 10-fold ($> 20 \text{ ng/ml}$). Furthermore, a detectable proportion of the neutrophil population appeared to be activated directly during moderate exercise, as evidenced by the two-fold increase in the circulating concentration of complexed elastase, but this also diminished significantly when the subjects consumed glucose during the exercise test. The magnitude of elastase release varied considerably between individuals, which suggests that the neutrophil activation threshold may also show considerable inter-individual variation. All of these cellular responses were due to exercise \textit{per se} and not to time-dependent variation.

During the course of this project, the acute and chronic effects of exercise on neutrophil microbicidal activity have also been examined by several independent groups, and by two PhD students who joined our group to study interval exercise at maximal intensity and the effect of concentric versus eccentric exercise on various aspects of neutrophil microbicidal activity. These studies are discussed in the next two paragraphs and are, where appropriate, compared with my own results.

The major conclusions of these studies, and of my own work, is that physical activity - depending on its type, intensity, frequency and duration - has differential effects on various functions of neutrophils. The basal phagocytic and chemotactic activities of neutrophils, and their capacity to kill \textit{in vitro} the fungus \textit{Candida albicans}, were significantly higher in cells isolated from female basketball players compared to cells from untrained controls (Ortega \textit{et al.}, 1993a). In contrast, Hack and colleagues (1992) reported that neutrophil phagocytic and chemotactic activities, and superoxide production, were not different in cells isolated from either
highly-trained runners and triathletes (during a moderate training period) or untrained controls, suggesting that tapering of the training program may have normalised these parameters. While phagocytic activity was not different between trained cyclists and untrained controls, Lewicki and colleagues (1987) reported that neutrophil bacterial killing capacity was significantly lower in the cyclists before exercise and this training effect was even greater after a session of maximal exercise. These differential training effects are sport-specific, presumably, because of diversity in training intensity, volume, and frequency. Endurance athletes, for example, undertake a much more demanding training schedule than basketball players. Our own group has shown in a recent three month longitudinal study that neutrophils isolated from elite swimmers at rest produce responses to OZ and PMA that are two- and four-fold lower, respectively, in the dihydrorhodamine-123 flow cytometric assay, compared to the values detected in cells from untrained controls (D.B. Pyne, unpublished results). This confirms my earlier work, using luminol-amplified chemiluminescence, which showed that the specific oxidative activities of neutrophils from trained cyclists at rest were significantly lower than in cells from non-training controls (Smith et al., 1990). In these longitudinal training studies, it is essential that an untrained control group be included in the experimental design, to account for seasonal and circadian variability, and that extraneous stress factors like general well-being, diet, and illness be recorded and controlled for as much as possible.

Single episodes of exercise also have intensity-dependent effects on neutrophil function. Progressive exercise to exhaustion increased neutrophil phagocytic capacity for the first 24 hours post exercise while superoxide production in stimulated cells fell immediately after exercise but increased one hour later and remained elevated for the following 24 hours (Hack et al., 1992). The chemotactic and phagocytic activities of neutrophils were enhanced significantly immediately after one hour of cycling at 50% VO₂ max but their Candicidal capacity was not altered from pre-exercise values (Ortega et al., 1993b). This confirms some of the conclusions reached here but suggests that the ability of neutrophils to kill various types of micro-organisms is primed differentially by moderate exercise. In contrast to my results, Macha and colleagues (1990) reported that the responsiveness to PMA of neutrophils isolated immediately after a similar exercise workload was decreased significantly. This may reflect differences in intra- and extra-cellular assays for H₂O₂ because only a small amount of the H₂O₂ generated may diffuse into the extracellular medium.
The intensity-dependent responses of neutrophils to exercise may be explained by the following mechanisms. In the case of moderate exercise, neutrophil-priming cytokines and hormones and/or substimulating concentrations of activating stimuli such as immune complexes and tissue debris may amplify the responsiveness of cells to stimulation in vitro and/or may convert non-responsive cells to a responsive state. With intensive exercise, however, the plasma concentrations of these agents may exceed the priming threshold leading to the direct activation in vivo of a subpopulation of neutrophils. The activated cells may not respond to secondary stimulation until a substantial recovery (refractory) period elapses. This may amplify the effects of immunosuppressive hormones such as adrenalin, ACTH and cortisol - which are released into the circulation once exercise intensity exceeds 60% VO$_2$ max - on the neutrophil oxidative burst and may explain the apparent refractory period found immediately after intensive submaximal and maximal exercise. The tendency for treadmill-running to induce the refractory period at moderate exercise intensity - below the threshold required to induce the secretion of immunosuppressive hormones into the circulation (see Section 3.2, Chapter 1) - may be due to minor tissue damage caused by footstrike that triggers activation. Because the moderate cycling protocol involved working at 60% VO$_2$ max, the inter-individual variability in the responses to moderate exercise may be due to a lower neutrophil activation threshold in some individuals and to durational effects, particularly in untrained people who showed significant increases in the plasma concentrations of ACTH (50%) and cortisol (20%) immediately after exercise. This could be investigated by using less intense and shorter exercise workloads (e.g. 20 to 40% VO$_2$ max for 20 to 40 min). It is unlikely that extraneous factors such as psychological stress played a significant role (compared to long-term training effects) in the acute responses to exercise in my studies, because the subjects were either age-matched male students or workers in the laboratory with similar lifestyles and with no recent symptoms of illness.

My studies and those of others have also shown that submaximal exercise induces activation of a detectable subpopulation of neutrophils, as indicated by the release of elastase into the circulation (Kokot et al., 1988; Dufaux and Order, 1989), and translocation of p$_{47}^{phox}$ to the membrane (A.B. Gray et al., unpublished results). Exercise appears to shift the equilibrium between non-responsive, primed and activated neutrophils in an intensity-dependent manner (Fig 1). While moderate exercise may shift a proportion of neutrophils from a quiescent to a responsive state and/or may amplify the microbicidal activity of responsive neutrophils, a
Figure 1

DISTRIBUTION OF NEUTROPHIL RESPONSES

- Normal state
- Primed state
- Suppressed state
- Refractory period
- Activation threshold
- Recovery period
proportion of the neutrophil population may become fully activated once exercise intensity exceeds a critical threshold. This may vary considerably between individuals of similar physical fitness levels. More intense exercise may lead to the premature activation and/or humoral factor-induced suppression of a much larger percentage of the circulating pool which, in turn, leaves them in a non-responsive refractory state for some hours during recovery (Prasad et al., 1991) or until spent cells can be replaced with recently-matured neutrophils from the bone marrow. The latter hypothesis may explain why large increases in the percentage of circulating neutrophils are found in many studies which employ maximal exercise protocols (McCarthy and Dale, 1988).

4.3 Exercise and immunoregulation
My decision to study the effect of moderate exercise on neutrophil microbicidal activity was prompted by a report that the putative neutrophil-priming cytokine interleukin-1 was elevated by one hour of cycling at 60% VO2 max (Cannon and Kluger, 1983; Cannon et al., 1986). Whilst I was unable to detect, using immunoassays, a significant change in the circulating concentration of interleukin-1β and several other cytokines (Smith et al., 1992), a role for cytokines in priming neutrophil oxidative activity during moderate exercise cannot be ruled out. Sprenger and colleagues (1992) found a substantial elevation in the concentrations of IFN-γ, TNF-α, IL-1-β, IL-6 and soluble IL-2 receptor in urine samples taken from well-trained runners after a 20 km road race despite the absence of corresponding changes in plasma. These cytokines may be synthesized and secreted from a variety of cell types including monocytes, lymphocytes and endothelial cells during exercise. Cytokine activity may be confined to regions outside the peripheral circulation or, alternatively, the cytokines released may be cleared selectively and rapidly from the circulation into the urine (Sprenger et al., 1992). Alternatively, these cytokines may be derived from cells in the lumen of the urinary tract (Ko et al., 1993). Soluble receptors for cytokines have been detected in urine from healthy human subjects (Novick et al., 1989). Because this is the first report of cytokine release into the urine in response to exercise, the work needs to be replicated and extended by others to examine whether moderate exercise affects the concentration of neutrophil-priming cytokines or their soluble receptors/inhibitors in urine.

Subsequent work by others has shown that growth hormone (GH) is a plausible candidate for exercise-induced priming of neutrophils because its concentration increases at least 10-fold after exercise and GH is a potent
neutrophil-priming agent in vitro (see Chapter 3.3). However, this hypothesis is far from being resolved. My initial attempt to solve this question has been equivocal. Whilst consumption of a glucose-enriched solution by exercising subjects substantially diminish the GH response to exercise, it did not entirely abolish it.

Quite unexpectedly, a modest rise in the plasma concentration of GH was associated with priming of neutrophils to generate $\text{H}_2\text{O}_2$ intracellularly in response to stimulation with PMA in vitro. Whilst the magnitude of the primed response increased progressively with rises in plasma GH up to three-fold, once the plasma concentration of GH reached and increased beyond a critical threshold, the intensity of the primed response fell progressively. To a lesser extent, similar relationships between moderate increases in GH and in the expression of C$_3$b$_1$ receptors and the release of elastase into the circulation were also found. The decline in these neutrophil activities may be due to a direct or potentiating effect of GH on a subpopulation of neutrophils leading to their full activation in vivo by stimuli such as immune complexes. Furthermore, the subsequent non-responsiveness of the activated cells to secondary stimulation (Prasad et al., 1991) may be sustained by hormones such as ACTH, adrenalin, and cortisol which are secreted into the bloodstream in response to activation of the immunosuppressive arm of the pituitary/adrenal axis via negative feedback by GH (Lanzi and Tannenbaum, 1992).

Alternative strategies are evidently required to study the effects of GH on neutrophils in vivo to resolve this question. Injection of recombinant GH into human subjects, sufficient to raise plasma GH to the same level as that achieved by exercise, in the presence and absence of glucose, could be tried initially. It is not known whether the kinetics of GH release into the circulation, and the development of primed neutrophil functions are correlated directly. This could be investigated by taking several blood samples post exercise at 20 to 30 minute intervals to determine when the priming response is maximal, and by carrying out the appropriate variability controls.

4.3.1 Other potential immunomediators
The highly-pleiotropic nature of the functions of individual humoral components of the neuroendocrine system suggests that additional regulatory factors that are released into the circulation in response to exercise are likely to be identified. In this study, while I have not been able to resolve whether GH is involved positively in exercise-induced priming of neutrophil microbicidal activity in vivo, I have been able to rule out the
involvement of some factors such as substance-P and prolactin. While glucose ingestion may attenuate the GH response to exercise, the release of atrial natriuretic peptide (ANP), which also primes neutrophils (Wiedermann et al., 1992), may compensate for this fall because its secretion is enhanced during hyperglycaemia (Clark et al., 1993) and may be related to the mandatory secretion of insulin. Several independent studies have shown that the plasma concentration of ANP increases significantly during moderate exercise (Schmidt et al., 1990; Follenius et al. 1991; Freund et al., 1991). It may be virtually impossible to prove a direct cause-and-effect relationship between changes in the concentrations of individual humoral factors that may be released into the circulation during moderate exercise and the priming of neutrophil microbicidal activity. However, expression of neutrophil receptors for specific humoral factors such as GH and other cellular markers may be a useful area to pursue.

The high turnover of ATP associated with vigorous exercise leads to the release of adenosine, which is immunosuppressive and raises the level of cAMP in neutrophils (Cronstein et al., 1988), into the circulation (Simpson and Phillis, 1992). This may be triggered by exercise-induced hypoxia because adenosine concentration in some tissues increases significantly during ischaemic muscle contraction (Ballard et al., 1987) and vigorous exercise (Belloni et al., 1979), primarily as a result of restricted blood flow. In the accompanying diagram (Fig. 2), a revised hypothesis based on current knowledge is outlined. This scheme is necessarily oversimplistic because the effects of exercise on other modulators of neutrophil function such as arachidonic acid metabolites, amino acids and nucleotides have not yet been addressed.

4.4 Physiological implications

A recent review has concluded that while "excessive training suppresses immunity, the changes are small, variable and difficult to relate to overtraining" (Shephard et al., 1991). Moderate exercise may have only transient or modest effects in the healthy subjects which have been used in nearly all published studies, while in contrast, intensive exercise may be detrimental only when a critical threshold is exceeded. The related question of whether moderate exercise has beneficial effects on resistance to infection needs to be investigated further for similar reasons. Furthermore, a reference range needs to be established which defines the limits of normal and abnormal responses of the major cell populations that make up the immune system so that the clinical consequences of responses that are above or below this range can be assessed; at present it is not known what
Figure 2

EXERCISE

ATRIAL NATRIURETIC PEPTIDE

HYPOTHALAMUS/PITUITARY

ACTH

ADRENAL CORTEX

ADRENALIN CORTISOL

MONOCYTE

CYTOKINES (IL-1, TNF)

GROWTH HORMONE

HEMATOCRIT

Positive

Negative
constitutes a genuine "normal" response. This question was raised previously in relation to neutrophil microbicidal activity (Chapter 2.2). Optimum neutrophil function confers resistance to opportunistic fungal and bacterial infections (Schaffner et al., 1986) which are quite common in athletes. Whilst priming is likely to be beneficial because it amplifies the potential of neutrophils to kill infectious agents, premature activation may result in release into the extracellular milieu of harmful ROS and enzymes with the potential to cause inflammatory damage to surrounding host tissues. This would effectively lower neutrophil protection because it would induce a significant refractory period during which the cells do not respond to secondary stimulation. This hypothesis may explain the cumulative negative effects of regular sessions of intensive endurance training on the neutrophil oxidative burst and, perhaps, on other immune cell activities.

To investigate more directly whether the link between exercise and immunity has functional consequences on resistance to infection, athletes or untrained subjects placed on appropriate exercise programs (varying from moderate to maximal intensity) could be challenged with antigens or vaccines under carefully defined conditions. This approach should include functional assays of various immunological parameters, incorporating cellular and humoral mechanisms of both specific and non-specific immunity (e.g. delayed hypersensitivity skin test, specific antibody production, lymphocyte mitogenesis, NK cell cytotoxicity, and neutrophil microbicidal activity). For instance, Cohen and colleagues (1991) showed that subjects with a high psychological stress index (assessed by questionnaire) were more susceptible to infection and the development of clinical symptoms than less-stressed individuals when inoculated with relatively harmless respiratory viruses. Alternatively, the same parameters of immunity could be investigated in people with a history of infectious illness indicative of a low level of immunity who are placed on moderate training programs and studied longitudinally.

Some pertinent questions on the controversial effects of exercise on resistance to infection have been raised recently (Cannon, 1993). In particular, Cannon suggested that "like any homeostatic system, the in-built redundancy of the immune system ensures that essential processes are carried out". Thus, changes in one immune parameter in response to exercise would not necessarily mean that resistance to infection is altered because potential compensatory responses in other immune parameters may occur that maintain normal function. This may not be the case under all conditions because, in general, moderate exercise potentiates, but
exercise at maximum effort attenuates, the activities of lymphocytes, NK cells, neutrophils, serum & salivary immunoglobulins and complement factors as well as resistance to infection (see Section 4, Chapter 1). However, some cell populations may be more susceptible to immunosuppressive factors than others, leading to considerable inter-individual variation. The key issue for athletes, for example, is whether the activities of these cellular and humoral components fall below a critical threshold during intensive training, resulting in increased susceptibility to infection. Alternatively, for people with significant immunodeficiency, does moderate exercise exert sufficient influence to push the activities of some key cell populations over that threshold?

4.5 Future work

All future experiments in this field must include a control group of non-exercising subjects to rule out time-dependent alterations as a source of variability in both cross-sectional and longitudinal studies. Furthermore, a number of additional post-exercise samples should be taken to account for intra-individual variability in the kinetics of neuroendocrine and immune responses to exercise. At present there is no unique cellular marker that is indicative of neutrophil priming, although several molecular markers of activation have been reported. Animal models may be needed to address many of the issues raised here because studies with human subjects are restricted to material obtained from blood samples and occasional tissue biopsies. Furthermore, diet and other lifestyle factors (e.g. psychological stress, sleep) may contribute to the variability immune cell activity found in longitudinal training studies and, possibly, in the responses of these cells to single episodes of exercise.

If circulating humoral factors modulate the responses of neutrophils to exercise, treatment of neutrophils isolated from non-exercised subjects with plasma taken after exercise may be expected to reproduce at least some aspects of the exercise response. Our initial attempt to perform this experiment did not show any priming effect with plasma isolated from subjects after moderate exercise, but the quenching of the luminol signal by albumin and other plasma proteins may have masked the response (Chapter 2.1). Alternatively, the plasma factors that mediate priming may have a short half-life. This experiment needs to be repeated, using the flow cytometric assay, under conditions in which the plasma is removed at the end of the incubation period to minimise interference in assays of the oxidative burst from proteins and other plasma constituents. At the cellular level, it may be possible to identify priming activity in neutrophils after
exercise by treating membranes prepared from neutrophils isolated before
exercise with cytosolic fractions isolated after exercise, providing these
factors are not inactivated during processing.

If priming is not mediated by plasma factors, it may occur through
endothelial cell-neutrophil contact in localized marginated pools. The
primed cells may then be expelled into the peripheral circulation during
exercise. Whilst our experiments with the maximal exercise protocol
provide some evidence that marginated cells are not intrinsically more
active than their circulating counterparts (Chapter 2.3), this hypothesis
cannot be rejected completely because the activities of marginated cells may
be influenced differentially by exercise intensity.

The competition between the opposing pathways of ROS and reactive
nitrogen species (RNS) generation in neutrophils should also be
investigated. The variability found in exercise-induced changes in
neutrophil production of ROS, and their differential responses to OZ and
PMA, may be due, partially, to the extent that ROS and RNS react with
oxidative probes and/or cellular enzymes, their secondary reactions, and
their distribution between the intra- and extracellular compartments. As
discussed in the introduction (Section 2.5.1.2; Chapter 1), RNS production
in non-activated neutrophils may be important in facilitating their
movement through the microcirculation, and these species may be
additional microbicidal products in activated cells (Malawista et al., 1992).
At present, there are no flow cytometric assays for detecting the
intracellular generation of RNS specifically. Competition between various
pathways and the non-specificity of assays used routinely to measure
various ROS also highlights the problems associated with interpretation of
the physiological consequences of exercise-induced changes. Therefore it
will be necessary to determine whether exercise and training alters the
microbial killing capacity of neutrophils over a wide time period to rule out
negligible transient changes induced by single episodes of exercise. I have
already reported that moderate exercise does increase neutrophil
cytotoxicity against Staphylococcus aureus (Chapter 2.3). Future work would
adapt the bacterial killing assay to a flow cytometric technique so that
changes triggered by exercise in the abilities of neutrophil subpopulations to
kill various types of micro-organisms (e.g. gram-positive & -negative
bacteria, fungi, protozoa, etc) can be identified more directly. As more
neutrophil surface markers continue to be identified, the relative
percentages of immature, primed and activated cells present in the
circulation, and the mechanisms by which these ratios are altered by
exercise, may be studied.
It is possible that changes in some neutrophil activities such as C₃b₁ receptor expression may not be directly related to immunity but may reflect clearance of toxins and/or a possible role in sequestering and inactivating humoral agents and, thus, maintenance of homeostasis, particularly during the recovery process.

4.6 Conclusions
Moderate exercise increases the percentage of high-activity neutrophils (which generate H₂O₂ intracellularly) and this was associated with their enhanced phagocytic and bacterial killing capacities. The intensity-dependent effects of exercise on some aspects of neutrophil microbicidal activity can be explained, partially, by the associated concentration-dependent increase in the plasma concentration of growth hormone (GH). While modest rises in plasma GH were associated with increased levels of priming, this declined progressively once plasma GH reached and increased beyond a critical threshold (20 ng/ml). The decline may be related to direct activation of a neutrophil subpopulation during exercise (as indicated by elastase release into the circulation) which leaves them subsequently in a non-responsive state during the recovery period after exercise. The net response to exercise would reflect a balance between priming and activation which are biochemically inseparable. Whilst the results of the immunoregulation studies are consistent with GH playing a role in the priming and activation of neutrophils that occurs during submaximal exercise, further work is required to establish this and the possible involvement of other regulatory mediators. Overall, this work sheds light on why the responses of various types of immune cells to exercise are highly variable and offers insights as to why the effect of exercise on immunity has been a highly-controversial field and indicate ways in which the controversy may be resolved.
REFERENCES


PART 2

ERYTHROCYTE FUNCTION
ABSTRACT

Many reports suggest that regular physical exercise accelerates the rate of red blood cell (RBC) destruction and, in some cases, leads to the so-called condition of "sports anaemia". In this project, I determined whether the large increase in \( \text{O}_2 \) uptake (and, therefore, oxygen free radical generation) and/or changes in RBC hydration induced by a single episode of submaximal exercise increased the susceptibility of RBCs to haemolysis by either osmotic or oxidative stress \textit{in vitro}. I also investigated whether lactic acid release into the circulation during maximal exercise affected the osmotic properties of RBCs. During the course of these studies, a luminol-amplified chemiluminescence assay was developed for monitoring, continuously, the generation of free radicals by RBCs treated with organic hydroperoxides.

The effects of single episodes of submaximal exercise on the vulnerability of RBCs to haemolysis induced by osmotic (hypotonic saline) and peroxidative stress (with \( \text{H}_2\text{O}_2 \) or organic hydroperoxides) \textit{in vitro} was examined in both trained and untrained men. Mean cell haemoglobin concentration (MCHC) and mean cell volume (MCV) were also assessed and, in some cases, RBC "subpopulations" were characterised by assessing their density distribution both before and after exercise. With the exception of the oxidative stress assays, the same techniques were used to investigate changes in the osmotic properties of RBCs induced by exercise at maximal effort. The effect of supplementation of the diet with ascorbate (1000 mg/day) and \( \alpha \)-tocopherol (1000 IU/day) on some of these parameters, and the effect of this regime on the responses of the subjects to submaximal exercise, was also examined.

Pre-exercise (resting) and post-exercise RBC osmotic fragility, MCHC and MCV were not influenced by training but vulnerability to peroxidative lysis was 30 % higher in untrained than in trained subjects (\( n = 9,11 \); \( P < 0.05 \)). Whilst no significant change in peroxidative lysis occurred immediately after one hour of cycling at 60% \( \text{VO}_2 \text{max} \), a 20% increase was found six hours later (\( P < 0.01 \)). The susceptibility of RBCs to osmotic haemolysis decreased by 15% (\( P < 0.001 \)) immediately after exercise, and this was sustained for six hours. This correlated with a small increase in MCHC (\( P < 0.05 \)). These responses are similar, but smaller in magnitude, to those detected in cells from thalassaemic patients and may be caused by incorporation of haem iron into the membrane which has the potential to short-circuit \( \alpha \)-tocopherol protection. In a follow-up study, treadmill running at 75% \( \text{VO}_2 \text{max} \) for 45 min (\( n = 20 \) trained men; 6 untrained men)
caused a 10% increase in the percentage of high-density RBCs (P < 0.05). This was associated with a significant decrease in both the induction (lag) period (an index of antioxidant protection) and maximal rate of O$_2$ uptake (an index of peroxidation) in RBCs challenged with cumene hydroperoxide in vitro (P < 0.01). In contrast to O$_2$ uptake, no significant changes in the induction time or maximum rate of luminol-amplified chemiluminescence (LCL) were found. The latter technique was shown to be a sensitive assay of free radical generation by RBCs treated in vitro with organic hydroperoxide because LCL was inhibited strongly by antioxidants but enhanced by pre-oxidative stress. These results suggest that, whilst a single episode of submaximal exercise increased the susceptibility of RBCs to oxidative stress, regular endurance training may confer a protective effect. These paradoxical changes may be due to acute or chronic alterations in the redox state of cellular glutathione, because RBC $\alpha$-tocopherol concentration did not change and plasma ascorbate increased by 25%. The percentage of high-density cells in the circulation also increased by 10% immediately after running (P < 0.001), but there was no associated increase in membrane-incorporated haem iron. In contrast, treatment of RBCs with low concentrations of oxidants (20-50 µM) in vitro caused much larger increases in cell density and significant membrane incorporation of haem iron. While antioxidant supplementation increased the membrane level of $\alpha$-tocopherol by three-fold, it did not alter RBC density at rest, but virtually abolished the exercise-induced increase in RBC density. The exercise-induced decrease in the time taken for peroxidation to commence in RBCs challenged with cumene hydroperoxide in vitro was also attenuated substantially by antioxidant supplementation.

In contrast to submaximal exercise, single episodes of exercise for one minute at maximum effort induced a 30% increase in osmotic fragility immediately after exercise (P < 0.05) which returned to resting levels within one hour. This correlated with a small decrease in MCHC (P < 0.05) and an increase in MCV (P < 0.05). Blood lactate increased ten-fold (i.e. from 1.5 mM to 15 mM) during this form of exercise and returned to resting levels one hour later. This increase was distributed evenly between the cells and the plasma. Density fractionation of the cells before and after exercise showed that the percentage of low-density RBCs increased at least two-fold immediately after exercise. The majority of the changes induced by maximal exercise could be simulated by treating whole blood from non-exercised subjects with 15 mM L-lactate in vitro with the pH maintained at 7.3. The
functional consequences of these changes are not known, but high blood lactate concentrations impair RBC deformability at both low and high shear rates. The associated increase in MCV may amplify this effect by restricting the passage of RBCs through the microcirculation, thereby limiting the delivery of $O_2$ to working muscles during strenuous exercise. This provides evidence that the lactate anion may contribute to fatigue independently of changes in pH.

In conclusion, single episodes of submaximal and maximal exercise have differential effects on the susceptibility of RBCs to osmotic stress. Submaximal exercise increases the susceptibility of RBCs to oxidative stress, possibly, by lowering the cellular concentration of reduced glutathione, while, in contrast, endurance training confers a protective effect. Supplementation of the diet with antioxidant vitamins ameliorates the effects of oxidative and osmotic stress during submaximal exercise. These results are consistent with the hypothesis that oxidative and osmotic stress both play a role in the increased rate of RBC turnover reported in athletes.
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>2,3-DPG</td>
<td>2,3-diphosphoglycerate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced form)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidized form)</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>OH$^\cdot$</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>LCL</td>
<td>Luminol-amplified chemiluminescence</td>
</tr>
<tr>
<td>MDA</td>
<td>Malonyldialdehyde</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean cell haemoglobin</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean cell haemoglobin concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean cell volume</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>VO$_2$ max</td>
<td>Maximum rate of oxygen consumption</td>
</tr>
</tbody>
</table>
CHAPTER 5

LITERATURE REVIEW

This chapter includes a summary and outline of the work presented in this section of the thesis.
1. Introduction

Efficient delivery of O₂ to exercising muscles by red blood cells (RBC) is an important determinant of peak performance by human athletes. RBCs have a limited life-span. There is a wide body of evidence which suggests that endurance training accelerates the rate of RBC destruction which may lead, in some cases, to the so-called condition of "sports anaemia". Destruction of RBCs as a result of physical exertion was first reported, in fact, in 1881 (see review by Szygula, 1990). The majority of reports of sports anaemia, however, base their conclusions on measurements of plasma indices of iron status (e.g. plasma ferritin concentration). RBC parameters that are indicative of more subtle damage (e.g. markers of osmotic and oxidative stress, deformability, etc) are now being examined during training because iron deficiency is not necessarily indicative of true anaemia (RBC deficiency) (Magnusson et al., 1984).

The aim of this chapter is to review the studies which are relevant to the results of my own project. The review begins by describing the biology of the RBC. The haematology of exercise and plasma indices of sports anaemia are then reviewed. This is followed by a discussion of the putative mechanisms involved in damaging the RBC (like osmotic and oxidative stress) and their possible effects on RBC deformability. The hypotheses that were tested are then presented following the Summary and Conclusions Section.

2. The biology and life cycle of the red blood cell

The major role of the RBC is to transport O₂ via haemoglobin (Hb), from the lungs to body tissues and return CO₂ to the lungs for expiration. Haemoglobin (Hb) constitutes 99% of the cytosolic protein in mature RBCs (Hebbel and Eaton, 1989). Mature anucleated RBCs are derived from nucleated erythroid precursors produced in the bone marrow. They are released as reticulocytes (which are immature cells containing RNA fragments) into the general circulation where they differentiate into mature RBCs that lack RNA and nuclear material. The production of RBCs is regulated by erythropoietin, a glycoprotein secreted principally by the kidney; the secretion of erythropoietin may be initiated by hypoxia (Krantz, 1991).

Mature RBCs are, however, more than a concentrated solution of Hb enclosed in a lipid/protein shell (Fig. 1). The plasma membrane is a complex four layer structure consisting of a single extracellular glycoprotein coat...
Fig. 1. Schematic illustration of a cross-section through the four-layer structure of the human erythrocyte plasma membrane. The glycocalyx (I) consists largely of glycoproteins. The two halves of the membrane lipid bilayers (II and III) consist mostly of phospholipids. Of these, a significant fraction is glycolipids. The lipid molecules of both the extracellular and protoplasmic halves of the lipid bilayer are in the fluid state. The thickness of the membrane lipid bilayer (2b) equals about 4.5 nm. The major component of the membrane skeleton (IV) is the water-soluble protein spectrin. The structure of this protein is believed to resemble that of a flexible necklace-like chain of rigid segments. In the membrane skeleton, the chain length is about 40 times the lipid bilayer thickness and about 100 times the average diameter of each of the individual chains. Ankyrin interconnects the membrane skeleton to the intramembrane particles. The glycoprotein glycophorin C is connected to the membrane skeleton by binding to protein 4.1. The distance from the lipid bilayer cytoplasmic surface to the attachment point of the membrane skeleton equals a. Coordinate z is the distance from this attachment point and defined to be positive for points located on the cytoplasmic side of the membrane skeleton.

Source: Elgsaeter and Mikkelsen (1991)
Membrane-spanning proteins such as band transporter and glycophorin are embedded in the lipid bilayer. These proteins are attached, via ankyrin and protein 4.1, to cytoskeletal components that contain a further 15 major proteins associated loosely with the plasma membrane; these proteins include spectrin and actin (Elgaeter and Mikkelsen, 1991). This organisation gives the RBC its remarkable physico-elastic properties that enable it to withstand shearing and deforming forces in the circulation and allow it to reshape itself in order to pass through capillaries and splenic sinusoids with diameters much smaller than the cell itself normally possesses in its biconcave discoid configuration (Waugh et al., 1992). Disruption of this physical network is potentially fatal to the cell.

RBCs have a limited life-span of 120 days in the circulation of human subjects and, under normal conditions, about one percent of the total RBC population (~2 x 10^{11} cells) is removed and replaced per day (Aminoff, 1988). The maintenance of an adequate O_{2} supply to the tissues requires the rate of replacement to keep pace with that of destruction. Upon its release into the circulation, the RBC undergoes various mechanical, metabolic, osmotic and physiological stresses over a journey estimated to be about 175 miles by the end of its circulating life-span (Piomelli, 1988). Whilst retention of its deformability appears to be the major determinant of RBC life-span (Mohandas et al., 1979), the exact factors that regulate the survival of RBCs and promote the sequestration of senescent RBCs by tissue macrophages are not known. Because the RBC is anuclear, it cannot replace proteins which have been damaged irreversibly, but it does possess a limited capacity to repair itself (see Section 5.2).

The modifications to RBCs that result in their senescence and subsequent destruction have been reviewed extensively (Clark, 1988; Kosower, 1993). Because normal senescent RBCs cannot be separated from young cells morphologically (Beutler, 1988a), nearly all investigators have used isolated high-density cells to study the mechanisms of RBC aging on the assumption that RBC density increases progressively with age. This theory has been challenged by several groups recently (Beutler, 1988b; Dale and Norenberg, 1990; Waugh et al., 1992). The radiolabelling or biotinylation of a cohort of isolated RBCs and their transfusion back into the original subject has been suggested as an alternative procedure for following their...
removal from the circulation (Clark, 1988; Dale and Norenberg, 1990). There are, however, considerable technical and ethical problems associated with the use of such procedures in human subjects, and animal models are unlikely to offer a suitable alternative because of the considerable interspecies variation in RBC turnover (Clark, 1988).

In high-density RBCs, putative markers of senescence include, inter alia, increases in: the band 4.1a:4.1b ratio (Mueller et al., 1987); membrane rigidity and impaired deformability (Fortier et al., 1988; Shiga et al., 1985); protein carboxyl methylation; glycosylated Hb (Clark, 1988); and binding to the cell surface of anti-band III and anti-α-galactosyl autoantibodies (Beppu et al., 1990). However, putative markers whose activities change progressively as the RBC ages have not been found so far (Clark, 1988). Irreversible modification to band III, the anion transport protein, appears to generate a "senescent antigen", to which IgG and C3b bind and promote phagocytosis by macrophages (Kay et al., 1988). The irreversible aggregation of the transmembrane proteins, band III and glycophorin, increases progressively with increasing RBC density (Corbett and Golan, 1993). Senescence is also associated with loss of sialic acid residues caused by the budding of membrane sections (as vesicles) which results in decreased surface area (Clark, 1988). Oxidative damage and/or non-enzymatic glycosylation of RBC membrane surface proteins are the most likely mechanisms involved in the generation of senescent antigens (Vlassara et al., 1987; Lutz et al., 1988). There is no conclusive evidence that metabolic depletion ages RBCs (Clark, 1988) because enzyme activities do not change significantly in mature RBCs (Beutler, 1988a,b) and Ca2+ or ATP levels are not depleted (Clark, 1988; Chiu and Lubin, 1989). These reports suggest that the ageing of RBCs is indeed a multifactor process (Danon and Marikovsky, 1988).

3. The haematology of exercise

The efficient transport and delivery of O2 to working tissues during physical activity is an important factor in peak sports performance. Thus, if the rate of RBC destruction exceeds the rate of replacement, the athlete may become anaemic to an extent that compromises performance. In fact, there have been numerous reports of "sports anaemia" (reviewed by Carlson and Mawdsley, 1986; Newhouse and Clement, 1988). Various hypotheses have been proposed to explain the aetiology of this condition. These include:
dietary iron deficiency; haemolysis caused by repeated mechanical trauma; and osmotic and oxidative damage to RBCs during intensive exercise. Considerable debate still exists, however, as to whether training-related indices of iron deficiency observed in some athletes designate true anaemia (RBC deficiency) or whether the symptoms are due to training-induced increases in plasma volume that cause a haemodilutional effect (pseudoanaemia) (O'Toole et al., 1988; Green et al., 1991). Moderate endurance exercise may increase the rate of RBC production concomitantly with accelerated turnover resulting in the presence of a steady-state population of “younger” RBCs in athletes. This may be advantageous to athletic performance, as younger RBCs are more deformable and deliver O₂ to working muscle more efficiently than older cells (Schmidt et al., 1988). This is partly due to their higher concentrations of 2,3-diphosphoglycerate (2,3-DPG) which serves, in turn, to decrease the affinity of Hb for O₂ at the partial pressures found in contracting muscle (Mairbaurl et al., 1983). A major purpose of altitude training - which is an important component of elite endurance athletes’ training programs - is to increase RBC 2,3-DPG levels. Thus, increased RBC destruction per se is unlikely to be detrimental to performance unless replacement fails to keep pace with it. It may, in fact, be advantageous if it results in the constant replenishment of a younger RBC population.

4. Plasma indices of "sports anaemia"

Sports anaemia is usually diagnosed with reference to significant reductions in blood parameters such as Hb concentration, circulating RBC counts and plasma ferritin (an iron-binding protein). The plasma ferritin concentration is the major index used clinically to assess the total iron status of individuals (Carlson and Mawdsley, 1986; Newhouse and Clement, 1988). Two thirds of the body's iron is contained within RBCs as Hb. Decreases in iron status may not be indicative of RBC deficiency, however, because iron availability may not be limiting for erythropoiesis. Iron intake can usually be manipulated by diet and/or supplementation but careful monitoring is required because iron may be toxic in high concentrations (see Section 5.1). Whilst 80 to 90 % of RBC destruction occurs extravascularly (e.g. RBCs are destroyed by macrophages within the spleen) without Hb release into plasma, the small amount generated from intravascular haemolysis is sequestered by high-affinity haem-binding glycoproteins (Schacter, 1988).
Reticuloendothelial cells such as macrophages provide most of the iron required for erythropoiesis under normal conditions (Fillet et al., 1989).

There is a wide and somewhat variable body of evidence which suggests that endurance athletes, particularly runners, may be vulnerable to sports anaemia. Marathon running, in particular, is associated with substantial RBC destruction. Significant reductions in RBC counts, Hb concentration and haematocrits have been detected in cyclists during the racing season in the absence of any change in plasma ferritin (Guglielmini et al., 1989). Because haemodilution may explain these changes, more specific indices of anaemia need to be examined. Haptoglobin and haemopexin are iron-binding proteins that shunt haem iron back to the liver and prevent that portion released from haemolysed RBCs from being excreted. Intravascular haemolysis, but not iron deficiency, is indicated by low plasma haptoglobin concentrations (Magnusson et al., 1984). Free Hb will only be detected in the plasma and urine if the iron-binding capacities of haptoglobin and haemopexin are saturated (Hunding et al., 1981). In addition, ferritin and haptoglobin are acute-phase proteins (Witte, 1991) whose plasma concentrations can increase markedly as a result of strenuous exercise. The maintenance of a chronic acute-phase response during periods of intensive training may mask iron deficiency and haemolysis in some athletes (Witte, 1991). Iron can also be lost in sweat, urine and through gastrointestinal bleeding (Seiler et al., 1989). Transition metal-binding proteins such as ferritin and ceruloplasmin prevent iron and copper ions from acting directly as catalysts in the formation of toxic free radicals (Krinsky, 1992) (see Section 5.2).

The mechanical trauma of footstrike may be a dominant factor in RBC changes observed after running. Downhill running, for example, causes much greater tissue damage than uphill running at the same intensity because of the greater emphasis on eccentric rather than concentric muscle contractions (Miller et al., 1988). The duration of physical exertion as well as its intensity is another important variable because intravascular haemolysis (assessed by the fall in plasma haptoglobin) occurs in virtually all triathletes competing in events that last longer than two hours (O'Toole et al., 1988), although the direct damage to RBCs may be confined to the running phase. Genuine intravascular haemolysis occurs during the course of a marathon because the significant decreases in plasma haptoglobin that are detectable immediately after the race are accompanied by reductions in Hb concentration, haematocrit, MCHC, MCV, ferritin and serum iron
(Casoni et al., 1985). Haematuria and significant falls in plasma haptoglobin were detected in the majority of 23 runners who showed evidence of substantial muscle damage after a marathon (Lijnen et al., 1988). The early onset of haemolysis during a marathon is likely in view of the rapid fall in plasma haptoglobin concentration detected after a 5 km run at maximal effort by 11 Olympic runners (Wolf et al., 1987).

Footstrike may not be the only component involved in the mechanical destruction of RBCs because intravascular haemolysis and iron depletion have also been reported after activities like distance swimming that involve no foot impact (Selby and Eichner, 1986). Even strength training has been reported to reduce plasma ferritin and haptoglobin concentrations significantly (Schobersberger et al., 1990). Endurance runners have lower plasma concentrations of ferritin and haptoglobin than matched untrained sedentary controls, elite cyclists and rowers (Dufaux et al., 1981) while, in contrast, both male and female swimmers have higher iron stores than sedentary controls (Pellicca and Di Nucci, 1987).

These studies show that mechanical trauma is clearly an important component of RBC damage and iron loss although the indices discussed so far rely on the haemolysis of RBCs. The removal of RBCs associated with normal turnover, however, is largely an extravascular process that occurs without haemolysis (Watts, 1989). Therefore, a much more sensitive index of RBC damage may be found by examining RBCs for more subtle markers of damage that precede haemolysis. Erythropoietin does not appear to be a useful index of RBC production because its serum concentration does not differ significantly between highly-trained cross country skiers and untrained subjects (Berglund et al., 1988) and large intra-individual variations occur over 24 hours as part of the normal circadian rhythm (Wide et al., 1989). Thus, it is not surprising that are no direct correlations between serum erythropoietin concentration, blood reticulocyte counts and Hb concentration (Klausen et al., 1991).

5. Cellular indices of sports anaemia

In this section, discussion will focus on changes at the cellular level that may be indicative of increased RBC destruction and vulnerability to sports anaemia. Because the RBC is subjected to increased oxidative, mechanical and osmotic stress during exercise, I will focus on these stressors as causative mechanisms that may be involved in exercise-induced RBC
damage and destruction. Recently, convincing direct evidence of increased RBC destruction during training was reported (Weight et al., 1991). Using $^{51}$Cr-labelled autologous RBCs, which were injected back into the donor, this group showed that the labelled cells were not detectable after 114 days in sedentary subjects compared with the disappearance after only 74 days in male and females running 50 to 129 km/week. The $^{51}$Cr-labelling technique can be criticized on the grounds that continuous elution of the label from the cells can occur which is kinetically indistinguishable from loss of label due to increased RBC destruction (Landaw, 1988). Furthermore, the half life of $^{51}$Cr-labelled cells does not appear to be directly proportional to RBC life-span perhaps because of the strong oxidative action of $^{51}$Cr (Dacie and Lewis, 1984). Despite these concerns, these studies suggest that RBC turnover in runners may be nearly double that of their sedentary counterparts.

5.1 Standard cellular parameters

Reticulocyte counts, the RBC content of zinc protoporphyrin and creatine may be useful in assessing the vulnerability of individual athletes to “sports anaemia” because these parameters only change as the ratio of young to old RBCs is altered (Labbe and Rettmer, 1989; Buysse et al., 1990). Reticulocyte counts have been shown to be elevated by 100% two days after a marathon, for example, and this increase was sustained for a further two days while the plasma haptoglobin concentration decreased significantly (Schmidt et al., 1989). The same group also reported that the rate of RBC destruction increased two-fold during a three week period of moderate endurance training by previously sedentary male subjects (Schmidt et al., 1988). Increased reticulocytosis and RBC creatine content were indicative of the presence of an increased proportion of younger RBCs which may have been responsible, in part, for the increased physical performance of these subjects at the end of the training period (Schmidt et al., 1988).

5.2 Oxidative stress

Oxidative stress occurs as a result of the metabolic generation of reactive oxygen species (ROS), most of which are free radicals; they are generated continually as by-products of aerobic metabolism. These species may damage body tissues if their production is not controlled precisely and cells are not protected adequately (Demopoulos et al., 1986). Irreversible oxidative damage to certain vulnerable biomolecules is thought
Fig. 2. The antioxidant network of erythrocytes.

Source: Hebbel (1986a)
to contribute to the degenerative processes associated with cell breakdown and ageing (Halliwell et al., 1992). Fortunately, a network of cellular defence systems (i.e. antioxidant compounds and free radical detoxification enzymes) have evolved which, together with dietary antioxidants (such as vitamins A, C, & E), function to prevent oxidative damage (Fig. 2). If these defence systems are impaired, or overwhelmed, highly-reactive radicals such as the hydroxyl radical (OH·) can induce irreversible chemical modifications and, thereby damage, to all major biomolecules including proteins, lipids, carbohydrates and nucleic acids (Halliwell et al., 1992).

Alterations to lipids and proteins occur in oxidatively-stressed RBCs (see Section 5.2.1). Peroxidation of cellular lipids by free radicals is the best characterized process of molecular oxidative damage (Fig. 3). It is initiated under appropriate conditions by free radicals (R') that are capable of abstracting hydrogen atoms from methylene groups situated between the cis double-bonds of unsaturated fatty acids (Maiorino et al., 1989). The resulting radicals undergo spontaneous molecular rearrangement, followed by reaction with O₂, to produce peroxy radicals, which attack adjacent membrane fatty acids and initiate a chain-reaction that cycles until self-termination (through reaction between two radicals) or interruption of the cycle by chain-breaking antioxidants (e.g. α-tocopherol) occurs. Lipid hydroperoxides, which are generated as by-products of α-tocopherol activity, can be detoxified by GSH peroxidase and this limits their availability for reinitiation of the peroxidation cascade (Maiorino et al., 1989). Reactive aldehydes such as malonyldialdehyde (MDA) and 4-hydroxynonenal are detectable end-products of the lipid peroxidation cascade (Gutteridge and Halliwell, 1990).

In contrast to lipid peroxidation, the mechanisms by which oxidative damage to proteins propagates are not well understood (Davies and Goldberg, 1987a). Protein oxidation can be initiated through hydrogen abstraction from thiol groups (which are extremely vulnerable to free radical attack) and, perhaps, from other amino acids such as methionine (Shechter et al., 1975) with exposed susceptible functional groups. This process may occur independently of, or be directly associated with, lipid peroxidation (Baysal et al., 1989). Oxidative modification of proteins disrupts their tertiary structure which, in turn, makes them highly-susceptible to proteolysis (Dean et al., 1992). Protein carbonyl derivatives are also generated during oxidative attack and these can be detected in vitro by chemical conversion to stable end-products (Oliver et al., 1987). Exposure of some amino acids and
Initiation

Removal of H· (can occur at several places in the chain)

Molecular rearrangement

Minor reactions

Attack on membrane proteins, crosslinking if two radicals meet

Major reaction

O₂

Lipid peroxy radical

H· abstraction from adjacent membrane lipid

Lipid hydroperoxide

Attack on membrane proteins; reaction of two peroxy radicals to cause singlet oxygen ('O₂)

formation

\[ \cdot\text{CHO}_2 + \cdot\text{CHO}_2 \rightarrow \cdot\text{C} = \cdot\text{O} \]

\[ + \cdot\text{C} - \cdot\text{OH} + \text{'O}_2 \]

Fig. 3. An outline of the mechanism of lipid peroxidation.

Source: Gutteridge and Halliwell (1990)
proteins to \( \gamma \)-irradiation or various free radical generating systems in vitro has been reported to form long-lived protein hydroperoxides (Gebicki and Gebicki, 1993). The exact role of these species in cell damage is unknown at present (Gebicki and Gebicki, 1993).

Nearly all cellular antioxidants and enzymes that interact with and detoxify free radicals are located in the cytosol, with the exception of \( \alpha \)-tocopherol which is the principal lipophilic antioxidant of all cell membranes. Retinoids and carotenoids are also lipophilic antioxidants but their presence in RBCs has not been reported. Ubiquinol may also be an important lipophilic antioxidant of nucleated cells (Frei et al., 1990) but the total blood concentration of ubiquinols and ubiquinone is very low (Lang et al., 1986; Constantinescu et al., 1993). Enzymes that detoxify free radicals nearly all work in collaboration. For example, \( \text{O}_2^- \) is dismutated to \( \text{H}_2\text{O}_2 \), either spontaneously or by the catalytic action of superoxide dismutase (SOD) which speeds up the reaction by \( 10^4 \)-fold (Hebbel, 1986a). However, \( \text{H}_2\text{O}_2 \) itself is toxic and must be reduced to water by either GSH peroxidase or catalase. The highly-reactive \( \cdot\text{OH} \) can be generated from \( \text{H}_2\text{O}_2 \) via the iron-catalysed “Fenton reaction” (Krinsky, 1992).

Lipid antioxidants such as \( \alpha \)-tocopherol function as membrane-localized “chain-breakers” that inhibit the autocatalytic peroxidation of vulnerable polyunsaturated fatty acids (Packer, 1991). In humans, \( \alpha \)-tocopherol is derived from dietary sources. This vitamin is transported in the blood via lipoproteins and is also stored in the liver (Warren et al., 1991). The liver pool may maintain the circulating pool which, in turn, replenishes the plasma membrane pool of \( \alpha \)-tocopherol in RBCs during oxidative assault. Because \( \alpha \)-tocopherol is the only lipid antioxidant in RBCs, GSH and ascorbate are thought to participate in a specific redox shuttle that maintains it in the reduced state (Stocker et al., 1986) but proof of this mechanism has not been found under conditions that prevail in vivo (Burton et al., 1990). Alternatively, \( \alpha \)-tocopherol in RBCs may be recycled from its tocopheroxy radical by an NADH-cytochrome b\(_5\) reductase (Constantinescu et al., 1993).

Glutathione in its reduced state (GSH) is a tripeptide with a free thiol group which acts as a “thiol buffer” that minimises oxidative damage to thiol-containing proteins. About 95% of intracellular glutathione is in its reduced form (GSH) in nearly all human cell types (Kretzschmar and Muller, 1993). GSH also inactivates various oxygen-, carbon-, nitrogen, and sulphur-centred free radicals either directly, or indirectly through the
selenium-dependent GSH peroxidase (Hebbel, 1986a). This enzyme also detoxifies lipid hydroperoxides and H₂O₂. The GSH oxidized to the disulphide form (GSSG) during this reaction is regenerated by GSSG reductase which utilises, as cosubstrate, NADPH supplied exclusively through the oxidative segment of the pentose phosphate pathway (Ouwererk et al., 1989). GSSG reductase can also cleave mixed disulphides between GSH and Hb but it cannot break protein-protein disulphides (Hebbel, 1986a). GSH is stored in high abundance in all mammalian cells, particularly in hepatocytes; the liver may replenish depleted GSH reserves in other cells (Lu et al., 1990).

5.2.1 Oxidative stress and red blood cells

Because of their continuous exposure to high O₂ fluxes, RBCs are extremely vulnerable to oxidative damage. RBCs contain large amounts of catalytic iron (i.e. two thirds of the body's iron is contained in Hb) and polyunsaturated fatty acids that are highly-susceptible to oxidative attack. Mature RBCs are unable to replace damaged proteins by synthesis de novo. There is considerable evidence that oxidative damage may be the primary mechanism by which RBCs age (Clark, 1988). ROS generation begins in the RBC by auto-oxidation of oxy-Hb to met-Hb (which cannot carry O₂). This reaction, which normally occurs at the rate of three percent/day (Hebbel and Eaton, 1989), culminates in the release of O₂⁻, the precursor highly-toxic free radicals such as OH⁻. As described already, OH⁻ may be generated by an Fe²⁺-catalysed reaction in which H₂O₂ is the substrate and the role of O₂⁻ is to recycle iron to its ferrous state by reducing Fe³⁺ (Hebbel, 1986). The accumulation of primary products of lipid peroxidation (e.g. MDA, conjugated dienes) and the formation of abnormal protein aggregates and fragments (e.g. spectrin-Hb complexes) are indicative of RBC oxidative damage. Slightly damaged RBCs may also be susceptible targets for attack by neutrophils in vivo (Hatherill et al., 1986) and, in doing so, they may have an "altruistic" role in extracellular antioxidant defence through protection of less vulnerable liver and lung tissue from oxidative damage (Winterbourn and Stern, 1987). RBCs may also be targeted for phagocytic attack because of their role in binding circulating immune complexes and shunting them to the liver for removal by macrophages (Emlen et al., 1992).

Oxidative damage has been implicated in the normal ageing of RBCs because the methionine sulfoxide content of "middle-aged" and senescent RBCs has been reported to be double that of recently matured cells.
Seppi et al., 1991). Many enzymes like the Ca\textsuperscript{2+}-ATPase (Moore et al., 1992) and GSH peroxidase (Pigeolet and Remacle, 1991) of RBCs, are extremely susceptible to oxidative damage, possibly because of exposed thiol groups. Senescent cells also contain increased amounts of glycosylated Hb (Clark, 1988) which may contribute to accelerated senescence after a certain point because the glycosylation of some membrane proteins increases their vulnerability to oxidative damage (Jain, 1989a; Birlouez-Aragon et al., 1990).

Many laboratories have reported data showing that the pathology of hereditary conditions such as sickle cell anaemia and \( \beta \)-thalassaemia involves oxidative damage to RBCs (reviewed by Chiu and Lubin, 1989; Hebbel, 1991). Hebbel (1986a,b) has put forward an attractive hypothesis to explain the pathophysiology of sickle cell anaemia which involves an acceleration of the normal RBC ageing process. Hb is a cytosolic protein which is segregated from the membrane under normal conditions. Hb\textsubscript{SS}, the abnormal form found in RBCs from people with the homozygous form of sickled cell anaemia (HbAA is the normal form), exhibits accelerated spontaneous auto-oxidation \textit{in vitro} and Hb polymers are formed readily under hypoxic conditions (Hebbel and Eaton, 1989). RBC membranes from sickled cells also contain excessive amounts of haem and non-haem iron that is genuinely bound (i.e. not just trapped during membrane preparation) to the membrane (Kuross et al., 1988; 1989). Haem iron binds to the membrane as haemichrome, denatured low-spin ferric Hb, which is formed from met-Hb. This is a reversible process but it becomes irreversible if haemichrome binds covalently to the cytoplasmic domain of band III (Kannon et al., 1988) and aggregates of these complexes coalesce into large deposits known as Heinz bodies (Fig. 4). Deficiencies of catalase and GSH in sickle cells further increase the potential for irreversible oxidative damage (Hebbel, 1986a).

Figure 4 shows the pathway of Hb denaturation under pathological conditions. Even under normal circumstances, however, where three percent of Hb is auto-oxidised daily, if the OH\textsuperscript{•} is generated in an environment that is segregated from cytoplasmic defences, the potential for irreversible oxidative damage must increase (Hebbel, 1986a,b). Furthermore, decompartmentation of iron from the cytosol to the membrane may exacerbate the process by short-circuiting \( \alpha \)-tocopherol protection if membrane-associated iron catalyses the decomposition of lipid hydroperoxides into highly-reactive alkoxy and hydroxyl radicals which
Fig 2. Proposed sequence of events in the oxidation and precipitation of unstable hemoglobin as Heinz bodies. Individual steps are discussed in the text. Heme-depleted hemoglobin may arise directly through deletion of heme-binding amino acids, or it may result from heme loss either from methemoglobin or after formation of irreversible hemichrome; hence its multiple points of entry into the sequence. It may precipitate directly or via hemichrome formation on the non-mutant chain. In some cases, it appears that chain dissociation precedes hemichrome formation. Heinz bodies may be the trigger for recognition although other modifications to the cell membrane may also be involved.

Fig. 4. Proposed pathway of haemoglobin oxidation and erythrocyte haemolysis.

Source: Winterbourn (1990)
reinitiate the chain propagation reactions that peroxidize lipids (Hebbel, 1986a,b).

Despite their potential vulnerability to oxidative stress, RBCs are well protected from immediate attack by high activities of the major free radical detoxification enzymes and large concentrations of ascorbate and α-tocopherol (Hebbel, 1986a,b). The importance of the enzyme networks that detoxify free radicals is illustrated by genetic deficiencies. Lack of glucose-6-phosphate dehydrogenase activity, for example, leads to early haemolysis because the NADPH necessary to maintain GSH in the reduced state cannot be synthesized by the oxidative segment of the pentose-phosphate pathway (Arese and De Flora, 1990). Deficiencies in met-Hb reductase and the glutathione-dependent enzymes have also been reported with similar consequences.

In RBCs, oxidation of proteins appears to precede the onset of lipid peroxidation (Davies and Goldberg, 1987a). Some oxidized proteins can be repaired. Met-Hb can be reduced to oxy-Hb by the NADH-dependent enzyme met-Hb reductase (Hebbel, 1986a) and thiol damage may be repaired to some extent by GSH-mediated reduction (Chiu et al., 1989). RBCs, in particular, contain large amounts of GSH at a ratio to GSSG of several hundred to one (Beutler, 1984). Alternatively, if protein oxidation is irreversible, the affected protein can, in some cases, be degraded to amino acids by proteases that function as “secondary antioxidants” and thereby prevent protein crosslinking (Sacchetta et al., 1990; Fagan and Waxman, 1991). In fact, the presence of free alanine in RBC extracts is indicative of proteolysis (Davies and Goldberg, 1987b). Degradation of damaged proteins may delay, but not prevent, protein crosslinking. The β-subunit of Hb, for example, is degraded by proteases more efficiently than are the α-subunits, leaving a greater number of unpartnered α-subunits to crosslink with spectrin (Joshi et al., 1983).

As with irreversibly-oxidized proteins, RBCs cannot replace all of their damaged phospholipids by synthesis de novo, but there is continual turnover of some phospholipid pools which allows a proportion of the peroxidized lipids to be replaced by exchange with plasma lipids (Chiu et al., 1989). This occurs mainly with phosphatidylcholine and sphingomyelin, since the plasma concentrations of most other phospholipids are very low (Chiu et al., 1989). Furthermore, the exchange of α-tocopherol between plasma and cellular lipids is rapid (Jain, 1989b). Because irreversibly damaged proteins cannot be replaced under any circumstances, protein
rather than lipid damage is likely to have the most detrimental effect on RBC function. Irreversible lipid damage may, however, increase membrane permeability and rigidity (Chiu et al., 1989) and thereby perturb cellular homeostasis.

5.2.2 Oxidative stress and exercise

Evidence accumulated during the last 15 years suggests that exercise can trigger antioxidant depletion and cause oxidative damage to a variety of cell types including muscle, liver, and RBCs; supplementation with dietary antioxidants such as vitamin-E has been reported to reduce this damage significantly (Jenkins, 1988). During exercise, the O$_2$ flux through the circulation increases at least 10-fold (Gohil et al., 1988; Duthie et al., 1990). Thus, auto-oxidation of Hb may increase in proportion to the O$_2$ uptake induced by exercise and, in some individuals, antioxidant defences may not keep pace.

While cycling is a low-impact activity compared to running, several reports suggest that it does cause oxidative damage to a "subpopulation" of RBCs. A significant increase in GSSG reductase activity in RBCs was detected, for example, immediately after 30 minutes of cycling at 75 % VO$_2$ max (Ohno et al., 1986). Increased GSSG reductase activity may have occurred in response to glutathione oxidation because a 60 % decrease in the RBC level of GSH, and a 100% increase in GSSG, has been reported immediately after 90 minutes of cycling at 65% VO$_2$ max (Gohil et al., 1988).

Whilst some laboratories have reported that human plasma contains negligible amounts of GSH (Gohil et al., 1988), Kretzschmar and colleagues (1991) reported that plasma GSH was significantly higher in young human subjects (27-35 yrs) compared with those aged between 36-57 yrs. They also found that endurance training increased the concentration of GSH in plasma but did not prevent its age-related decline. Furthermore, the baseline concentration of plasma lipid peroxides was not affected by age or training (Kretzschmar et al., 1991) but the plasma concentration of MDA increased significantly after a single session of vigorous exercise (Mena et al., 1991). These workers also reported that the concentration of GSH in plasma fell after maximal exercise in trained subjects only, possibly because of their greater power output. Rises and falls of this magnitude in plasma may follow from the secretion of GSH from the liver (Lu et al., 1990).

Exercise-induced changes in the glutathione system may explain why isolated RBCs become more vulnerable to oxidative stress in vitro after
exercise. This system may be the "first-line-of-defence" against the oxidative stress induced by exercise because even a half marathon caused no changes in the activities of glucose-6-phosphate dehydrogenase, SOD, GSH peroxidase, and catalase, or the redox state of α-tocopherol (Duthie et al., 1990). The vulnerability of RBCs to peroxidative lysis by H₂O₂ increased significantly 24 hours after a half-marathon although no increase was detected immediately after the race in spite of a significant fall in GSH (Duthie et al., 1990); no corresponding rise in GSSG was detected but the authors suggested that this may have been due to the binding of GSSG to RBC cytoskeletal proteins.

Release of antioxidants such as α-tocopherol from adipose tissue or GSH from the liver into plasma during intensive exercise may conserve antioxidant protection in muscle, liver and RBCs. α-Tocopherol levels increased by 20% in plasma and doubled in RBCs immediately after maximal exercise but both returned to baseline values 10 minutes later (Pincemail et al., 1988). The plasma concentration of GSH increased by 30% immediately after maximal exercise in trained, but not untrained, subjects while no change in plasma GSSG or lipid peroxides occurred (Kretzschmar et al., 1991). The concentration of ascorbate in plasma increased by 25% during a 21 km running race and this rise correlated with increased plasma cortisol (Gleeson et al., 1987). It is possible that these events are connected and that this hormone may initiate the release of ascorbic acid from the liver and other tissue stores into the circulation.

Endurance training (5 km/day for 10 weeks) increases the activities of GSSG reductase and catalase in RBCs (Ohno et al., 1988). Twenty weeks of endurance-training increased blood GSH by 50% in previously sedentary men and this was accompanied by a significant increase in GSSG reductase activity (Evelo et al., 1992). Furthermore, the activities of GSH peroxidase and catalase in RBCs have been reported to increase progressively with training distance in endurance runners (Robertson et al., 1991), and RBC isolated from professional cyclists have much higher catalase and GSH peroxidase activities than cells isolated from matched sedentary subjects (Mena et al., 1991). The concentrations of vitamin-E and GSH in RBCs also increase in trained runners (Robertson et al., 1991). These increases in the steady-state concentrations of RBC antioxidants were not considered to be due to the presence of younger cells because no correlations were found with RBC creatine or reticulocyte counts (Robertson et al., 1991). Training-induced adaptations may occur at the level of nucleated bone
marrow RBC precursors because the selection pressure of high oxidative stress (e.g. that associated with ß-thalassaemia) leads to an increase in the activities of some RBC free radical detoxification enzymes such as superoxide dismutase and GSH peroxidase (Gerle et al., 1987). In contrast, one study with rodents suggests that RBCs produced under accelerated conditions of erythropoiesis and/or metabolic rate have reduced survival times (Landaw, 1988). Endurance training of rats, however, increases the activities of several antioxidant enzymes in both skeletal muscle and heart and it delays the onset of RBC haemolysis (Aikawa et al., 1984).

Oxidative damage to RBCs and muscle may limit aerobic performance because the endurance capacity of human subjects is reduced substantially by vitamin-E deficiency (Gohil et al., 1986) but prolonged by the regular ingestion of vitamin-E and/or free radical spin-trapping agents (Novelli et al., 1990). Vitamin-E also protects against the detrimental effects of physical exertion at high altitude (Simon-Schnass and Korniszewski, 1990). The age-related decline in athletic performance may due, in part, to RBC oxidation because RBCs from older rats (> 27 months) are affected more severely by oxidative stress than cells isolated from young rats (< 8 months), possibly because their RBCs have lower activities of GSSG reductase and SOD (Glass and Gershon, 1984). The activities of some antioxidant enzymes are markedly diminished in RBCs isolated from elderly people (Kosower, 1993).

As discussed previously, sickled RBCs are more vulnerable to oxidative damage than normal cells (Section 5.2.1). Sickle cell trait (the heterozygous form, HbAS) increases the risk of sudden death during intense physical exertion, perhaps through irreversibly-sickled cells causing vascular obstruction (Kark et al., 1988). Remarkably, this condition does not affect anaerobic and aerobic exercise performance (Gozel et al., 1992) and elite athletes with sickle cell trait are still capable of achieving world class results (Konotey-Ahulu, 1974). To my knowledge, RBC turnover has not been examined in athletes with sickle cell trait but this may be a fruitful area for future studies. A recent report suggests that HbAS cells are more vulnerable than normal cells to oxidation during exercise and, furthermore, the percentage of dense RBC membranes increases substantially in HbAS, but not HbA2, cells after exercise (Das et al., 1993).
5.3 Osmotic effects

With each trip around the circulation, the RBC undergoes a shrinking and swelling cycle which may lead to physical damage due to normal "wear-and-tear". Therefore changes in blood osmolality may adversely affect RBC deformability and their passage through the microcirculation (see Section 6).

5.3.1 Osmotic homeostasis and red blood cells

RBCs are normally biconcave discocytes with a large surface area to volume ratio which gives them great "filterability" properties (Boucher et al., 1985). Filterability refers to the ability of isolated RBCs to pass through filters with pore diameters ~ 3 µm under constant pressure in vitro (Chien, 1987). The high content of water (70%) in the cytoplasm of normal RBCs contributes to their low internal viscosity (Stuart and Ellary, 1988). The surface area of the RBC remains constant during osmotic volume changes (Canham and Parkinson, 1970). Plasma and intraerythrocyte osmolality are positively correlated, but this relationship does not hold under acidic conditions in which RBC swelling is induced by lactate accumulation and a lower pH (Buono and Faucher, 1985). Thus the RBC does not behave like a perfect osmometer under all conditions (Van Beaumont et al., 1981) and some studies suggest that plasma osmolality may not be the key factor in regulating RBC volume (Staubli and Roessler, 1986) because RBCs do not respond to small changes in osmolality of the order of 10-12% (Van Beaumont and Rochelle, 1974). Some hormones (e.g. catecholamines) may also influence the osmotic properties of RBCs by regulating cation transport (Staubli and Roessler, 1986; Bodemann et al., 1987). β-adrenoceptor stimulation, for example, activates active Ca\(^{2+}\) transport into the cell to counterbalance the release of K\(^+\). Low physiological concentrations of adrenalin and PGE\(_2\) decrease the deformability of RBCs and increase their vulnerability to haemolysis under hypotonic conditions (Rasmussen et al., 1975).

Cation homeostasis within the RBC is maintained under substantial gradients for monovalent cations and Ca\(^{2+}\) (Hebbel, 1991). These gradients are controlled by active transport carriers and various mechanisms for water and ion passage that are not yet clearly defined. Transport of anions (e.g. Cl\(^-\)) occurs via the band III transmembrane carrier and lactate is transported mainly via a specific monocarboxylate carrier by a H\(^+\)-lactate symport mechanism (Poole and Halestrap, 1993). Cellular dehydration, which may
occur through the loss of cations, and thus water, increases cell density and impairs the ability of RBCs to deform in the microcirculation (Wiley, 1990). As normal RBCs age, they lose $K^+$, and thus water, and thereby show increased mean cell haemoglobin concentration (MCHC) and density (Usami et al., 1971). The intracellular $K^+$ content is, in fact, critical to the maintenance of cell volume within normal limits (Brugnara et al., 1987).

Two transport systems appear to be the major regulators of RBC volume. The $K^+ / Cl^-$ co-transport system is responsible for volume decreases in hypotonic conditions, while the $Na^+ / H^+$ antiporter can produce volume increases in both hypertonic or acid conditions (Fabry et al., 1991). Cytoplasmic viscosity increases exponentially once MCHC increases above 32 g/dl (Stuart and Ellary, 1988) in dehydrated cells that are more sensitive to shear stress (Platt et al., 1981). Shrinkage may allow opposing sides of the membrane to contact and establish covalent bonds through disulphide linkage (Fischer et al., 1985) and these cells could become particularly vulnerable to oxidative damage if haem iron, which is a potent catalyst of free radical formation, became associated with the lipid phase of the membrane due to Hb binding to membrane components (Snyder et al., 1981). While the mechanism(s) responsible for dehydration are not understood, oxidative damage may be a contributing factor because of the increased $K^+$ leakage observed following lipid peroxidation (Jain et al., 1989).

5.3.2 Osmotic stress and exercise

Substantial osmotic changes can occur in the blood during exercise. These changes depend primarily on the intensity of the workload. Workloads up to 75% $VO_2$ max cause a small linear decrease in plasma volume as the exercise progresses because of the movement of plasma water into the extravascular space and its loss through perspiration (Wilkerson et al., 1977). However, it is unlikely that significant osmotic adjustments occur in circulating RBCs in response to this because vasopressin secretion, which normally rises in proportion to the increase in plasma osmolality, does not increase significantly after moderate exercise (Wade, 1984; Freund et al., 1991). Furthermore, intraerythrocyte $K^+$ concentration does not change unless the workload exceeds 80% $VO_2$ max (Hespel et al., 1986).

Although large changes in plasma osmolarity can alter RBC density, MCHC varies within narrow limits in normal individuals with about 95% of the RBC population falling within the range 29-35 g/dl and only 5% exceeding 37 g/dl (Evans et al., 1984). Regular endurance training has been
reported to decrease the percentage of high-density RBCs (Mairbaurl et al., 1983) and to increase MCV without changing RBC mass (Green et al., 1991). These changes are consistent with the accelerated removal of the oldest cells. Participation in a half-marathon, in contrast, caused a significant redistribution of RBCs to the heavier fraction (Robertson et al., 1988) suggesting that a subpopulation acquired one of the properties of “aged” cells during the race. These changes were unlikely to be mediated osmotically because the decrease in MCV detected after a full marathon did not correlate with increased plasma osmolality (Staubli and Roessler, 1986).

Exercise at maximum capacity may also affect RBC osmotic properties. For example, treatment of RBCs with lactic acid in vitro increases their MCV and osmotic fragility (Beutler et al., 1982). Acidosis also causes movement of \( H^+ \) and \( Cl^- \) ions into RBCs (Van Beaumont, 1973). However, a compensatory osmotic response to acidosis may explain why progressive exercise to exhaustion does not alter MCHC or MCV significantly despite a 7% increase in intraerythrocyte and plasma osmolality (Buono and Faucher, 1985; Van Beaumont and Rochelle, 1974).

6. Blood Rheology and red blood cell deformability

Blood rheology is determined by the combined influences of plasma viscosity, haematocrit and blood cell deformability. Plasma viscosity is controlled largely by the concentration of macroproteins, of which fibrinogen and the globulins exert the primary influence (Klug et al., 1974). Interaction of fibrinogen with RBCs may lead to their aggregation. The flow of blood cells may be impeded by the vascular endothelium, particularly in the microcirculation where - because capillary diameter may be smaller than that of both leucocytes and RBCs - blood cells have to deform to pass through. Leucocytes are much larger than RBCs and move through capillaries with a slower velocity. Activated leucocytes can also adhere to endothelial cells and plug capillaries, giving rise to local hypoxia and fatigue. Non-activated leucocytes secrete nitric oxide, which facilitates their passage through capillaries by dilating the vascular endothelium (Vallance et al., 1989).

The deformability of RBCs is controlled by the internal fluidity, surface area to volume ratio and the physical properties of the plasma membranes of individual cells (Klug et al., 1974). Declining deformability of RBCs leads to their sequestration, particularly in the spleen where the RBC
must negotiate extraordinarily narrow passages (Sutera et al., 1985). RBC fluidity is regulated osmotically by Na\(^+\) and K\(^+\). The extracellular release of these cations causes dehydration which increases cytoplasmic viscosity, and thereby, impairs cell deformability (Wiley, 1990). Fluidity is also influenced by the phospholipid composition and physical properties of the membrane (Kamada et al., 1993). Optimal fluidity and integrity of the cytoskeleton and membrane are critical because the RBC passes through fine capillaries by extending and folding (Evans et al., 1984). Changes in the surface area of the normal RBC may produce, without affecting volume, either stomatocytes (with decreased surface area) or echinocytes (with increased surface area) with impaired deformability (Chein, 1987). Numerous studies have shown that older (high-density) RBCs are less deformable under shear stress than younger (low-density) RBCs (Shiga et al., 1985; Sutera et al., 1985; Morse and Warth, 1990). Membrane viscosity increases independently of increased internal viscosity during ageing, making shape recovery slower in older RBCs; this may be caused by the binding of Hb to membrane components (Nash and Meiselmann, 1983). As osmotic properties of the RBC have been discussed previously (see Section 5), I will only discuss membrane and cytoskeletal properties here.

Various factors, including oxidative and osmotic damage, surface alterations and ATP depletion may be responsible for the impaired deformability that accompanies RBC senescence (Clark, 1988). When membrane/cytoskeletal proteins are crosslinked by sulphhydryl agents the cells become non-deformable (Chassis and Schrier, 1989). Protein crosslinking associated with the oxidation of Hb also impairs deformability (Chien, 1987). Lipid peroxidation decreases the deformability of both normal and sickle RBCs (Jain et al., 1990) but \(\alpha\)-tocopherol supplementation reduces the impact of oxidative stress on RBC deformability (Kon et al., 1983). Loss of deformability as the cell ages may be due to reduced surface area of the plasma membrane caused by the selective removal of damaged components as the cell passes through the spleen; this process also decreases cell volume (Waugh et al., 1992).

6.1 Deformability of red blood cells and exercise

There is a considerable body of evidence which suggests that regular exercise confers a “rheological advantage” on blood flow in trained individuals. Plasma viscosity is lower in endurance athletes (Charm et al., 1979), and their RBCs are more deformable than those isolated from their
sedentary counterparts (Ernst et al., 1985; Silva, 1988). Increased membrane fluidity improves O₂ diffusion. It has been suggested that these changes may benefit the athlete by leading to better perfusion and O₂ supply to working muscles. One hour of exercise daily may be sufficient to confer this rheological advantage and, consequently, to reduce the risk of cardiovascular disease (Ernst et al., 1985). RBC fluidity is significantly higher in RBCs isolated from endurance athletes, which correlates with higher concentrations of polyunsaturated fatty acids and, perhaps, a greater proportion of younger cells (Kamada et al., 1993).

In contrast to the benefits of regular endurance training, single episodes of intensive exercise may cause transient detrimental changes in blood rheology. Short-term maximal exercise causes significant haemoconcentration, which may be one of the primary mechanisms responsible for its detrimental effect (Ernst et al., 1985). Haemoconcentration during exercise may increase blood viscosity to the point where, at haematocrits above 60%, the ease or difficulty with which RBCs deform may limit blood flow through the microcirculation (Weed, 1970). Marathon running, for example, causes a transient transformation of a proportion of normal discocytes into stomatocytes that may manifest as impaired filterability (Reinhart and Chien, 1985). In contrast, after a 100 km race, a significant increase in RBC filterability was found in RBCs isolated from runners; this suggests that a significant proportion of the oldest cells may have haemolysed during the longer race (Reinhart et al., 1983). Animal studies suggest sequested senescent RBCs such as echinocytes may be released into the general circulation during high-intensity workloads (Boucher et al., 1985), although this does not appear to be the case in humans (Costill and Fink, 1974).

Sweating during intense exercise may be an additional factor which contributes to impaired blood flow. Blood viscosity increases significantly during one hour of cycling because of increases in haematocrit and plasma viscosity that occur in the absence of impaired RBC deformability and aggregation; drinking during the ride attenuates the increase in blood viscosity to some degree (Vandewalle et al., 1988). In contrast, one hour of cycling at an altitude of 3000 meters, but not at sea level, has been reported to decrease RBC deformability; this may have been due to a combination of aggregation of RBCs and lipid damage because six weeks of dietary fish oil supplementation prevented these detrimental changes (Guezennec et al., 1989; Ernst et al., 1990).
Dehydration may, by impairing RBC deformability, be a substantial factor contributing to the fatigue and exhaustion which occurs in response to vigorous exercise. Recently, atrial natriuretic peptide (ANP), which regulates fluid homeostasis (Freund et al., 1991), was reported to increase the filterability of RBCs by elevating their membrane mobility (Zamir et al., 1992). Because the plasma concentration of ANP increases significantly during moderate exercise (Follenius et al., 1989), this hormone may contribute to the regulation of O₂ delivery to working muscle by maintaining or increasing RBC deformability. The role of hormones on RBC haemodynamics, metabolism and deformability has received little experimental attention but may be a fruitful area for future studies.
7. Summary and conclusions

The mechanisms which may cause an increase in RBC turnover in athletes have been examined. RBCs have a limited life-span of about 120 days in humans but the factors that normally control RBC turnover are poorly understood. Furthermore, senescent RBCs cannot be separated unequivocally from their younger counterparts. The evidence reviewed suggests that some athletes undertaking intensive training programs may be vulnerable to "sports anaemia". While this condition is usually diagnosed by plasma indices (e.g. ferritin, erythropoietin), neither iron deficiency nor low plasma erythropoietin concentration are necessarily indicative of RBC deficiency.

Attention is now turning to cellular indices that may be more indicative of exercise-induced damage, particularly that induced by oxidative and osmotic stress. RBCs are vulnerable to oxidative damage because of their continuous exposure to $O_2$, and their high concentrations of unsaturated fatty acids and haem iron which is a potent free radical catalyst. There is considerable evidence that exercise can deplete antioxidants in muscle, liver and RBCs, whilst supplementation with dietary antioxidants can prevent this to some extent. Furthermore, oxidative damage to RBCs may alter their osmotic status by adversely affecting cation homeostasis and, thereby facilitating cellular dehydration. Exercise-induced increases in mean cell density have been reported. The short-term physiological effect of exercise-induced oxidative and osmotic damage is that deformability of RBCs is likely to be impaired, thereby impeding their passage through the microcirculation. This may lead to hypoxia in working muscle during single episodes of exercise and, perhaps, in the long-term, to an increased rate of destruction of RBCs.
8. Scope of this thesis

The aims of this project were to assess:

(i) whether single episodes of submaximal exercise increased the susceptibility of isolated RBCs to oxidative and osmotic stress \textit{in vitro}, and whether these responses were influenced by regular endurance training and/or antioxidant supplementation; and

(ii) whether lactic acid release into the circulation during maximal exercise affected RBC susceptibility to haemolysis by osmotic stress;

During the course of these studies, a luminol-amplified chemiluminescence assay for monitoring the generation of free radicals continuously in oxidatively-stressed RBCs was developed.

The specific hypotheses tested in this part of the thesis are presented in the separate introductions to each experimental paper.
9. References


CHAPTER 6

EXPERIMENTAL

(Exercise-induced damage to erythrocytes and its prevention)
CHAPTER 6.1

Free radical generation in hydroperoxide-treated erythrocytes monitored continuously by luminol-amplified chemiluminescence
FREE RADICAL GENERATION IN HYDROPEROXIDE-TREATED ERYTHROCYTES 
MONITORED CONTINUOUSLY BY LUMINOL-AMPLIFIED 
CHEMILUMINESCENCE

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SUMMARY

Organic hydroperoxides induce oxidative damage to mammalian cells. We describe how 
luminol-amplified chemiluminescence can be used to monitor free radical generation (following 
treatment of erythrocytes in vitro with organic hydroperoxides) throughout the entire time-course 
of oxidative stress. Enrichment of erythrocyte α-tocopherol levels increased the induction time by 
25% and led peak chemiluminescence fall of 30%. Furthermore, ascorbate loading reduced the 
signal four-fold during the induction period. The catalytic role of haemoglobin was shown by the 
abolition of chemiluminescence by azide and a low (but detectable) signal in haemoglobin- 
depleted erythrocyte ghosts. Luminol-amplified chemiluminescence enables the kinetics of free 
radical generation to be monitored continuously. Furthermore, it may enable features of the 
mechanism of interaction between cellular antioxidants and antioxidant enzymes to be elucidated.

INTRODUCTION

The oxidative damage caused by exposure of mammalian cells to organic hydroperoxides (ROOH) in vitro has been used widely as models to study the mechanisms of oxidative stress and the prevention of oxidative damage by dietary antioxidants and detoxification enzymes (1-6). These cellular defence mechanisms are thought to interact because, for example, ascorbate and reduced glutathione (GSH) in the cytosol together maintain α-tocopherol (the only significant lipid antioxidant in erythrocytes) in the reduced state (7). Exposure of erythrocytes or microsomes to ROOH initiates a cascade of reactions which cause irreversible lipid and protein damage once antioxidant protection is overwhelmed (1-4). Homolytic scission of the ROOH (by haemoproteins) generates alkoxy and peroxy radicals that are detected by ESR (6). These radicals initiate lipid peroxidation by abstracting a hydrogen atom from the methylene carbon in

*Abbreviations: butylated hydroxyanisole (BHA), cumene hydroperoxide (Cum-OOH), reduced glutathione (GSH), Hanks' balanced salts solution (HBSS), luminol-amplified chemiluminescence (LCL), met-haemoglobin (met-Hb), organic hydroperoxide (ROOH), phosphate-buffered saline (PBS), phenazine methosulphate (PMS), t-butyl hydroperoxide (t-BuOOH).
unsaturated fatty acids (8). This activates a chain-reaction that cycles until self-termination or interruption by chain-breaking antioxidants (e.g. α-tocopherol). Lipid hydroperoxides, by-products generated by this process can be detoxified by glutathione peroxidase, thereby reducing their availability in the peroxidation cascade (8). In contrast to lipid peroxidation, the mechanism(s) by which oxidative damage to protein substrates is propagated are not well understood.

The majority of techniques used to assess the effects of oxidative stress measure loss of substrate and/or consumption of antioxidant activity on the one hand, or the accumulation of secondary products like malondialdehyde (MDA) or oxidation of thiol groups on the other (8). Alternatively, continuous assays like the monitoring of oxygen uptake (1,2), loss of parinaric acid fluorescence (7), or low-level (non-amplified) chemiluminescence (2,9) have been employed. None of these techniques, however, detect events during the induction period during which no measurable responses are detectable. Because amplified chemiluminescence assays have been used to measure free radical generation in a number of experimental systems (10), we determined whether luminol-amplified chemiluminescence (LCL) could be used to measure free radicals generated during treatment of erythrocytes with ROOH. The major aim of this study was to determine: (i) if free radical generation could be monitored continuously throughout oxidative stress using LCL; and (ii) if antioxidants and free radical scavengers would inhibit LCL. We also determined the separate contributions of Hb (free radical catalyst) and the erythrocyte membrane (target) to LCL triggered by ROOH treatment.

MATERIALS AND METHODS

Materials. Cumene hydroperoxide (95%, w/w aqueous solution), t-butyl hydroperoxide (70%, w/w aqueous solution), butylated hydroxyanisole (BHA), human haemoglobin-A0, luminol, phenazine methosulphate, Tween-80 (polyoxyethylene-sorbitan monooleate) and vitamin-E (dl-α-tocopherol) were all obtained from Sigma (St. Louis, MO, USA). L-Ascorbic acid, sodium azide and sodium dithionite were purchased from BHA chemicals (Poole, England). Triethylamine was purchased from Ajax chemicals (Sydney, Australia). The 0.22 μm filters were purchased from Millipore (Bedford, MA, USA). The protein assay kit was obtained from Bio-Rad (San Lorenzo, CA, USA). 2,2'-Azobis (2-aminopropane) hydrochloride (AAPH) was a gift from Dr. R. Stocker of the Heart Research Institute, Sydney, Australia. Hank's balanced salt solution, pH 7.3 (HBSS) and phosphate-buffered saline, pH 7.3 (PBS) were prepared by conventional methods. BHA was dissolved in 50% ethanol at a stock concentration of 100 mM and diluted further with HBSS. The α-tocopherol emulsion was prepared by adding one part of α-tocopherol (w/w) to two parts of Tween-80 and warming the emulsion at 40°C until clear (~30 min) followed by dilution with HBSS under mild sonification to achieve the desired stock concentrations (11). Ascorbate was prepared as a 10 mM stock solution by dissolving L-ascorbic acid in HBSS. The pH of 7.3 was restored by adding NaOH. AAPH was dissolved in HBSS to a stock concentration of 750 mM. A stock solution of luminol (2mg/ml) was prepared in HBSS containing 8 μM triethylamine and sonicated for one minute.

Preparative techniques. Blood was obtained by venipuncture from healthy human volunteers after informed consent. Erythrocytes were isolated from whole blood (drawn into K2-EDTA tubes) by centrifugation (1000 x g for 15 min at 4°C). The "buffy coat" was discarded and plasma retained for incorporation of α-tocopherol into erythrocytes (see below). The erythrocytes were washed three times in PBS before resuspension in HBSS containing 5 mM D-
glucose. Erythrocytes were counted with a haemocytometer. The haemoglobin (Hb) solution was prepared by dissolving Hb-A_0 in HBSS. Erythrocyte membranes (i.e. ghosts) were prepared by hypotonic lysis using the technique described by Kuroos and colleagues (12). Between each wash, the ghosts were pelleted by centrifugation at 50,000 x g and 4°C. White ghosts (i.e. those essentially free of Hb contamination) obtained after three to four washes were finally resuspended in HBSS. The protein content of ghosts was determined with a Bio-Rad protein assay kit using bovine serum albumin as the standard.

Ascorbate and α-tocopherol were incorporated into erythrocytes by the following procedures. Equal volumes (1.0 mL) of packed erythrocytes, filtered (0.22 µm filter) autologous plasma and one mL of α-tocopherol emulsion at the required concentration were mixed and incubated for 30 min at 37°C (11) by an adaptation of a similar method (13). Ascorbate was incorporated into erythrocytes (separately or together with α-tocopherol) (14). Control samples included erythrocytes incubated in the absence of ascorbate and α-tocopherol with buffer or Tween-80 only. Non-incorporated vitamins were removed by several washes. In control experiments, HBSS and Tween-80 replaced α-tocopherol. In some experiments, erythrocytes were pretreated with an oxidant (phenazine methosulphate (PMS)) for 30 min at 37°C to diminish endogenous antioxidant protection (15). PMS was removed by washing.

**Oxygen Uptake.** Oxygen uptake was monitored in a Clark-type electrode (Rank, England) (1,2) calibrated with sodium dithionite. Erythrocyte (or erythrocyte ghost) suspensions (final volume, 3.0 mL) were stirred continuously at 37°C in a water-jacketed incubation chamber. Baseline oxygen uptake was recorded for five min prior to the addition of ROOH. Total oxygen uptake was then recorded until completion of the reaction (i.e. until baseline rate was re-established).

**Chemiluminescence.** LCL of ROOH-treated erythrocytes (or ghosts) was monitored continuously at 37°C in a six-channel Lumicon-luminometer (Hamilton, Bonaduz, Switzerland) in duplicate. Erythrocytes (or ghosts) were added to 12 x 75 mm glass vials containing HBSS and luminol (225 µM). The reaction mixture was preincubated for five min before addition of ROOH (final volume, 1.0 mL). Light output in each individual chamber (expressed in cpm) was recorded for six sec during each cycle (Hamilton, Bonaduz, Switz).

**Other assays.** Established techniques were used to measure the concentration of α-tocopherol in erythrocyte ghosts by HPLC (16). Erythrocyte susceptibility to lysis by H_2O_2 was assessed by the following method. Erythrocytes (10^8 in 1.0 mL) were incubated for two hr at 37°C in a tube containing HBSS with azide (1 mM) and 1.4 mM H_2O_2. The cells were pelleted by a five sec centrifugation in an microcentrifuge (Eppendorf, Hamburg, Germany) and Hb released into the supernatant was measured spectrophotometrically at 410 nm.

**RESULTS**

**Kinetics of oxygen uptake and chemiluminescence.** The typical kinetic patterns of O_2 uptake and LCL generated following treatment of erythrocytes with organic hydroperoxides (ROOH) are shown in Figure 1. When ROOH was added to the erythrocyte suspension, a distinct induction (lag) phase was observed before the onset of O_2 uptake (Fig 1a) and the consequent oxidation of oxy-Hb to met-Hb. While an induction phase was also observed in the LCL assay, free radical generation was clearly detectable as LCL from the moment ROOH was added to the cells (Fig 1b). In both assays, the length of the induction phase decreased and the maximum rates increased in response to rising ROOH concentration (data not shown). No induction period was observed when the cells were subjected to a second treatment with hydroperoxide, and the initial rates of O_2 uptake and LCL were maximal under these conditions (data not shown). This
suggests that exhaustion of the initiating ROOH was responsible for the cessation of O2 uptake and LCL at the end of the first addition and that antioxidant protection was depleted completely by this procedure. Cum-OOH, which is strongly hydrophobic, was a more potent oxidant than the amphipathic r-butyl hydroperoxide (r-BuOOH) added at equimolar concentration. Because the O2 uptake assay required 10-fold higher cell numbers and ROOH concentrations than the LCL assay to be detectable, the kinetics of the two responses were quantitatively different. Despite this, the induction phase was always inversely proportional (and the maximum rates directly proportional) to ROOH concentration.

Optimization of experimental procedures. Initial experiments showed that the presence of luminol amplified the chemiluminescence of ROOH-treated erythrocytes 100-fold and thereby eliminated background “noise”. Chemiluminescence was not accurately quantifiable without luminol. Furthermore, lucigenin [a chemiluminogetic probe with a reported specificity for superoxide (10)] did not amplify the signal significantly. No LCL was detectable above background levels when erythrocytes or Hb were omitted from the incubation mixture. We determined the optimum number of erythrocytes for the LCL assay to minimise the quenching effect of Hb on light output which is observed when related assays (e.g. LCL assays of phagocyte activity) are performed with whole blood (10). This was established as 1.5 x 10^7 RBCs in 1.0 ml when 75 µM of Cum-OOH was used. RBC concentrations above this optimum value caused a progressive decline in LCL. At optimum cell number and Cum-OOH concentration, LCL increased linearly as the luminol concentration was increased within the range 0.01 to 1 mM (r > 0.9).

Fig. 1. Kinetics of (a) oxygen uptake and (b) luminol-amplified chemiluminescence generated following treatment of erythrocytes with organic hydroperoxides.
(a) Erythrocytes (3 x 10^8 in 3.0 ml) were treated with 1.2 mM organic hydroperoxide.
(b) Erythrocytes (1.5 x 10^7 in 1.0 ml) were treated with 75 µM organic hydroperoxide.
The results represent the mean ± SE of triplicate determinations. The kinetic patterns were typical for all experiments reported in this paper. Data are plotted at one minute intervals for convenience.
The specificity of the radical species responsible for the luminol signal was not elucidated in this study. Preliminary studies indicated that peroxy radicals, which are generated at a constant rate by decomposition of the thermolabile compound 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) (17), produced a weak LCL signal ($10^4$ cpm with 3 to 75 mM AAPH) compared to that generated by hydroperoxide-treated erythrocytes ($10^5$-$10^6$ cpm). Spontaneous decomposition of AAPH also induced oxygen uptake at a constant rate with no lag phase (data not shown).

**Effect of antioxidant and oxidant treatment.** In these studies, we tested whether externally-added antioxidants were able to quench LCL generated by Cum-OOH-treated erythrocytes. Cum-OOH was used in these (and all subsequent) experiments because of its strong lipophilic properties and greater oxidative potency. LCL (measured at peak velocity) was inhibited by the exogenous addition of the free radical scavengers BHA (0-30 mM) or α-tocopherol (0-500 ppm) in a concentration-dependent manner. LCL was virtually abolished at high concentrations of these antioxidants.

In the next group of experiments, α-tocopherol and ascorbate were incorporated into erythrocytes either separately or together. The incorporation procedure increased the concentration of plasma membrane-localized vitamin-E three-fold as determined by HPLC (16) (from 3 mg/ml packed cells to 10 mg/ml packed cells, n = 4) and substantially reduced the susceptibility of the cells to peroxidative lysis by 1.4 mM H$_2$O$_2$ (70% ± 15 to 1.2% ± 0.73, n = 4). The procedure utilised to enrich cellular ascorbate levels has been reported to increase cytosolic ascorbate two- to three-fold in erythrocytes (14). Figure 2a shows that incorporation of α-tocopherol into RBCs reduced peak-LCL by 30-40% and lengthened the induction period by 30% (n = 10). LCL intensity was reduced further (two- to three-fold) during the induction period (static LCL for 10 minutes) as a result of ascorbate incorporation, but peak LCL and the length of the induction period were similar to cells supplemented with α-tocopherol only. No significant changes in the kinetics of O$_2$ uptake could be detected as a consequence of antioxidant incorporation (Fig 2b).

In contrast to antioxidant enrichment, pretreatment of the cells with an oxidant (PMS), which generates O$_2^-$ intracellularly (15), substantially decreased the length of the induction period (~ 40%) and increased the peak intensity (~ 20%) of the LCL observed after a second challenge with 75 µM Cum-OOH; a similar kinetic pattern was detected in the O$_2$ uptake assay (data not shown). These experiments demonstrate unequivocally that the kinetics and intensity of LCL are dependent upon both the type of initiator and the presence of antioxidants.

**Mechanism(s) responsible for the LCL response of erythrocytes to ROOHs.**

**Role of haemoglobin.** Cum-OOH treatment of pure Hb (at the same concentration as that contained in cellular assays) generated both LCL and O$_2$ uptake (Fig 3a,b). The peak LCL response of pure Hb (or Hb released in the presence of other components from lysed cells) was
Fig. 2. Effect of antioxidants incorporated into erythrocytes on the kinetics of (a) LCL and (b) $O_2$ uptake. (a) LCL was initiated by adding 75 $\mu$M Cum-OOH to the erythrocyte suspension ($1.5 \times 10^7$ in 1.0 ml) while (b) $O_2$ uptake was activated by adding 1.2 mM Cum-OOH to a separate cell suspension ($3 \times 10^8$ in 3.0 ml). Antioxidants were incorporated into the cells using procedures described in the Materials and Methods section. The results represent typical duplicate determinations (mean $\pm$ SE) from ten separate experiments.

five-fold greater than that obtained with intact erythrocytes (Fig 3a). The majority of the initiating radicals appear to be detected under these cell-free conditions. The $O_2$ uptake responses of pure Hb or lysed erythrocytes were only about half of those obtained with intact cells at an equivalent Hb concentration (Fig 3b). These data show that, while the induction phase was negligible with Hb only, a distinct induction phase was observed in both assays when pure Hb was replaced with that released from lysed cells, suggesting the presence of "considerable" antioxidant protection. Furthermore, the kinetics of LCL and $O_2$ uptake were much slower with intact cells. The obligatory role of haem iron in catalysing ROOH breakdown is inferred from the inhibitory effect of prior azide treatment, which blocks ROOH access to the haem moiety (18). Both LCL
In the case of free Hb, the concentration used was equal to that in the cellular assays (i.e. 30 pg/cell). The azide concentration was 10 µM (i.e. Hb + azide). All other experimental conditions are described in the Materials and Methods section.

and O₂ uptake were reduced by 80% in the presence of azide (Fig 3a,b). A similar inhibitory response was observed with azide pretreatment of erythrocytes. As with intact cells, LCL increased in proportion to Hb concentration, but high levels were strongly inhibitory (data not shown).

Role of the membrane. LCL can also be quantified accurately from erythrocyte membranes (ghosts) treated with Cum-OOH (Fig 4). In unwashed ghosts, the responses were kinetically similar to those found with intact cells, even though most of the cytosolic water-soluble antioxidants will be lost during preparation. Successive washing of the ghosts further diminished
Fig. 4. Effect of successive washing of erythrocyte membranes (ghosts) on LCL intensity. Erythrocyte ghosts, prepared by the technique of Kuroos and colleagues (12), were washed successively to remove Hb. After three washes white ghosts (with no visible Hb) were obtained. The ghosts (1 mg protein) were treated with 75 µM Cum-OOH. All other experimental conditions are described in the Materials and Methods section. The results of a typical experiment, performed in triplicate, are shown. The inset shows the results obtained (using an expanded vertical scale) with white ghosts (ghosts-3) which are not clearly visible from the main figure.

LCL, with removal of virtually all Hb (to yield white ghosts) reducing light output by three orders of magnitude to barely detectable levels. This may be due to the absence of accessible catalytic haem iron. Although the LCL signal was three orders of magnitude lower in white ghosts, it was still accurately quantifiable (see inset, Fig 4). With white ghosts, LCL increased linearly with membrane concentration up to 0.4 mg protein. No O₂ uptake could be detected under any conditions in the white ghosts, in spite of the generation of LCL.

The effect of α-tocopherol incorporation on ghost LCL activated by Cum-OOH was also assessed. When very low concentrations of Hb (0.2 µg/ml) were added back to white ghosts, the LCL of the reconstituted system was about two-fold greater than the control (Hb only) value. However the LCL generated from ghosts containing incorporated α-tocopherol was three- to four-fold lower than the values obtained with untreated control white ghosts [from 2.2 x 10⁴ cpm ± 0.87 (controls) to 0.52 x 10⁴ cpm ± 0.019 (α-tocopherol)] (n = 4). These experiments provide further evidence that LCL is due to organic free radicals generated by haem iron catalysed breakdown of ROOH and/or secondary free radicals generated during subsequent protein and lipid oxidation.
DISCUSSION

LCL is a sensitive and reproducible technique for the continuous monitoring of free radicals generated during the treatment of erythrocytes (or membranes) with ROOHs. The involvement of free radicals was indicated by the ability of antioxidants to quench (and pre-oxidative stress to enhance) LCL intensity. The responses to ROOH treatment could be detected by LCL over the entire time-course of the reaction, including the induction period which preceded O₂ uptake. Because LCL is dependent on the existence of a complex balance between initiators and terminators of free radical formation, the mechanism(s) of interaction between antioxidants in the target (erythrocytes) are accessible to elucidation. These interactions could be further investigated using specific inhibitors for other antioxidant systems and/or agents to deplete cofactors (e.g. GSH and ascorbate). In addition, because LCL is dependent on the presence of haem, the contamination of apparently Hb-free washed (white) ghosts with catalytically-accessible haem iron can be detected rapidly. This has important practical implications because sickled erythrocytes have excessive amounts of membrane-associated iron which may be responsible for the pathology associated with this condition (12).

Low-level (unamplified) chemiluminescence assays have been used in a number of studies to measure free radical generation by ROOH-treated erythrocytes (2,9,19). The kinetics of t-BuOOH decomposition to t-butanol, met-Hb formation and the accumulation of MDA correlates with increased chemiluminescence (9). In Cum-OOH-treated erythrocytes (measured at 30 min intervals only), the chemiluminescence kinetics correlated with increased MDA formation and loss of protein thiols (19). In both of these studies, however, chemiluminescence was not accurately quantifiable with ROOH concentrations lower than 0.5 mM. Furthermore, the kinetics of light output generated by treatment with either Cum-OOH or t-BuOOH in this concentration range were similar (2,9,19). Therefore, amplification of the chemiluminescent signal with luminol is necessary to characterise the individual kinetic patterns of free radical generation induced by Cum-OOH and t-BuOOH and it enables lower concentrations of ROOHs to be used.

In contrast to LCL, established techniques like the measurement of O₂ uptake (1,2,4) and the loss of parinaric acid fluorescence (7) give little information about the events that precede the involvement of oxygen and/or the damage to lipids and proteins. The induction time that precedes O₂ uptake and the loss of parinaric acid fluorescence (where negligible changes occur) is thought to represent the time taken to overwhelm endogenous antioxidant protection (1,2,7). The LCL generated during the induction period most likely represents a balance between the rate of free radical generation and the extent of antioxidant quenching. This indicates that, although free radicals are being generated, no net peroxidation of membrane lipids occurs. Antioxidant enrichment also extends the lag phase of parinaric acid oxidation in erythrocyte ghosts, and the combination of α-tocopherol and ascorbate has a much greater protective effect than the sum of their individual contributions (7). In contrast, our results suggest that the presence of ascorbate does not prolong the protective effect of α-tocopherol. The substantial reduction of LCL we
found during the induction phase suggests that ascorbate scavenges luminol-reactive free radicals directly. Whilst we found that \( \alpha \)-tocopherol incorporation \textit{in vitro} did not significantly extend the induction phase prior to \( O_2 \) uptake activated by Cum-OOH, supplementation of \( \alpha \)-tocopherol \textit{in vivo} extended the induction phase of both low-level chemiluminescence and \( O_2 \) uptake in rat erythrocytes treated with 0.75 mM \( t \)-BuOOH (2). The differences may be due to more effective \( \alpha \)-tocopherol uptake under physiological conditions.

The different kinetic patterns of LCL generation and \( O_2 \) uptake by Cum-OOH- and \( t \)-BuOOH-treated erythrocytes may be due to differences in ROOH solubility properties, ROOH binding to Hb and/or the reactivity of secondary free radicals generated (20). With \( t \)-BuOOH treatment, the first peak of LCL may be due to protein thiol oxidation (18), corresponding to exhaustion of GSH or the overwhelming of glutathione peroxidase activity (18), while the second peak (and the single Cum-OOH peak) may signify lipid peroxidation. The long induction phase detected in the \( O_2 \) uptake assay with \( t \)-BuOOH suggests the the first peak of LCL may not involve peroxidation (i.e. it may be \( O_2 \)-independent). In contrast, because the majority of the Cum-OOH may be retained in membrane lipids, the slow initial rate of LCL may be due to the absence of catalytically-accessible haem iron within the membrane (20). Thiol oxidation of cytosolic Hb leads to Hb attachment to the membrane (18,20), thereby enhancing the susceptibility of membrane lipids to peroxidation and perhaps increasing the rate of Cum-OOH-activated LCL substantially once \( \alpha \)-tocopherol is consumed. In fact, Cum-OOH-induced MDA formation is preceded by loss of membrane thiol groups (3). There is a distinct relationship between the amount of ROOH added to microsomes and the subsequent uptake of \( O_2 \) and MDA formation (4). In fact, MDA accumulation accounts for only 10% of the total \( O_2 \) consumption (4). This supports our observations showing that Hb oxidation alone accounts for over half the \( O_2 \) uptake detected in Cum-OOH-treated erythrocytes. Oxygen may also be consumed during the oxidation of cytoskeletal proteins (e.g.band III) and lipids which are not detected by MDA measurements.

The intensity of LCL was much greater with Hb only (compared with intact cells) either because terminating antioxidants were not present or they were not in close contact with Hb at their appropriate intracellular concentrations. Furthermore, the induction period was negligible with free Hb but significant with lysed cells. Because LCL was inhibited substantially by azide, our results suggest that haem iron is an essential component of the free radical generating reactions responsible for producing LCL. This is not surprising since lipid peroxidation occurs at negligible rates in Hb-depleted ghosts (21). The \( O_2 \) uptake associated with Cum-OOH treatment of Hb suggests that peroxy radicals are produced as a result of ROOH scission or that Hb itself is peroxidized. This result was unexpected since \( O_2 \) uptake has been claimed by some workers to be due solely to lipid peroxidation (1,2,4). Treatment of haemoprotein (e.g. cytochrome-c) with \( t \)-BuOOH, however, induces both low-level chemiluminescence and \( O_2 \) uptake (22).

The reactions involved in luminol excitation are likely to be complex. For example, the reaction of two \( t \)-BuOO· radicals yields singlet \( O_2 \) (22) and the oxidation of Hb thiols generates
thiyl radicals (5) which may contribute to light output in low-level chemiluminescence and LCL assays. Our failure to detect chemiluminescence in the absence of luminol was possibly due to the emission of light in the red region of the spectrum which was not detectable with the luminometer used in this study. The ability of haemoproteins to catalyse ROOH breakdown has been utilized to measure lipid hydroperoxides in blood by HPLC using LCL as a detection system (23). Luminol is likely be excited by a variety of free radicals (23) and thus, in our system, it acts as a non-specific indicator of total free radical generation (i.e. initiating and secondary free radicals) in addition to providing an estimate of quenching by endogenous erythrocyte defences.

In conclusion, we have reported that LCL is a sensitive technique for the continuous monitoring of free radicals generated from the breakdown of organic hydroperoxides. The major advantage of LCL is that free radicals can be detected from the moment very low concentrations of hydroperoxides are added to a cell or membrane suspension while, in contrast, current techniques do not detect responses until a considerable induction period has elapsed. Thus, LCL enables the interaction between antioxidants and detoxification enzymes to be studied in whole cell or cell-free systems during oxidative stress.

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CHAPTER 6.2

Oxidative and osmotic changes in red blood cells following submaximal exercise
OXIDATIVE AND OSMOTIC CHANGES IN RED BLOOD CELLS FOLLOWING SUBMAXIMAL EXERCISE

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The effect of submaximal exercise on the susceptibility of red blood cells (RBC) to oxidative and osmotic stress in vitro was investigated in trained and untrained men. RBCs from untrained subjects were more vulnerable to peroxidative lysis by H₂O₂ than cells from trained individuals both before and after exercise (P < 0.01). Training did not influence any other parameter measured. Whilst no significant change in peroxidative lysis occurred immediately after one hour of cycling at 60% VO₂ max, a 20% increase was found six hours later (P < 0.05). RBC osmotic fragility decreased by 15% immediately after exercise and this was sustained for six hours (P < 0.001). A significant increase in mean cell haemoglobin concentration occurred concomitantly (P < 0.05). These changes bear striking similarities to the somewhat larger responses found in RBCs isolated from people with β-thalassaemia and may be linked to incorporation of haem iron into membrane lipids which has the potential to short-circuit membrane antioxidant protection by vitamin-E. In a second study to test this hypothesis, running at 75% VO₂ max for 45 min caused a significant reduction in the time taken for O₂ uptake (peroxidation) to begin (consistent with reduced antioxidant capacity), and an increase in the maximum rate of O₂ uptake (consistent with increased peroxidation) in RBCs challenged with cumene hydroperoxide (75 μM) in vitro (P < 0.001). The percentage of high-density cells in the circulation also increased by 10% immediately after running (P < 0.001) but there was no increase in membrane-incorporated haem iron. In contrast, treatment of RBCs with low concentrations of oxidants (20-50 μM) in vitro caused large increases in cell density and significant membrane incorporation of haem iron. These results provide further evidence that exercise causes significant increases in the susceptibility of RBCs to oxidative and osmotic stress which may increase their turnover rate during athletic training.
INTRODUCTION

Efficient delivery and release of oxygen to exercising muscles by red blood cells (RBC) is an important determinant of peak athletic performance. Many reports suggest that intensive physical training accelerates the rate of RBC turnover and can, in some cases, lead to the so-called condition of "sports anaemia" which may result in a decreased capacity for physical activity (reviewed by Szygula et al., 1990). Evidence that exercise may cause significant RBC damage and haemolysis includes data showing significant exercise-induced reductions in haemoglobin concentration and circulating RBC counts (Guglielmini et al., 1989), as well as reductions in the plasma concentrations of iron-binding proteins such as ferritin and haptoglobin (Selby and Eichner, 1986). At the cellular level, regular exercise elevates reticulocyte counts (Schmidt et al., 1989) and the activities of some RBC antioxidant enzymes (Ohno et al., 1988; Robertson et al., 1991). Training also reduces RBC density, which is indicative of an increase in the percentage of younger cells (Mairbaurl et al., 1983). While the mechanical trauma associated with footstrike has been suggested as a cause of intravascular haemolysis during running, low impact activities such as cycling and swimming also induce significant levels of RBC destruction (Schmidt et al., 1988; Selby and Eichner, 1986).

Oxidative and osmotic stress to RBCs during exercise may enhance their susceptibility to irreversible damage and, thus, to premature ageing and destruction. Because of their continual exposure to $O_2$, and their high content of haem iron and polyunsaturated fatty acids, RBCs are particularly vulnerable to oxidative stress (Hebbel and Eaton, 1989). Association of haem iron, which is normally confined to the cytosol, with membrane lipids may increase the vulnerability of RBCs to oxidative stress substantially because of its potential to short-circuit membrane vitamin-E protection. Auto-oxidation of haemoglobin to met-haemoglobin (which occurs normally at the rate of 3%/day) is accompanied by free radical generation. This may increase in proportion to the increased rate of whole body oxygen uptake (10-20-fold) that occurs during exercise (Duthie et al., 1990). Oxygen radicals may challenge and overwhelm antioxidant defences and attack polyunsaturated lipids and proteins in RBCs. Single episodes of submaximal exercise have been reported to have differential effects on the redox status of blood glutathione (Gohil et al., 1988; Ji et al., 1993). In addition to oxidative damage, changes in RBC hydration may occur because submaximal exercise decreases plasma volume through perspiration loss (Wilkerson et al., 1977). Intensive exercise also triggers leakage of intraerythrocyte K$^+$ (Hespel et al., 1986) and, thus water,
which increases cell density (Robertson et al., 1988). These changes may impair the ability of RBCs to enter and move through the microcirculation (Wiley, 1990).

Because several studies have reported that both single episodes of moderate exercise and regular training influence the antioxidant status of RBCs (Gohil et al., 1988; Robertson et al., 1991), we have investigated whether a single episode of moderate aerobic exercise and/or endurance training alters the susceptibility of isolated RBCs to oxidative and osmotic stress in vitro. Furthermore, we have determined whether RBC density, which is considered by many authors to be proportional to RBC “age” (Clark, 1988), and mean cell volume, are affected by exercise, either acutely or chronically.
MATERIALS AND METHODS

Materials
Cumene hydroperoxide (95%, w/w aqueous solution), tert-butyl hydroperoxide (70%, w/w aqueous solution), human haemoglobin-A, luminol, meglumine diatrizoate, sodium diatrizoate and phenazine methosulphate were all obtained from Sigma (St. Louis, MO, USA). Percoll® was purchased from Pharmacia (Uppsala, Sweden). The protein assay kit was obtained from Bio-Rad (San Lorenzo, CA, USA).

Human Subjects and exercise protocols
Group 1. Eleven trained [maximal aerobic capacity (VO₂ max) 71.4 ± 6.0 ml/kg<sup>*</sup>; mean age 22 yrs] and nine untrained men (VO₂ max 48.1 ± 9.0 ml/kg<sup>*</sup>; mean age 22 yrs) were recruited for the initial phase of the work (*data are means ± SEM). The trained subjects were road cyclists competing at national level who trained about 25 hours/week. In contrast, the untrained subjects devoted less than 3 hours/week to physical activity. Each subject exercised continuously for one hour on a cycle ergometer at 60% of their predetermined (VO₂ max) as described previously (Smith et al., 1990). The blood withdrawal and RBC isolation procedures have been described previously (Smith et al., 1992).

Group 2. Twenty highly-trained men (VO₂ max 71.5 ± 4.5 ml/kg<sup>*</sup>; mean age 24 yrs) and six untrained men (VO₂ max 52.0 ± 6.0 ml/kg<sup>*</sup>; mean age 34 yrs) were recruited for the second study (*data are means ± SEM). The trained subjects were triathletes, cross country skiers and runners competing at national level and training for 10 to 20 hours per week. The untrained subjects devoted less than 4 hours/week to physical activity. Each individual ran on a treadmill (level gradient) for 40 minutes at a workload equivalent to approximately 75% of their VO₂ max. At least a week before the test, the VO₂ max of each subject was determined on a treadmill. A continuous incremental protocol was used in which the gradient of the treadmill was increased each min until exhaustion.

Haematological Parameters
Red and white blood cell counts, haematocrit, mean cell volume (MCV), haemoglobin concentration, mean cell haemoglobin concentration (MCHC), and mean cell haemoglobin (MCH) were determined on single blood samples (100 μl) aspirated into an automated haematology analyser (Coulter Electronics, Hialeah, FL, USA). Met-haemoglobin was measured spectrophotometrically in RBC supernatants by the technique of Winterbourn (1983).
Osmotic fragility
Heparinised whole blood (100 µl) was added to hypotonic phosphate-buffered saline (pH 7.4) in the concentration range of 0.2 to 0.5 % (w/v) of saline (Simmons, 1972). The tubes were mixed by inversion, allowed to stand for one hour at room temperature, mixed again and centrifuged for five minutes at 1000 x g to pellet the ghosts. The optical density of each sample was measured in the supernatants by spectrophotometry at 540 nm. The percentage of haemolysis was calculated with reference to the haemolysis induced by incubating cells in distilled water under the same conditions.

Density Fractionation and Scanning
The RBC population was fractionated on a self-forming Percoll®/diatrizoate density gradient using the original technique described by Vettore and colleagues (1980) with the modifications introduced by Snyder and colleagues (1983). Specifically, RBCs were washed in PBS three times before the addition of packed cells (200 µl) to the Percoll®/diatrizoate mixture (10 ml) in a Corex tube. The suspension was mixed by inversion and centrifuged for 20 minutes at 35,000 x g at 4°C using an angle-rotor (SS-34) in a RC-2B centrifuge (Sorvall, USA).

After centrifugation, each tube was photographed, using an evenly illuminated light box as background, with 25 ASA film (Kodak Technical Pan). The film was exposed for six seconds at f16, developed in Kodak Technidol, and the continuous-tone negatives printed on multigrade paper (Ilford, Multigrade 3) using a zero-grade filter. Optimum black-and-white photography conditions were chosen so that the maximum density of the exposed image did not exceed an optical density of 4.0 which represented the upper linear detection limit of the laser densitometer. The density of each band was quantitated by scanning laser densitometry (LKB Ultrascan XL™, Bromma, Switz.). Individual density profiles were integrated using the Gelscan XL™ software package (LKB, Bromma, Switz.) in the attached microprocessor.

Oxidative stress assays
Peroxidative lysis, oxygen uptake and luminol-amplified chemiluminescence were monitored as described previously (Smith et al., 1992). RBC membranes (ghosts) were prepared by the method of Kuross and colleagues (1988). In membrane assays, ghost protein (0.3mg) was added to a total volume of 1.0 ml. Spectrophotometric methods were used to measure the concentrations of haem iron (Kuross et al., 1988) and haemichrome (Campwala and Desforges, 1982) in RBC ghosts.
Statistical analysis

The results were analysed by Student's $t$ test for paired (exercise effects) and unpaired (training effects) data.
RESULTS

Oxidative responses

Group 1. The H$_2$O$_2$ peroxidative lysis test represents one of the oldest and simplest methods for assessing the antioxidant capacity of RBCs (Mino et al., 1978). Haem iron catalyses the formation from H$_2$O$_2$ of highly-toxic hydroxyl radicals (Clemens and Waller, 1987) that induce haemolysis once the antioxidant protection mechanisms of the cells have been overwhelmed. As expected, exposure of RBCs to H$_2$O$_2$ induced haemolysis in a concentration-dependent manner. Figure 1a shows a typical individual response where, after one hour of cycling at 60% VO$_2$ max, RBCs showed increased vulnerability to peroxidative lysis at six hours after cessation of exercise. Statistical analysis of the mean pre-exercise values at a single concentration point (1 mM H$_2$O$_2$) showed that the exercise-induced increases in peroxidative lysis only became significant at six hours post exercise in both trained and untrained subjects (P < 0.05; Fig. 1b). RBCs isolated from the trained individuals were much more resistant to haemolysis induced by H$_2$O$_2$ than cells from their untrained counterparts both before and after exercise (P < 0.01; Fig. 1b).

Group 2. As a follow-up to this preliminary study, the vulnerability of RBCs to oxidative stress induced by either H$_2$O$_2$ or organic hydroperoxides (ROOH) in vitro was examined before and immediately after exercise in a separate group of highly-trained (n = 20) and untrained male subjects (n = 6) using a more intense workload (40 minutes of running at 75% VO$_2$ max). As shown for the moderately exercised subjects, there was no significant change in the susceptibility of RBCs to peroxidative lysis by H$_2$O$_2$ immediately after exercise (data not shown).

In addition to the peroxidative lysis test, the vulnerability of RBCs to oxidative stress induced by organic hydroperoxides in vitro was assessed by monitoring oxygen uptake and luminol-amplified chemiluminescence (LCL) in separate assays. The typical kinetic patterns of O$_2$ uptake and LCL following treatment of RBCs with either Cum-OOH (a lipophilic agent) or t-BuOOH (which is amphipathic) are shown in Figures 2a,b & 3a,b. As reported previously (Smith et al., 1992), Cum-OOH was the more potent oxidant in both assays. The lag period which precedes both the uptake of O$_2$, caused by peroxidation (Lissi et al., 1986), and, possibly, the onset of accelerated LCL may be representative of total cellular antioxidant capacity (Smith et al., 1992).

The O$_2$ uptake data for each individual are presented in four separate panels (Fig. 4a-d). These panels show the induction times and maximum
Fig. 1. Effect of moderate cycling on RBC susceptibility to peroxidative lysis.

(a) A typical individual profile of the concentration-dependent haemolysis of isolated RBCs caused by exposure to H$_2$O$_2$ in vitro.

(b) The mean effect of single episodes of exercise and long-term training on RBC peroxidative lysis induced by 1 mM H$_2$O$_2$. The results are presented as means ± SEM.

The experimental procedures and data analysis are described in the Materials and Methods section.
Fig. 2. Typical effect of treadmill running on the kinetics of RBC $O_2$ uptake.

The typical kinetics of $O_2$ uptake following treatment of RBCs with either (a) Cum-OOH or (b) tBuOOH in vitro. The concentration of both ROOHs was 1.2 mM.
Fig. 3. Typical effect of treadmill running on the kinetics of RBC luminol-amplified chemiluminescence.

The typical kinetics of luminol-amplified chemiluminescence generated following treatment of RBCs with either (a) Cum-OOH or (b) tBuOOH in vitro. The concentration of both ROOHs was 75 µM.
Fig. 4. The effects of treadmill running on RBC peroxidation in each individual.

These panels show each individual result, plotted in ascending order of the pre-exercise value, for (a,c) induction time and (b,d) maximum rate of $O_2$ uptake following exposure of isolated RBCs to either (a,b) Cum-OOH or (c,d) tBuOOH in vitro (1.2mM). Each individual is identified by an upper case letter.
Figure 4

(c) Pre-exercise
Exercise

Induction time (min)

Pre-exercise - Exercise

(d) Pre-exercise
Exercise

Oxygen uptake (nmol/min)
rates of O₂ uptake induced by exposure of RBCs to both Cum-OOH and t-BuOOH at identical concentrations (final concentration: 1.2 mM). Since there were no significant differences between the athletes and untrained subjects in any of the parameters measured either before or after exercise, the data were pooled for statistical analysis of the exercise effects. Exercise caused a significant decrease in the induction period in cells challenged with either Cum-OOH (P < 0.001) or t-BuOOH (P < 0.05; Table 1). With Cum-OOH only, the decreased lag phase was accompanied by a significant increase in the maximum rate of O₂ uptake (P < 0.01; Table 1). In some subjects (n = 9), blood samples were also taken 24 hours after exercise. There were no significant differences between the pre-exercise and 24 hour post-exercise induction times and the maximum rates of O₂ uptake in either Cum-OOH- or t-BuOOH-treated cells.

As with O₂ uptake, the training status of the individual subjects did not affect any of the parameters measured by LCL, so the data were again pooled for statistical analysis. In contrast to O₂ uptake, however, no significant changes in the induction times or maximum rate of LCL (i.e. peak-LCL) were detected after exercise in response to either ROOH (final concentration: 75 µM) (Table 2). This was also the case when the integrated areas under the kinetic curves were analysed statistically (Table 2). There were also no significant differences from pre-exercise values in any of the parameters measured in Cum-OOH-treated cells isolated 24 hours after exercise (n = 15) although integrated-LCL tended to decrease [rest: 206 ± 91.2 x 10⁶ cpm; to 144 ± 73.9 x 10⁶ cpm, 24 hours post exercise (P = 0.074)]. However, in cells stressed with t-BuOOH, both peak- and integrated-LCL fell significantly at 24 hours after exercise [peak-LCL: rest 90.8 ± 27.7 x 10⁴ cpm; to 68.2 ± 15.1 x 10⁴ cpm, 24 hours post exercise (P = 0.008)]; [integrated-LCL: rest 186 ± 68.3 x 10⁶ cpm to 139 ± 40.5 x 10⁶ cpm, 24 hours post exercise (P = 0.015)] (data are means ± SEM). The same trends were found when the cells were treated with the organic hydroperoxides at concentrations of 115 µM or 150 µM; the induction times decreased, and peak- and integrated-LCL increased in proportion to the ROOH concentration (data not shown).

**Osmotic responses**

**Group 1 only.** The osmotic fragility test measures the physical state of the membrane which is dependent on the surface area/volume ratio of the cell (Beutler et al., 1982). A typical individual result (Fig 5a) shows that one hour of cycling at 60% VO₂ max reduced RBC susceptibility to osmotic fragility immediately after exercise and to even a greater extent six hours later. When the mean responses of the trained and untrained subjects were analysed...
Table 1. Induction time and maximum rate of $O_2$ uptake immediately before and after 45 minutes of treadmill running

<table>
<thead>
<tr>
<th>State</th>
<th>Cum-OOH Induction time (mins)</th>
<th>Cum-OOH Max-rate (nmol $O_2$/min)</th>
<th>tBuOOH Induction time (mins)</th>
<th>tBuOOH Max-rate (nmol $O_2$/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>4.15 ± 0.32</td>
<td>108 ± 24</td>
<td>9.58 ± 0.66</td>
<td>85.5 ± 64</td>
</tr>
<tr>
<td>Exercise</td>
<td>3.91 ± 0.35†</td>
<td>115 ± 26†</td>
<td>9.43 ± 0.73†</td>
<td>87.1 ± 11</td>
</tr>
</tbody>
</table>

*RBCs were treated with either cumene hydroperoxide (Cum-OOH) or $t$-butyl hydroperoxide (tBuOOH) at a concentration of 1.20 mM. $O_2$ uptake was measured as described in the Materials and Methods section. The results are expressed as means ± SD.

† significantly different from pre-exercise ($P < 0.05$)
Table 2. Chemiluminescence parameters determined immediately before and after 45 minutes of treadmill running

<table>
<thead>
<tr>
<th>State</th>
<th>Cum-OOH Induction time (mins)</th>
<th>Peak-LCL (x 10^4 cpm)</th>
<th>Integral-LCL (x 10^6 cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>21.9 ± 5.22</td>
<td>119 ± 73.5</td>
<td>185 ± 85.4</td>
</tr>
<tr>
<td>Exercise</td>
<td>22.0 ± 4.45</td>
<td>122 ± 62.0</td>
<td>177 ± 79.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>tBuOOH</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>18.4 ± 5.57</td>
<td>103 ± 56.1</td>
<td>172 ± 64.8</td>
</tr>
<tr>
<td>Exercise</td>
<td>17.3 ± 5.03</td>
<td>94.2 ± 42.3</td>
<td>163 ± 55.2</td>
</tr>
</tbody>
</table>

RBCs were treated with either cumene hydroperoxide (Cum-OOH) or t-butyl hydroperoxide (tBuOOH) at a concentration of 75 µM. The results are expressed as means ± SD.
Fig. 5. Effect of moderate cycling on RBC susceptibility to osmotic lysis.

(a) A typical individual profile of the concentration-dependent haemolysis of isolated RBCs caused by exposure to hypotonic saline in vitro.

(b) The mean effect of single episodes of exercise on RBC osmotic fragility induced by 0.40% saline. The results are presented as means ± SEM.

The experimental procedures and data analysis are described in the Materials and Methods section.
statistically at one concentration point (0.40% saline), no differences attributable to training were found either before or after exercise. Thus, the data were pooled for statistical analysis of the exercise differences. The mean 15% decrease found immediately after exercise ($P < 0.001$) was sustained for at least six hours ($P < 0.001$; Fig. 5b).

**Effect on RBC density**

**Group 2 only.** There were no significant differences in the exercise responses between the trained and untrained subjects. Figure 6 shows a typical individual profile of density-fractionated RBCs before and after exercise, together with a laser scan of the gradient. The percentage of RBCs found in the densest half of the gradient increased by, on average, 10% (i.e. sum of quadrants 1 & 2) immediately after exercise ($n = 17; P < 0.001$) and this shift was accompanied by a similar decrease in the percentage of low-density RBCs in quadrants 3 & 4 (Fig. 7a). In the trained group before exercise (i.e. at rest), there was a significantly lower percentage of the low-density cells in quadrant 4 in RBCs isolated before exercise ($P = 0.009$) accompanied by uniform increases in the percentages of cells in the other three quadrants, which were, however, not significant statistically (Fig 7b).

We also examined whether the exercise-induced increase in RBC density could be reproduced by conditions that are known to cause oxidative damage *in vitro*. Normal RBCs isolated from non-exercised subjects were treated with phenazine methosulphate (PMS) (0.05mM) for 45 minutes in order to simulate the auto-oxidation of haemoglobin (Hebbel *et al.*, 1989). There was a much greater increase in the percentage of high-density cells in PMS-treated cells than in those induced by exercise (Fig. 8). This increase was even greater when RBCs were challenged with a low concentration (20 µM) of lipophilic Cum-OOH.

**Haematological parameters**

**Groups 1 & 2.** Statistical analysis of the mean values for each haematological parameter showed no significant differences between the trained and untrained groups so the data were pooled for analysis of the exercise effects alone. The decrease in osmotic fragility immediately after one hour of cycling at 60% VO$_2$ max was accompanied by a significant increase in mean cell haemoglobin concentration (MCHC) ($P < 0.05$). Whilst the mean cell volume (MCV) decreased consistently after exercise, this difference was not statistically significant. There was no significant change in mean cell haemoglobin (MCH) (Table 3). In contrast, there were no significant changes
Fig. 6. Typical individual profile of RBC density distribution immediately before and after treadmill running.

The laser densitometry scan of the accompanying photograph represents an overlay of the profiles analysed immediately before (A: unhatched) and after exercise (B: hatched). Each profile was analysed by division of the panel into four equal quadrants and calculation of the relative percentages of cells within each area to determine shifts in density caused by exercise.
Fig. 7. Effect of training status, and a single session of running, on RBC density.

(a) The density distribution of RBCs both before and immediately after exercise from the untrained group is presented as means ± SEM.

(b) The relative distribution of RBC density in cells isolated from non-exercised untrained and trained men. (means ± SEM)
Fig. 8. Changes in RBC density caused by oxidative stress in vitro.

The left panel shows an overlay of the density distribution of the untreated cells (A: unhatched) and the cells challenged with 20 µM cumene hydroperoxide (B: hatched). The right panel compares the effect of cell treatment with 50 µM phenazine methosulphate (C: cross-hatched) with the untreated cells (A: unhatched). Untreated cells were incubated in phosphate-buffered saline under the same conditions as the cells treated with oxidants (see Materials and Methods). The profiles were analysed using the procedure described in the legend to Figure 6.
Table 3. Haematological parameters measured immediately before and after submaximal exercise

<table>
<thead>
<tr>
<th>State</th>
<th>MCV (fL)</th>
<th>MCHC (g/dL)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cycling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>89.2 ± 2.9</td>
<td>33.23 ± 1.2</td>
<td>29.50 ± 1.8</td>
</tr>
<tr>
<td>Exercise</td>
<td>88.5 ± 2.9</td>
<td>34.47 ± 1.0*</td>
<td>30.23 ± 1.6</td>
</tr>
<tr>
<td>Post exercise (6h)</td>
<td>88.9 ± 2.8</td>
<td>34.17 ± 1.0*</td>
<td>29.84 ± 1.7</td>
</tr>
<tr>
<td><strong>Running</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>90.3 ± 1.9</td>
<td>34.30 ± 0.7</td>
<td>30.90 ± 0.8</td>
</tr>
<tr>
<td>Exercise</td>
<td>90.3 ± 1.8</td>
<td>34.30 ± 0.7</td>
<td>30.91 ± 1.6</td>
</tr>
</tbody>
</table>

* The results are expressed as means ± SD.
† Significantly different from pre-exercise values (p < 0.05)
We exercise-induced changes may due free radical generation catalysed by an increase in haem-iron bound to the plasma membrane. This hypothesis was proposed because RBCs isolated from individuals with β-thalassaemia or sickle cell anaemia also show increased vulnerability to peroxidative lysis and decreased susceptibility to osmotic fragility when compared to RBCs isolated from normal individuals (Snyder et al., 1981). Sickled cells contain excessive concentrations of haem-iron that is truly membrane-incorporated (Kuross et al., 1988). No significant changes in membrane-bound haem-iron were found in ghosts prepared from RBCs isolated either immediately after treadmill running (Table 4) or three hours later (data not shown), irrespective of whether this was measured by spectrophotometric or chemiluminescent methods. Furthermore, no significant changes in met-Hb concentration were detected after exercise (pre-exercise $0.78 \pm 0.78^\circ \%$ to exercise $0.24 \pm 0.26 \%$; n = 8). In contrast to the negligible effect of exercise, cells stressed oxidatively with PMS (50 μM; n = 4) showed a substantial increase in membrane-associated haem iron (control $1.27 \pm 0.53$ nmol/mg to PMS 1.66 nmol/mg) accompanied by peak- and integrated-LCL signals that were two-fold stronger in washed ghosts from PMS-treated ($86.6 \pm 33.0 \times 10^4$ cpm) than from untreated cells ($47.6 \pm 9.60 \times 10^4$ cpm) [all data are means ± SEM].
Table 4. Individual measurements of membrane-associated haem iron before and after treadmill running

<table>
<thead>
<tr>
<th>Subject #</th>
<th>Haemichrome (µM/mg protein)</th>
<th>Haem-iron (nmol/mg protein)</th>
<th>Peak-LCL (cpm x 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
<td>Exercise</td>
<td>Rest</td>
</tr>
<tr>
<td>1</td>
<td>0.408</td>
<td>0.507</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0.426</td>
<td>0.474</td>
<td>1.96</td>
</tr>
<tr>
<td>3</td>
<td>0.419</td>
<td>0.429</td>
<td>1.48</td>
</tr>
<tr>
<td>4</td>
<td>0.341</td>
<td>0.352</td>
<td>2.64</td>
</tr>
<tr>
<td>5</td>
<td>0.297</td>
<td>0.308</td>
<td>6.89</td>
</tr>
<tr>
<td>6</td>
<td>0.308</td>
<td>0.264</td>
<td>7.67</td>
</tr>
<tr>
<td>7</td>
<td>n m</td>
<td>n m</td>
<td>n m</td>
</tr>
<tr>
<td>8</td>
<td>n m</td>
<td>n m</td>
<td>n m</td>
</tr>
</tbody>
</table>

*Haemichrome, haem-iron and LCL were measured in washed ghosts using the procedures described in the Materials and Methods section. LCL was initiated by adding Cum-OOH (75 µM) to the reaction mixture which contained the ghosts (1.0 mg protein) and luminol (225 µM) in HBSS. The final volume was 1.0 ml. nm = not measured.
DISCUSSION

The results presented here show that single episodes of submaximal exercise (60 to 75% VO₂ max) increase the susceptibility of RBCs to oxidative stress in vitro. Depletion of endogenous antioxidant protection is the most likely explanation for this altered behaviour because the very small amount of haem iron already incorporated into the membrane in cells from each individual before exercise did not change up to three hours post exercise. Paradoxically, the vulnerability of RBCs to osmotic stress decreased after exercise, and this was associated with increased MCHC and a concomitant shift towards a greater percentage of high-density cells. The cumulative effects of repeated oxidative damage may contribute, along with mechanical and osmotic stress, to an increased rate of RBC destruction in endurance athletes. In the steady-state, the presence of a younger (and thus more flexible) RBC subpopulation (Weight et al., 1991), which may increase endurance capacity, could explain why RBCs from trained men are more resistant to peroxidative, but not to osmotic, stress compared to cells isolated from untrained men. The oxidative and osmotic results are discussed separately.

Oxidative stress

Since several laboratories have reported that single episodes of submaximal exercise deplete RBC antioxidant protection and cause detectable lipid peroxidation (see next paragraph), we have used assays that measure the total antioxidant capacity of RBCs rather than the individual components of antioxidant networks or the activities of free radical detoxification enzymes. Antioxidant capacity may have to fall below a critical threshold before RBCs become vulnerable to significant oxidative damage in vivo and in vitro. We also attempted to simulate the effects of exercise-induced oxidative stress in vitro by treating RBCs from non-exercised subjects with a low concentration of the superoxide generating reagent PMS (50 µM) (Hebbel et al., 1989). This work showed clearly that the oxidative stress of exercise was insufficient to cause haem iron incorporation into RBC membranes although this phenomenon could be demonstrated, and was associated with increased LCL, when membranes prepared from RBCs treated with PMS were challenged with Cum-OOH. These results indicate that exercise results in minor oxidative damage to RBCs (compared to pathological situations such as β-thalassaemia which are associated with haem iron incorporation into the membrane) and this may involve cells in the senescent subpopulation. Thus, assays that can analyse antioxidant status and other footprints of oxidative
damage at the single cell level are required to identify the affected RBC population.

The work of other groups suggests that depletion and/or uncoupling of antioxidant protection is the most plausible explanation for the increased susceptibility of RBCs to peroxidation in vitro after exercise. Cycling for 90 minutes at an intensity of 65% VO₂ max has been shown to deplete the whole blood concentration of GSH by 60% and to increase GSSG by 100% in recreational athletes (Gohil et al., 1988). This group also reported that blood GSH was replenished during recovery, which is consistent with the increased activity of GSSG reductase detected in RBCs immediately after exercise of similar intensity (Ohno et al. 1986). In contrast, however, Ji and colleagues (1993) reported that blood GSH concentration in a group of highly-trained endurance cyclists increased progressively during two hours of cycling at 70% VO₂ max, possibly as a result of GSH efflux from liver stores (Kretschmar and Muller, 1993). The discrepancy between these observations may be due to the training status and the exercise stress experienced by each group. In addition to GSH, vitamin-E is stored in the liver (Kayden and Traber, 1993) and, possibly, in adipose tissue (Pincemail et al., 1988). These stores may contribute to the whole blood pool during exercise-induced oxidative stress. A two-fold increase in expired pentane and a small increase in the serum concentration of MDA, both found immediately after 30 minutes of treadmill running at 60% VO₂ max, are indicative of lipid peroxidation (Kanter et al., 1993). Depletion of other intracellular antioxidants such as ascorbate and α-tocopherol may also occur during exercise. In support of this suggestion, we have found that supplementation of the diet with these antioxidants virtually abolishes the exercise-induced increase in RBC density and the decrease in the induction time of O₂ uptake in RBCs challenged with CumOOH in vitro (I. Gillam and J.A. Smith, unpublished observations).

In addition to the 10- to 20-fold increase in oxygen flux during exercise, the elevated rate of oxygen consumption post exercise, which may not return to basal levels until 12 hours after a workload of this magnitude (Bahr and Sejersted, 1991), may contribute, along with secondary oxidative reactions, to the gradual appearance of increased susceptibility of RBCs to peroxidative lysis several hours after exercise has ceased. Replacement of oxidatively-damaged polyunsaturated fatty acids by plasma exchange (Chiu et al., 1989) with species such as arachidonate during the six hour period immediately after exercise may also increase the vulnerability of RBCs to lipid peroxidation (Clemens and Waller, 1987). Other groups have found delayed increases in markers of oxidative damage after exercise. No increase in lipid peroxidation products is detectable in serum until six hours after downhill
running (Maughan et al., 1989). Duthie and colleagues (1990) reported that the vulnerability of RBCs to peroxidative lysis by H_2O_2 increased significantly 24 hours after the subjects had completed a half-marathon, although no increase was detected immediately after the race in spite of a significant fall in blood GSH and an unexpected increase in RBC membrane α-tocopherol. It is possible that decreases in the activities of various free radical detoxification enzymes such as GSH peroxidase were responsible for the increased susceptibility of RBCs to peroxidation in vitro.

There is considerable evidence that cellular changes that occur in response to repeated oxidative stress may be a causal mechanism of increased RBC turnover in athletes. Endurance training (5 km/day for 10 weeks) increases the activities of GSSG reductase and catalase in circulating RBCs (Ohno et al., 1988). Twenty weeks of endurance-training increases the concentration of whole blood GSH by 50% in previously sedentary men and this is accompanied by a significant increase in GSSG reductase activity in RBCs (Evelo et al., 1992). The activities of glutathione peroxidase and catalase in RBCs have been reported to increase progressively with training distance in endurance runners (Robertson et al., 1991) and RBCs isolated from professional cyclists have much higher activities of catalase and glutathione peroxidase compared to cells from sedentary people (Mena et al., 1991). The concentrations of vitamin-E and GSH in RBCs are also higher in trained runners (Robertson et al., 1991), although diet may be a partial explanation for the increase in vitamin-E. While these increases in the steady-state concentrations of RBC antioxidants are indicative of “younger” cells, no correlations were found with the RBC concentration of creatine or reticulocyte counts in the one study that has examined these relationships (Robertson et al., 1991).

Training-induced adaptations may occur in humans at the level of nucleated marrow RBC precursors because the selection pressure of high oxidative stress, such as that associated, for example, with β-thalassaemia leads to increases in the activities of free radical detoxification enzymes such as superoxide dismutase and GSH peroxidase in mature RBCs (Gerle et al., 1987). One contrasting study with rats suggests that RBCs produced under conditions of accelerated erythropoiesis and/or metabolic rate have reduced survival times in these animals (Landaw, 1988).

**Osmotic stress**

RBCs are normally biconcave discocytes with a large surface area to volume ratio which endows them with strong filterability properties (Boucher et al., 1985). With each trip through the circulation, the RBCs
undergo a shrinking and swelling cycle as they pass through the microcirculation which may lead to damage associated with "wear-and-tear"; this may be exacerbated by the accelerated haemoconcentration associated with regular exercise. There are two possible mechanisms which may lead to decreased osmotic fragility coupled with the increased MCHC and an increased percentage of high-density cells after submaximal exercise: (i) cellular dehydration; and (ii) protein crosslinking which may reinforce the ability of the cytoskeleton to withstand hypotonic shock. Whilst there is no evidence from our study to support the latter explanation, GSSG may bind to cytoskeletal proteins (Duthie et al., 1990), which may lead to the crosslinking of cytosolic and membrane proteins.

Cellular dehydration is the most likely explanation for the increased percentage of high-density cells found immediately after running. Whilst a small significant increase in MCHC (and non-significant decrease in MCV) were found after cycling, the more intense running protocol did not alter MCHC or MCV significantly. This may reflect a variable rate of recovery of normal cell volume when the cells are transferred to isotonic buffer for Coulter counter measurements of these parameters (Staubli and Roessler, 1986). While K⁺ leakage is normally associated with decreased osmotic fragility (Snyder et al., 1981), exercise at the intensities used in this study is unlikely to have been sufficiently stressful to reduce intraerythrocyte K⁺ concentration which occurs only when the workload exceeds 80% VO₂ max (Hespel et al., 1986). Failure to detect a significant change in MCV is indicative of this outcome because a stable intracellular K⁺ content is, in fact, critical to the maintenance of cell volume (Brugnara et al., 1987). Because the increase in high-density cells was about 10%, calculation of mean changes in RBC volume and haemoglobin concentration in the entire population may mask subtle changes in subpopulations. Thus, analysis of volume and haemoglobin concentration of the cells individually by flow cytometry or a similar technique, or of subpopulations isolated from density gradients, may resolve this apparent conflict in our data. Increased RBC density may restrict the transport of O₂ in the microcirculation to working muscles during exercise because numerous studies have shown that older (high-density) RBCs are less deformable under shear stress than younger (low-density) RBCs (Shiga et al., 1985; Sutera et al., 1985; Morse and Warth, 1990). Therefore, intensive aerobic exercise may accelerate the onset of the RBC "ageing process" because, as normal RBCs age, they lose K⁺ and water which results in increased MCHC and density (Usami et al., 1971).

Our results suggest that the percentage of RBCs with the lowest density decrease significantly in the long-term in the blood of triathletes and runners.
compared to the profiles obtained with untrained subjects. Cycling, running and cross country skiing, in contrast, have been reported to cause a loss of high-density cells, thereby decreasing the mean density of RBCs (Mairbaurl et al., 1983) and increasing MCV without changing total RBC mass (Green et al., 1991). The reason for the discrepancy between our results and those of Mairbaurl's group (1983) is not clear but it may involve the type and intensity of training the subjects were undertaking when tested (i.e. whether "ageing" of the cells exceeded the rate of destruction and replacement, or vice versa). Recent work by our group has shown that the percentage of blood reticulocytes analysed at the single cell level by flow cytometry is two to three times higher in some groups of athletes compared to untrained controls (R.D. Telford et al., unpublished data). This is further evidence of increased RBC turnover during athletic training. Schmidt and colleagues (1988) have also reported that even three weeks of endurance training increased the percentage of blood reticulocytes significantly. These reports are consistent with accelerated removal of the oldest cells during athletic training. Weight and colleagues (1991) have shown, by injecting $^{51}$Cr-labelled autologous RBCs back into donors, that these cells survived for 114 days in sedentary subjects compared to only 74 days in male and females running 50 to 129 km/week. If applicable more generally, these studies indicate that the rate of RBC turnover in runners may be nearly double that of sedentary subjects.

In agreement with our results, participation in a half-marathon caused a significant redistribution of circulating RBCs to the denser fractions (Robertson et al., 1988), suggesting that a subpopulation of RBCs "aged" or lost fluid during the race. These changes may not be mediated osmotically because RBCs do not respond to changes in plasma osmolality that are as small as 10 to 12% (Van Beaumont and Rochelle, 1974), and decreases in MCV detected after a marathon do not correlate with increased plasma osmolality (Staubli and Roessler, 1986). Whilst we did not measure changes in plasma osmolality, several studies suggest that small changes in this parameter may not affect RBC volume (Staubli and Roessler, 1986). Although large changes in plasma osmolality can alter RBC density, MCHC varies within narrow limits in normal individuals, with about 95% of the RBC population falling within the range 29-35 g/dl and only 5% exceeding 37 g/dl (Evans et al., 1984). The increases in MCHC and RBC density detected after exercise are unlikely to be caused by the small increase in plasma osmolality associated with a workload of similar magnitude (Gore et al., 1992).

Because RBC volume changes were not significant and high-density RBCs have a high surface area/volume ratio, membrane contact with haemoglobin caused by osmotic shrinkage in hypertonic regions of the
circulation (e.g. in peritubular capillaries) or when the cells pass through narrow splenic slits (Arese and De Flora, 1990) may lead to increased surface area which, in turn, increases RBC density and resistance to osmotic stress. This may also lead to crosslinking of cytoskeletal proteins to haemoglobin. Transient shrinkage during exercise may be a compensatory response to increased haematocrit that enables RBCs to maintain a state of optimum deformability (Staubi and Roessler, 1986) providing the MCHC does not fall below a critical threshold. Atrial natriuretic peptide, which is secreted into the circulation during exercise (Follenius and Brandenberger, 1988), has been reported to increase membrane flexibility (Zamir et al., 1992) and this may optimise the deformability of the high-density cells and thus O₂ delivery to working muscles. In this way, hormones released into the circulation during exercise may be able to modulate the RBC responses to oxidative and osmotic stress (Kanaley and Ji, 1991).

It is well known that sickled RBCs are more susceptible than normal cells to oxidative damage. Sickle cell trait (the heterozygous form, HbAS) increases the risk of sudden death during intense physical exertion, perhaps through irreversibly-sickled cells causing vascular obstruction (Kark et al., 1988). A recent report suggests that HbAS cells are more vulnerable than normal cells to oxidation during exercise and, furthermore, that the percentage of "dense RBC membranes" increases substantially in HbAS, but not HbAA, cells after exercise (Das et al., 1993). The authors did not investigate whether these so-called dense membranes contained aggregates of incorporated haem iron.

**Conclusion**

Our results show that, whilst a single episode of submaximal exercise increases the susceptibility of RBCs to oxidative stress, regular endurance training may confer a protective effect. Furthermore, acute exercise increases MCHC and the percentage of high-density RBCs, which are both characteristic of cells that may have undergone accelerated "ageing". While the causal mechanisms do not involve incorporation of haem iron into the membrane, the most plausible explanation involves depletion of specific antioxidants and/or uncoupling of co-operative interactions between the different antioxidants and free radical detoxification enzymes. These results are consistent with the hypothesis that the increased oxidative and osmotic stress encountered by the RBC during endurance training may shorten its life-span considerably.
ACKNOWLEDGMENTS

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REFERENCES


Exercise-induced increases in erythrocyte density and susceptibility to oxidative stress are attenuated by dietary antioxidant supplementation.
EXERCISE-INDUCED INCREASES IN ERYTHROCYTE DENSITY AND SUSCEPTIBILITY TO OXIDATIVE STRESS ARE ATTENUATED BY DIETARY ANTIOXIDANT SUPPLEMENTATION.

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Australia.
The large increase in oxygen uptake during endurance exercise may increase the susceptibility of muscle, liver and red blood cells (RBC) to oxidative damage by lowering antioxidant protection. The rate of RBC turnover is increased dramatically in endurance athletes and oxidative stress may play a major role. To test this hypothesis, we supplemented the normal diets of male endurance athletes and untrained controls (n = 15,5) with vitamin-C (1000mg/day) and vitamin-E (1000 IU/day) added simultaneously, or a placebo (soybean oil), over two six week periods using a double-blind crossover design. We also determined changes in RBC density, and the susceptibility of RBCs to oxidative stress in vitro, immediately after 45 min of running at 75% VO2 max. The plasma and RBC membrane concentrations of α-tocopherol increased three-fold in supplemented subjects (P < 0.001) but they did not change in the unsupplemented group. There was also a 25% increase in the plasma concentration of ascorbate in supplemented subjects only (P = 0.11). While supplementation did not alter RBC density in non-exercised subjects, it virtually abolished the exercise-induced increase in the percentage of high density RBCs. The decrease in time taken for peroxidation to commence in RBCs challenged with cumene hydroperoxide in vitro found after exercise was also substantially attenuated by antioxidant supplementation. These results suggest that supplementation of the diet of endurance athletes with antioxidant vitamins may reduce the rate of exercise-induced RBC damage and this may, in turn, increase endurance capacity and mitigate against the appearance of symptoms of “sports anaemia".
INTRODUCTION

Oxidative stress during exercise may occur as a result of the increased generation of reactive oxygen species (ROS) as by-products of aerobic metabolism in working muscle (Davies et al., 1982) and the auto-oxidation of haemoglobin to met-haemoglobin in red blood cells (RBC) (Hebbel and Eaton, 1989). Excessive generation of ROS may lead to the depletion of the body's antioxidant defences, leading to irreversible damage to vulnerable and, possibly, irreplaceable biomolecules (Demopoulos et al., 1986). The antioxidant defence network consists of a number of chemical antioxidants such as α-tocopherol (vitamin-E), ascorbate (vitamin-C), glutathione, and the free radical detoxification enzymes which include glutathione peroxidase and catalase. Because α-tocopherol is the only lipid antioxidant in RBCs, GSH and ascorbate are thought to participate in a specific redox shuttle that maintains it in the reduced state (Stocker et al., 1986). Thus, it may be important to supplement the diet with both of these antioxidant vitamins. Irreversible oxidative damage to proteins and lipids may result in increased membrane permeability, ageing and cell disruption. Peroxidation of polyunsaturated fatty acids generates detectable by-products such as malonyldialdehyde (MDA) which accumulates in RBCs or is released into the circulation, while protein thiol oxidation leads to the formation of disulphide-linked aggregates (Gutteridge and Halliwell, 1990).

Evidence accumulated during the last 15 years suggests that exercise can trigger antioxidant depletion and cause oxidative damage to a variety of cell types including muscle, liver, and RBCs, probably because of the large increase in oxygen flux through these tissues (Gohil et al., 1988; Duthie et al., 1990). Thus, under normal circumstances, the generation of ROS may increase in proportion to the oxygen uptake induced by exercise, but if the existing antioxidant defences are unable to cope, damage to susceptible body tissues may occur. Dietary supplementation with antioxidants such as vitamin-E (the major lipid-soluble antioxidant) has been reported to significantly reduce ROS generation and cellular oxidative damage during exercise (Dillard et al., 1978; Sumida et al., 1989). Oxidative damage to RBCs and muscle may limit aerobic performance because endurance capacity is reduced substantially by vitamin-E deficiency (Gohil et al., 1986) but prolonged by the ingestion of vitamin-E and/or free radical spin-trapping agents (Novelli et al., 1990).

Efficient delivery of oxygen to exercising muscles by red blood cells (RBC) is an important determinant of peak performance by human athletes. RBCs have a limited lifespan of 120 days in humans. There is now
considerable evidence which suggests that endurance training accelerates the rate of RBC destruction (Weight *et al*., 1991), leading to depletion of body iron stores and the condition often referred to as “sports anaemia” (Newhouse and Clement, 1988).

The aim of this study was to determine whether supplementation of the diet with vitamins C and E protected RBCs against oxidative damage induced by a single session of endurance exercise. We focussed on RBCs because previous work by our group (*Smith et al*., 1993a) and others (Gohil *et al*., 1988; Duthie *et al*., 1990) had shown that exercise increased the vulnerability of RBCs to oxidative damage *in vitro*.
MATERIALS AND METHODS

Materials
Cumene hydroperoxide (95%, w/w aqueous solution), tert-butyl hydroperoxide (70%, w/w aqueous solution), luminol, meglumine diatrizoate, and sodium diatrizoate were all obtained from Sigma (St. Louis, MO, USA). Percoll® was purchased from Pharmacia (Uppsala, Sweden). The α-tocopherol and ascorbate “Goldmark” standards for the HPLC analyses were purchased from Sigma (St. Louis, MO, USA). Soft-gel capsules (R.P. Scherer, Melbourne, Aust.) were prepared that contained either natural source α-tocopherol (250 IU) generously provided by Henkle Fine Chemicals (La Grange, IL, USA), and calcium ascorbate (250 mg) purchased from R.P. Scherer (Melbourne, Aust.), or soybean oil (Placebo).

Supplementation and exercise protocols
Fifteen highly-trained men (VO₂ max 72.3 ± 4.8 ml/kg; mean age 24 yrs) and five untrained men (mean VO₂ max 51.2 ± 5.3 ml/kg; mean age 33 yrs) completed the 16 week study (data are means ± SEM). The trained subjects were triathletes, cross country skiers and runners competing at national level who trained for 10 to 20 hours per week. The subjects were supplemented for two six week periods with either placebo or the allocated vitamin capsules using a double-blind crossover design. Four capsules per day were consumed. A four week “washout period” separated the two treatment periods to allow blood levels of these dietary antioxidants to return to baseline levels for those subjects allocated the vitamin supplement first.

Blood was taken by venepuncture at the start and completion of each treatment period when the subjects performed the exercise test. Each individual ran on a treadmill (level gradient) for 40 minutes at a workload equivalent to approximately 75% of their maximum oxygen uptake (VO₂ max). Blood was taken immediately before and after the exercise test. The VO₂ max of each subject was determined on a treadmill at least one week before the exercise test. A continuous incremental protocol was used in which the gradient of the treadmill was increased each minute until exhaustion. Gas analysis was performed using the procedure described previously (Smith et al., 1990). The blood withdrawal and RBC isolation procedures have also been described previously (Smith et al., 1992).

Assays
The oxygen uptake and luminol-amplified chemiluminescence assays were carried out as described previously (Smith et al., 1992). The
density fractionation procedure has also been described previously (Smith et al., 1993a). The concentration of total ascorbate (in both reduced & oxidized forms) in plasma was measured by HPLC using electrochemical detection (Kutnink et al., 1988) and α-tocopherol concentration in plasma and RBC membranes was measured by HPLC using spectrophotometric detection as described by Catignari and Bieri (1983). RBC membranes were prepared for α-tocopherol analysis using the procedure described by Burton and colleagues (1985). Briefly, RBCs (1.0 ml packed cells) were haemolysed in hypotonic phosphate-buffered saline (pH 7.2; 5 mM) containing ascorbate (5 mM) in a 50 ml tube which was centrifuged at 20,000 x g for 30 mins at 4°C. Supernatants were aspirated carefully to leave pelleted RBC ghosts. The ghosts were emulsified in SDS (0.1 mM), deproteinized in pure ethanol, and re-extracted in heptane under vigorous shaking. Extracts were then stored at -20°C for subsequent α-tocopherol analysis as for plasma.

Statistical analysis

The effects of exercise and the differences between the unsupplemented and supplemented results for each individual were analysed using a one-way analysis of variance. Correlations were determined by regression analysis.
RESULTS

The major results of this study were that antioxidant supplementation increased the plasma concentrations of ascorbate and α-tocopherol, and the levels of α-tocopherol in RBC membranes, by about three-fold. Supplementation prevented the increases in the percentage of high-density (older) cells and the susceptibility of isolated RBCs to peroxidation in vitro found in unsupplemented individuals immediately after exercise. There were no differences attributable to training in any parameter measured either before or after exercise. Thus the data from both groups were pooled for all the analyses reported below.

Plasma and RBC membrane antioxidants. The concentration of α-tocopherol in plasma was three times higher than the membrane values determined both before and after the supplementation program. There were no differences between the basal values of plasma and membrane α-tocopherol in either group before the program commenced. The plasma and membrane concentrations of α-tocopherol increased approximately three-fold in both the trained and untrained subjects (P < 0.001) while these values did not change significantly in unsupplemented subjects (Fig. 1a,b). There was no significant short-term change in the concentration of plasma or membrane α-tocopherol in response to exercise in either the supplemented or non-supplemented groups.

Supplementation also increased the mean plasma concentration of ascorbate by 25% but this was not significant (P = 0.11). In contrast, there was a significant increase in the plasma concentration of ascorbate immediately after exercise in both the supplemented and non-supplemented individuals (Fig. 2; P = 0.02). The intracellular concentration of ascorbate in RBCs was not measured.

RBC density. Cell density is thought to increase progressively as RBCs age (Clark, 1988). Antioxidant supplementation substantially decreased the percentage of RBCs that shifted to the densest half of the profile immediately after exercise in nearly all individuals (P < 0.001). When the exercise-induced change in the percentage of high-density cells was plotted against membrane-associated α-tocopherol for each individual in both groups, a hyperbolic relationship was clearly apparent in which the largest exercise-induced changes in RBC density were found when the concentration of membrane-associated α-tocopherol was very low (Fig. 3).
Fig. 1. The plasma and RBC membrane concentrations of \( \alpha \)-tocopherol.

(a) Plasma; (b) RBC membranes.

Blood samples were taken from both supplemented and unsupplemented individuals before the supplementation program commenced (baseline), and then, after six weeks, immediately before (Pre) and after (Post) the exercise test.

The results are presented as means \( \pm \) SD;
Fig. 2. The plasma concentration of ascorbate.

The sampling and testing procedures, and the presentation of data, are identical to those described in the legend to Figure 1.
Fig. 3. The relationship between RBC membrane \(\alpha\)-tocopherol concentration and the exercise-induced change in RBC density.

The data are individual values.
Supplementation had no significant effect on the density distribution of RBCs in the samples taken immediately before exercise (data not shown).

Effect of antioxidant supplementation on RBC susceptibility to oxidative stress.

We also examined, using two independent assays, whether supplementation of the diet with α-tocopherol and ascorbate protected RBCs against peroxidation induced by challenging of the cells with organic hydroperoxides (ROOH) in vitro.

Oxygen uptake. The time that elapses before O₂ is taken up from the extracellular medium (induction or lag time) when cells are exposed to ROOHs is thought to be proportional to their total antioxidant capacity (Lissi et al., 1986; Berry et al., 1988). In these experiments, isolated RBCs were challenged with the same concentration (1.20 mM) of either Cum-OOH or tBuOOH in separate assays both before and after exercise. There was no significant difference in the induction times between the two groups before exercise (Table 1). Immediately after exercise, there was a significant decrease in the induction (lag) time of O₂ uptake in unsupplemented subjects (P < 0.001) but the apparent decrease detected in supplemented individuals did not approach significance (P = 0.16; Table 1). There was also a significant linear correlation between RBC α-tocopherol and the exercise-induced change in the induction time (r = 0.37; P = 0.007). Since there was no significant change in the maximum rate of O₂ uptake after exercise, and no additional effect of supplementation, it is not surprising that no significant correlation was detected between RBC α-tocopherol and the exercise-induced change in the maximum rate of O₂ uptake.

In contrast to the significant decrease in the induction time found when RBCs were challenged with the hydrophobic compound cumene hydroperoxide (Cum-OOH), no significant changes in either the induction time or the maximum rate of O₂ uptake were found when the cells were challenged with the amphipathic compound tert-butyl hydroperoxide (tBuOOH) (Table 2).

Chemiluminescence. We reported recently that luminol-amplified chemiluminescence can be used to detect the generation of ROS produced as a result of the catalytic decomposition of ROOH by haem iron in RBCs; furthermore, the induction time may also be representative of cellular antioxidant protection (Smith et al., 1992). These experiments were only carried out in the first phase of the crossover study (n = 13 placebo;
Table 1  Induction time of, and maximum rate of oxygen uptake in RBCs exposed to Cum-OOH*

<table>
<thead>
<tr>
<th>State</th>
<th>Placebo</th>
<th>Supplemented</th>
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<tbody>
<tr>
<td></td>
<td><em>Induction time (min)</em></td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>4.14 ± 0.08</td>
<td>4.15 ± 0.08</td>
</tr>
<tr>
<td>Exercise</td>
<td>3.91 ± 0.08†</td>
<td>4.03 ± 0.09</td>
</tr>
</tbody>
</table>

*Maximum rate (nmol O₂/min)*

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<tr>
<th>State</th>
<th>Placebo</th>
<th>Supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>111.7 ± 5.19</td>
<td>110.4 ± 6.50</td>
</tr>
<tr>
<td>Exercise</td>
<td>116.2 ± 6.38</td>
<td>114.2 ± 6.40</td>
</tr>
</tbody>
</table>

*The data are presented as means ± SD.
†Significantly different from pre-exercise values (P < 0.001)
Table 2: Induction time of, and maximum rate of oxygen uptake in RBCs exposed to tBuOOH*.

<table>
<thead>
<tr>
<th>State</th>
<th>Placebo</th>
<th>Supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*Induction time (min) *</td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>8.80 ± 0.49</td>
<td>9.14 ± 0.35</td>
</tr>
<tr>
<td>Exercise</td>
<td>8.66 ± 0.44</td>
<td>9.06 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>*Maximum rate (nmol O₂/min) *</td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>105.0 ± 9.6</td>
<td>96.9 ± 6.7</td>
</tr>
<tr>
<td>Exercise</td>
<td>108.8 ± 9.8</td>
<td>97.9 ± 6.7</td>
</tr>
</tbody>
</table>

* The data are presented as means ± SD.
7 supplemented). As reported previously (Smith et al., 1993a), there were no significant changes in either the induction time or peak-chemiluminescence intensity immediately after exercise when the cells were challenged with either Cum-OOH or tBuOOH in vitro. Before exercise, the induction time tended to be longer in Cum-OOH-treated RBCs (P = 0.27) from the supplemented subjects but the peak-LCL values were unaffected by antioxidant supplementation (Tables 3 & 4). There was no significant differences in either of these parameters when the cells from the supplemented and unsupplemented individuals were challenged with tBuOOH (Tables 3 & 4).
Table 3  Induction time of chemiluminescence in RBCs challenged with ROOHs*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cum-OOH</th>
<th>tBuOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>20.9 ± 5.59 min</td>
<td>16.0 ± 3.13 min</td>
</tr>
<tr>
<td>Supplemented</td>
<td>23.2 ± 4.48 min</td>
<td>17.2 ± 3.10 min</td>
</tr>
</tbody>
</table>

* The data are presented as means ± SEM. The concentration of each ROOH was 75 µM.
Table 4  Peak chemiluminescence of RBCs challenged with ROOHs*.

<table>
<thead>
<tr>
<th>State</th>
<th>Placebo</th>
<th>Supplemented</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Supplemented</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cum-OOH</td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>1390 ± 158</td>
<td>1328 ± 256</td>
</tr>
<tr>
<td>Exercise</td>
<td>1376 ± 150</td>
<td>1141 ± 216</td>
</tr>
<tr>
<td></td>
<td>Cum-OOH</td>
<td></td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>873 ± 65</td>
<td>783 ± 90</td>
</tr>
<tr>
<td>Exercise</td>
<td>808 ± 62</td>
<td>825 ± 89</td>
</tr>
</tbody>
</table>

* The data are presented as means ± SEM. The units are cpm x 10^4. The concentration of each ROOH was 75 µM.
DISCUSSION

Because of their continual exposure to $O_2$, and their high contents of haem iron and polyunsaturated fatty acids, RBCs are particularly vulnerable to oxidative stress, which is initiated under normal conditions by the auto-oxidation of haemoglobin which occurs at the rate of three percent per day (Hebbel and Eaton, 1989). This may increase in proportion to the increased rate of whole body oxygen uptake (10-20-fold) that occurs during exercise (Duthie et al., 1990). Our results show clearly that supplementation of the diet with $\alpha$-tocopherol (and possibly ascorbate) protects RBCs against exercise-induced increases in RBC density and the susceptibility of these cells to peroxidation in vitro. In contrast, there were no detectable differences in RBC susceptibility to peroxidation between the supplemented and non-supplemented groups before exercise and no significant changes in total $\alpha$-tocopherol or ascorbate concentrations in plasma, and $\alpha$-tocopherol levels in the RBC membrane, after exercise. However, the reduced/oxidized ratios of these antioxidants may decrease. It is also possible that endogenous antioxidants such as GSH may be consumed during the recycling of the $\alpha$-tocopherol and ascorbate radicals to their reduced states because a 60% decrease in the RBC concentration of GSH, and a 100% increase in GSSG, have been found immediately after 90 minutes of cycling at 65% VO$_2$ max (Gohil et al., 1988).

Paradoxically, high-density (old) RBCs contain a higher $\alpha$-tocopherol/lipid ratio than low-density (young) cells (Burton et al., 1986). In high-density RBCs, putative markers of senescence associated with oxidative stress include, inter alia, increases in: the band 4.1a:4.1b ratio; membrane rigidity and impaired deformability; and protein carboxyl methylation (reviewed by Clark, 1988). Thus the enhanced susceptibility of the high-density cells to peroxidation in vitro may be a function of their higher concentration of haemoglobin and the proximity of haem iron to the membrane and, thus, its potential to short-circuit detoxification of lipid radicals via $\alpha$-tocopherol (Hebbel, 1986). It is possible that membrane lipids were altered by exchange with plasma pools during exercise in some subpopulations of RBCs which may explain their increased susceptibility to peroxidation despite a lack of significant change in their $\alpha$-tocopherol levels. Thus, our results suggest that, in vitamin-E-sufficient men, these detrimental changes in RBCs during exercise may still contribute to reduced endurance capacity and accelerate the onset of fatigue during intensive exercise. Supplementation may have increased the $\alpha$-tocopherol levels in...
other body tissues because a strong correlation has been reported between
the plasma and liver concentrations (Mino et al., 1985).

These results raise the question as to whether the currently
recommended dietary allowance of vitamin-E (15 IU/day) (Packer, 1991)
and, perhaps, vitamin-C (60 mg/day) should be increased substantially in
sportspeople. In humans, vitamin-E is derived from dietary sources only.
The vitamin is transported in the blood via lipoproteins and may also be
stored in the liver and adipose tissue (Warren et al., 1991). The three-fold
increase in the plasma and RBC membrane pools of α-tocopherol may be
the maximum possible because of the limiting supply of an α-tocopherol-
binding protein in plasma which prevents excretion of the free form
(Kayden and Traber, 1993). Mobilization of tissue antioxidant stores during
intensive exercise may maintain the cellular pools. α-Tocopherol levels
increase by 20% in plasma and 100% in RBCs immediately after maximal
exercise but both return to baseline values 10 minutes later (Pincemail et al.,
1988) indicating that cellular exchange may be rapid. However, rapid
exchange of spent α-tocopherol may not prevent some damage from
occurring or being repaired, and may explain why we found no change in the
total concentration of α-tocopherol in the plasma or RBC membrane. GSH
release into plasma also increases by 30% immediately after maximal
exercise in trained subjects (Kretzschmar et al., 1991). The 20% increase in
plasma ascorbate concentration found immediately after exercise here was
not surprising considering an increase of the same magnitude occurred
during a 21 km running race; this rise correlated with increased plasma
cortisol (Gleeson et al., 1987). It is possible that these events are connected
and that cortisol may initiate the release of ascorbate and, perhaps, other
antioxidants from the liver and other storage sites into the circulation.
Neutrophils, for example, contain a high concentration of ascorbate
(Halliwell et al., 1987) that may become available to neighbouring RBCs
upon activation of neutrophils during exercise (Smith et al., 1993b). The
concentrations of vitamin-E and GSH in RBCs also increase chronically in
trained runners (Robertson et al., 1991). The increase in α-tocopherol is
likely to be due to diet and not to training per se. Release of antioxidants
such as α-tocopherol or GSH from tissue stores into the circulation during
intensive exercise may conserve antioxidant protection in muscle and RBCs
during oxidative assault. Furthermore, damaged portions of the membrane
may be removed as vesicles and therefore cause no alteration in the lipid to
α-tocopherol ratio.
Despite some conflicting reports, in general \( \alpha \)-tocopherol deficiency increases the susceptibility of various cell types to exercise-induced oxidative stress and reduces endurance capacity, while supplementation confers a protective effect (reviewed by Witt et al., 1992; Goldfarb, 1993). Evidence of a positive effect of \( \alpha \)-tocopherol supplementation on RBC function includes prevention of impaired RBC filterability caused by extended stays at high altitude (Simon-Schnass and Korniszewski, 1990) and a substantial reduction in the lipid peroxidation of both normal and sickle RBCs (Jain et al., 1990), although it has adverse effects on cell deformability (Kon et al., 1983). In contrast to our results, vitamin-A, -C & -E supplementation did not prevent increases from occurring in expired pentane and serum MDA in response to 30 min of running at 60\% VO\(_2\) max followed by 5 min at 90\% VO\(_2\) max despite some benefits being found at rest in the supplemented group (Kanter et al., 1993). The expiration of pentane increased two-fold during cycling for one hour at 60\% VO\(_2\) max and this was attenuated by daily supplementation with \( \alpha \)-tocopherol (Dillard et al., 1978). However, the parameters measured refer to whole body oxidative stress and may not be related directly to oxidative damage of RBCs. While \( \alpha \)-tocopherol is thought to be exclusively a lipophilic antioxidant, recent evidence suggests that it may also prevent protein oxidation in muscle during exercise (Reznick et al., 1992). Thus the protective effects of supplementation may also extend to protein oxidation, which may precede lipid peroxidation in RBCs (Davies and Goldberg, 1987).

Future work needs to determine whether these beneficial effects on RBCs were due to \( \alpha \)-tocopherol alone. Whilst ascorbate may have contributed to the maintenance of \( \alpha \)-tocopherol in the reduced state, enzymatic recycling may also be involved in RBCs (Constantinescu et al., 1993). The lipid composition of the RBC as well as the proportional association of \( \alpha \)-tocopherol with unsaturated lipids may be altered in supplemented subjects (Goldfarb, 1993). Because both ascorbate (Chen, 1981) and \( \alpha \)-tocopherol (Bowry et al., 1993) can act as pro-oxidants under conditions where their radical derivatives cannot be recycled to the reduced state, the optimal doses of these vitamins will have to be determined, before the recommended daily allowances can be increased substantially. The ratios at which these vitamins are added to the diet may be crucial because high doses of ascorbate lead to oxidative damage of RBCs in rodents unless adequate levels of vitamin-E are also included (Chen, 1981), While high concentrations of \( \alpha \)-tocopherol have generally been found to be non-toxic...
and have few side effects in the short-term (Bendich and Machlin, 1988), α-tocopherol can inhibit the proliferation of some cell types through its ability to block protein kinase-C activity (Boscoboinik et al., 1991). Thus, the long-term effects of high α-tocopherol intake are unknown. Because RBCs have low concentrations of ubiquinol (Constantinescu et al., 1993) and, possibly, vitamin-A, it is impossible to predict whether supplementation with these compounds would provide additional antioxidant protection. Finally, antioxidant supplementation may produce even more striking benefits in veteran sportspeople because the rate of RBC turnover may increase substantially with advancing age (Kosower, 1993).

In conclusion, supplementation of the diet with vitamins C & E conferred, simultaneously, substantial protection against the exercise-induced increase in RBC density and the susceptibility of these cells to peroxidation. This work provides clear evidence that supplementation of a normal diet with vitamin-C and -E may reduce the rate of RBC turnover in endurance athletes. This may prolong endurance capacity and protect against “sports anaemia”.

ACKNOWLEDGMENTS

We are grateful to the subjects who participated in this study. We also thank Dr. Roland Stocker (Heart Research Institute, Sydney, Aust.) for performing the ascorbate assay. Financial support was provided by the Faculties Research Fund of the Australian National University. IG and JAS were both recipients of Australian Postgraduate Research Awards. IG was also the recipient of Australian Institute of Sport Research Fellowship.
REFERENCES


CHAPTER 7

EXPERIMENTAL

(Maximal exercise)
CHAPTER 7.1

Red blood cell uptake of lactate during maximal exercise may contribute to fatigue independently of pH.
RED BLOOD CELL UPTAKE OF LACTATE DURING MAXIMAL EXERCISE MAY CONTRIBUTE TO FATIGUE INDEPENDENTLY OF pH.

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Fax: 61-6-249 0313
Blood lactate concentration increases substantially once the anaerobic threshold is exceeded during vigorous exercise. We investigated whether uptake of lactate by red blood cells (RBC) during maximal exercise altered certain cellular properties including volume, density and osmotic fragility. Irrespective of whether the subjects undertook one minute of maximal exercise or progressive exercise to exhaustion, whole blood lactate concentration increased by about 10-fold ($P < 0.001$) above pre-exercise values. The plasma and cellular concentrations of lactate increased uniformly and maintained a constant plasma/cell gradient ($P < 0.01$). Exercise-induced increases in RBC osmotic fragility and mean cell volume (MCV; $P = 0.028$), and decreases in mean cell haemoglobin concentration (MCHC; $P = 0.036$) and cell density, accompanied the increased blood lactate concentration ($P = 0.005$). The exercise-induced cellular responses could be simulated by treating blood taken from non-exercised subjects with L-lactate (15 mM) under conditions in which normal pH was maintained. We conclude that the uptake of lactate, and not $H^+$ ions, by RBCs during vigorous exercise alters certain cellular properties which may, in turn, impair cell deformability and lead, possibly, to capillary obstruction and the onset of fatigue.
INTRODUCTION

Lactic acid is released into the circulation from working muscles during prolonged exercise and may accumulate when energy demands exceed those associated with the capacity of the tricarboxylic acid cycle to oxidize pyruvate. Lactate efflux from working muscle is the major factor contributing to its increased concentration in the blood during maximal exercise. The lactate produced may be taken up and oxidized immediately by neighbouring slow-twitch muscle fibres and/or oxidation may occur upon reperfusion during the recovery phase (Mazzeo et al., 1986). Circulating lactate is also a major substrate for hepatic gluconeogenesis, but liver uptake accounts for only 10% of the lactate generated during exercise (Astrand et al., 1986). Because removal of lactate from the blood may fail to keep pace with its rate of generation, particularly during maximal exercise, high blood lactate concentrations and an associated acidosis may occur, the latter being associated with fatigue (Sahlin, 1992).

Lactate released into the blood is also taken up by red blood cells (RBC). Transport of lactate into (or out of) RBCs occurs principally via a specific monocarboxylate transporter, but non-ionic diffusion of the free acid, and anion transfer via band III, are also involved to a small extent (reviewed by Poole and Halestrap, 1993). The specific system involves a H⁺-lactate symport mechanism which is strongly pH-dependent (Dubinsky and Racker, 1978). Because glycolysis is the exclusive pathway of energy metabolism in RBCs, lactate is generated continuously as an end-product (Tilton et al., 1991). Under normal (non-exercise) conditions, there is a significant pH gradient between the RBC (7.2) and plasma (7.4) which conforms to the Donnan distribution of H⁺ ions caused by the negative charge of haemoglobin (Harris and Dudley, 1989). Several studies have reported that the distribution of lactate between plasma and RBCs varies under different physiological conditions; in fact, a substantial gradient exists after maximal exercise when lactate concentration in plasma exceeds that in the RBC by two- to three-fold (Buono and Yeager, 1986; Foxdale et al., 1990). The functional and metabolic consequences of lactate uptake by RBCs are not known.

The purpose of this study was: (i) to investigate the effects of lactate accumulation on mean cell volume and density of RBCs; and (ii) to determine the susceptibility of the cells to haemolysis. By investigating these changes independently of pH, it may be possible to relate lactate accumulation per se, as distinct from that of H⁺ ions, with fatigue.
MATERIALS AND METHODS

Materials.
L-lactic acid, meglumine diatrizoate and sodium diatrizoate were all obtained from Sigma (St. Louis, MO, USA). Percoll® was purchased from Pharmacia (Uppsala, Sweden).

Human Subjects and exercise workloads.
Twelve highly-trained male cyclists participated in the initial study; their physical characteristics have been described previously (Gray et al., 1992). Exercise involved one minute of cycling at maximal effort which was preceded by a ten minute submaximal warm-up at approx. 60% VO₂ max (Gray et al., 1992).

Three highly-trained male rowers and five downhill skiers (4 males, 1 female) were recruited for the second phase. Each subject underwent a progressive test to exhaustion on a rowing (or cycle) ergometer. Blood for the lactate studies in vitro was taken from six non-exercised volunteers (5 males, 1 female).

Lactate analysis
The blood withdrawal and RBC isolation procedures have been described previously (Smith et al., 1992). The plasma and cells were aspirated into separate tubes. The cells were washed three times in phosphate-buffered saline (PBS), pH 7.3, and resuspended in PBS at an haematocrit of 50%. Lactate concentrations in whole blood, plasma and RBCs were measured using an YSI Model 23L L-lactate analyser calibrated with a YSI 2747 L-lactate standard (Yellow Springs Instrument Company, Yellow Springs, OH, USA). The samples (25 μl) were added to a solution (50 μl) containing buffer, a RBC lysing agent (YSI 2357) and sodium fluoride (1 mg/ml) (Gray et al., 1992). Blood pH was measured with a Ciba Corning automated blood gas analyser (Diagnostic Corporation, Medfield, MA).

To simulate the effects of exercise, L-lactate was added to whole blood (final concentration, 15 mM) which was incubated for 20 minutes at 37°C. The pH of the stock L-lactate solution (3M) was adjusted to 7.3 with NaOH. We also investigated the separate role of pH on RBC density by replacing lactate with PBS adjusted to pH 6.8. At the end of the incubation period, lactate or acidic PBS was removed by three washes with normal PBS (pH 7.3) at room temperature and the cells were resuspended in PBS at the original haematocrit.

Haematological Parameters
Red and white blood cell counts, haematocrit, mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin
(MCH) and haemoglobin concentration were determined in single blood samples (100 µl) aspirated into an automated haematology analyser (Coulter Electronics, Hialeah, FL, USA).

**Osmotic fragility**

Heparinized whole blood (100 µl) was added to hypotonic phosphate-buffered saline (pH 7.4) in the concentration range of 0.2 to 0.5 % (w/v) of saline (Simmons, 1972). The tubes were mixed by inversion, allowed to stand for one hour at room temperature, mixed again and centrifuged for five minutes at 1000 x g to pellet the ghosts. The optical density of each sample was measured in the supernatants by spectrophotometry at 540 nm. The percentage of haemolysis was calculated with reference to the haemolysis induced by incubating cells in distilled water under the same conditions.

**Density Fractionation and Scanning**

The RBC population was fractionated on a self-forming Percoll®/diatrizoate density gradient using the original technique described by Vettore and colleagues (1980) with the modifications introduced by Snyder and colleagues (1983). Specifically, RBCs were washed in PBS three times before the addition of packed cells (200 µl) to the Percoll®/diatrizoate mixture (10 ml) in a Corex tube. The suspension was mixed by inversion and centrifuged for 20 minutes at 35,000 x g at 4°C using an angle-rotor (SS-34) in a RC-2B centrifuge (Sorvall, USA).

After centrifugation, each tube was photographed, using an evenly illuminated light box as background, with 25 ASA film (Kodak Technical Pan). The film was exposed for six seconds at f16, developed in Kodak Technidol, and the continuous-tone negatives printed on multigrade paper (Ilford, Multigrade 3) using a zero-grade filter. Optimum black-and-white photography conditions were chosen so that the maximum density of the exposed image did not exceed an optical density of 4.0, which represented the upper linear detection limit of the laser densitometer. The density of each band was quantitated by scanning laser densitometry (LKB Ultrascan XL™, Bromma, Switz.). Individual density profiles were integrated using the Gelscan XL™ software package (LKB, Bromma, Switz.) in the attached microprocessor.

**Statistical analysis.**

The significance of the differences between the paired data were assessed by Student's t test.
RESULTS

Exercise for one minute at maximal effort.

The stress of the workload at maximal effort was shown by the transient six-fold increase in blood lactate concentration (from $1.9 \pm 0.23$ mM$^*$ after the warm-up to $12.3 \pm 0.69$ mM immediately after exercise; $P < 0.001$). The blood lactate concentration before exercise was $1.2 \pm 0.13$ mM$^*$. It was still twice as high as the pre-exercise values one hour after exercise ($P = 0.0075$) ($^*$data are means ± SEM).

The effect of this large exercise-induced increase in blood lactate concentration on RBCs was examined by measuring changes in mean cell volume (MCV) and osmotic fragility, the latter being dependent, in part, on the cell surface area to volume ratio (Beutler et al., 1982). Table 1 shows that maximal exercise caused a significant increase in MCV ($P < 0.001$) and small concomitant fall in mean cell haemoglobin concentration (MCHC) ($P < 0.001$). There was no significant change in mean cell haemoglobin (MCH). Figure 1a shows a typical individual result in which a substantial increase in the osmotic fragility of RBCs occurred immediately after exercise but returned to a value slightly below its pre-exercise level one hour later. When the mean responses of the trained and untrained subjects were analysed statistically (at one concentration point in the linear region of the curve, 0.40% saline), no differences attributable to training were found either before or after exercise, which confirms the results of our previous study (Smith et al., 1993). Therefore the data from untrained and trained subjects were pooled for statistical analysis of the individual exercise differences. The mean 20% increase in RBC osmotic fragility that occurred immediately after maximal exercise ($P < 0.001$) was followed by a 10% decrease from pre-exercise values one hour later ($P < 0.001$; Fig. 1b). These responses followed the kinetics of lactate accumulation and clearance from the circulation.

Progressive exercise to exhaustion.

To investigate whether the uptake of lactate into RBCs during exercise was responsible for the increased MCV and osmotic fragility induced by one minute of maximal exercise, we measured the concentrations of lactate in whole blood, plasma and washed RBCs after a progressive exercise test to exhaustion. The progressive exercise bout produced a similar increase in MCV ($P = 0.028$) and decrease in MCHC ($P = 0.036$), and no significant change in MCH occurred (Table 2). Figure 2 shows that this workload also produced a 10-fold increase in blood lactate concentration which was accompanied by a significant fall in blood pH.
Table 1: Haematological parameters measured before and after one minute of maximal exercise†

<table>
<thead>
<tr>
<th>State</th>
<th>MCV (fL)</th>
<th>MCHC (g/dL)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>91.4 ± 2.8</td>
<td>33.81 ± 0.6</td>
<td>30.92 ± 1.1</td>
</tr>
<tr>
<td>Exercise</td>
<td>93.9 ± 3.0*</td>
<td>33.23 ± 0.5*</td>
<td>31.13 ± 1.1</td>
</tr>
<tr>
<td>Post exercise (1h)</td>
<td>91.7 ± 2.6</td>
<td>33.92 ± 0.6</td>
<td>31.12 ± 1.2</td>
</tr>
</tbody>
</table>

† The results are presented as means ± SEM.
* Significantly different from pre-exercise values (P < 0.001)
Fig. 1  Effect of maximal exercise on RBC susceptibility to osmotic haemolysis.

(a) A typical individual profile of the concentration-dependent osmotic fragility of isolated RBCs caused by exposure to hypotonic saline in vitro.

(b) The mean effect of single episodes of exercise on RBC osmotic fragility induced by 0.40% saline. The results are presented as means ±SEM.
Table 2: Haematological parameters measured before and after progressive exercise to exhaustion†.

<table>
<thead>
<tr>
<th>State</th>
<th>MCV (fL)</th>
<th>MCHC (g/dL)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-exercise</td>
<td>87.1 ± 0.61</td>
<td>33.1 ± 0.32</td>
<td>28.8 ± 0.60</td>
</tr>
<tr>
<td>Exercise</td>
<td>88.3 ± 0.75*</td>
<td>32.6 ± 0.17*</td>
<td>28.8 ± 0.63</td>
</tr>
</tbody>
</table>

† The results are presented as means ± SEM.
* Significantly different from pre-exercise values (P < 0.04)
**Fig. 2** The distribution of blood lactate before and after progressive exercise to exhaustion.

L-Lactate concentration was measured in whole blood, plasma and RBCs isolated from each individual immediately before and after the exercise test. The results are presented as means ± SEM. The experimental procedures are described in the Materials and Methods section.
(from 7.38 ± 0.01* pre-exercise to 7.15 ± 0.03 immediately after exercise; *data are means ± SEM). We also determined the separate contributions of plasma and RBC lactate concentrations to the whole blood value. The concentrations of lactate in plasma and whole blood before exercise were significantly higher than in RBCs (P = 0.01), giving rise to a positive gradient (plasma/cells = 1.68) that was not substantially affected by exercise (plasma/cells = 1.77; Fig. 2). The concentration of lactate in plasma immediately after exercise, however, was 20% higher than that of whole blood (P = 0.028) and 100% higher than the RBC value (P = 0.001).

We also investigated whether the uptake of lactate by RBCs during exercise influenced RBC density. Figure 3 shows a typical profile of density-separated RBCs isolated both before and after exercise. The accompanying laser scan of the photograph was analysed in quadrants. These data (n = 5 subjects) show that the percentage of low-density RBCs increased substantially immediately after exercise (Fig. 4). Statistical analysis showed that exercise caused a two- to three-fold increase in the percentage of RBCs in the least dense fraction (quadrant 4; P = 0.005) and a concomitant two-fold decrease in the percentage of cells in the densest fraction (quadrant 1; P = 0.021). Only small exercise-induced shifts, which were not significant statistically, occurred in quadrants 2 and 3.

**Effect of lactate treatment in vitro.**

To test the hypothesis that lactate uptake by RBCs was responsible for the increased MCV and decreased density, we attempted to simulate the exercise-induced changes by incubating in vitro whole blood from non-exercised subjects with L-lactate for 20 minutes (i.e. for about the same time that RBCs were exposed to lactate during exercise) at the same concentration as that generated physiologically by maximal exercise (15 mM). The pH of the control and lactate-treated blood samples was maintained at 7.3. Figure 5 shows that a lactate concentration eight-fold above the value of the untreated cells was achieved in treated whole blood and that this increase was distributed uniformly between the plasma and cells (P < 0.001). A plasma to RBC lactate concentration gradient of a similar order of magnitude was detected in both untreated (plasma/ cells = 4.76) and lactate-treated RBCs (plasma/cells = 3.80; P < 0.001). This was twice as high as the gradient detected in the in vivo exercise study reported here. The difference is unlikely to be accounted for by loss of intracellular lactate during the washing procedure since the same lactate concentration was detected in unwashed RBCs immediately after the end of the incubation period. Furthermore, no visible release of haemoglobin, and thus no loss of membrane integrity, was detected.
Typical individual profile of RBC density distribution immediately before and after progressive exercise to exhaustion.

The laser densitometry scan of the accompanying photograph represents an overlay of the profiles analysed immediately before (A: unhatched) and after exercise (B: hatched). Each profile was analysed by division of the panel into four equal quadrants and calculation of the relative percentages of cells within each area to determine shifts in density caused by exercise.
Fig. 4. The density distribution of RBCs immediately before and after progressive exercise to exhaustion.

The relative distribution of RBC density in cells both before and after exercise. The results are presented as means ± SEM.
Fig. 5  The distribution of blood lactate before and after exposure to L-lactate in vitro.

Lactate concentration was measured in whole blood, plasma and RBCs immediately before and after treatment of whole blood isolated from non-exercised subjects with either PBS (control) or L-lactate (15 mM) for 20 min at 37°C. The results are presented as means ± SEM. The experimental procedures are described in full in the text.
during the washing procedure and no significant differences in the density banding patterns were detected between washed and unwashed cells. The osmotic behaviour of the cells therefore appeared to be unaltered by replacing plasma with buffer.

Table 3 shows that the MCV increased and MCHC decreased, without altering MCH, to levels similar to those caused by maximal exercise ($P = 0.007$). Furthermore, a substantial increase in the percentage of low-density cells was found, although the distribution pattern was quantitatively different to that found after exercise (Fig. 6). Lactate treatment, like maximal exercise, caused a two- to three-fold increase in the percentage of RBCs in the least dense fraction (quadrant 4; $P < 0.001$), and this was accompanied by significant decreases in the percentage of RBCs in the other three quadrants ($P = 0.027$), particularly quadrant 3 ($P < 0.001$). The fall in blood pH per se normally associated with lactate accumulation was not responsible for the decrease in RBC density because when a low pH (6.8) was established by HCl addition, RBC density tended to increase (data not shown).
Table 3: Haematological parameters measured before and after exposure of RBCs to L-lactic acid *in vitro.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCV (fL)</th>
<th>MCHC (g/dL)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>91.9 ± 0.69</td>
<td>34.0 ± 0.12</td>
<td>31.3 ± 0.27</td>
</tr>
<tr>
<td>L-lactate</td>
<td>93.4 ± 0.42*</td>
<td>33.4 ± 0.16*</td>
<td>31.5 ± 0.34</td>
</tr>
</tbody>
</table>

* RBCs were incubated with either PBS (untreated) or L-lactate (15 mM) under conditions described in the Materials and Methods section. The results are presented as means ± SEM.

* Significantly different from untreated values (P < 0.007)
The density distribution of RBCs before and after treatment with L-lactate *in vitro*.

The presentation of data are identical to those in Figure 4. The experimental procedures are described in the legend to Figure 5 and in the text.
DISCUSSION

We have shown that the uptake by RBCs of lactate released into the circulation during exercise at maximal effort was accompanied by a substantial increase in MCV and a corresponding decrease in cell density. These changes were mediated by RBC uptake of the lactate anion and not by decreased pH. In fact, the intracellular concentration of H\(^+\) ions increases by 8% only after maximal exercise (Harris and Dudley, 1989). These exercise-induced responses were associated with a substantial increase in the susceptibility of the cells to haemolysis under the challenge of hypotonic osmotic stress in vitro. The functional consequences of these changes are not known, but high concentrations of blood lactate are known to impair the deformability of RBCs at both low and high shear rates (Shand, 1986; Brun et al., 1991). The associated increase in MCV may amplify this effect and significantly impair the delivery of O\(_2\) to working muscle during strenuous exercise. This may, in turn, accelerate the onset of fatigue. These exercise-induced changes are transient because blood lactate concentration, MCV, MCHC and osmotic fragility all returned to their respective pre-exercise values one hour after exercise.

We found that, while a positive concentration gradient of lactate between plasma and RBCs was detectable before and after exercise, the magnitude of the gradient did not change significantly. Other groups have reported the same finding (Harris and Dudley, 1989; Juel et al., 1990), but Buono and Yeager (1986) detected the gradient only after exercise. The measured plasma to RBC lactate gradient is not due to errors introduced by the dilutional effect of water occupying the extracellular space in packed cells, because this has been calculated to be only 4% (ICSH, 1980). RBCs stored at 4°C for 42 days slowly accumulate lactate intracellularly to a concentration of 45 mM, which increases their MCV and osmotic fragility substantially; the same outcome can be replicated by treating fresh RBCs with a concentration of L-lactate sufficient to achieve an intracellular concentration of 45 mM in vitro (Beutler et al., 1982). The present results show that physiological concentrations of lactate generated transiently during maximal exercise can also cause increases of a similar nature in MCV and osmotic fragility, together with a greater tendency toward haemolysis. The difference in the magnitude of the plasma to RBC lactate gradients in vivo and in vitro is surprising considering that whole blood was used in both experiments. Lactate uptake by RBCs may be more efficient in vivo where the turbulence associated with blood flow causes more effective mixing of the cells with lactate-rich plasma and where
other tissue sinks contribute to the rapid removal of lactate and the stabilization of its plasma concentration.

There are several reasons why RBCs may take up or generate lactate during intensive exercise. RBCs under mechanical stress have an increased requirement for glucose, which may reflect accelerated glycolytic flux (Kodicek, 1986) to supply ATP to support ATPases involved in ionic transport and, perhaps, deformability processes as cells move through the microcirculation. The relative concentrations of exogenous lactate and pyruvate may influence the activity of met-haemoglobin reductase (Sullivan and Stern, 1983) by increasing the NADH/NAD⁺ ratio. This may lower the potential for cellular oxidative damage because haemoglobin is maintained in the ferrous (reduced) state.

The acidosis associated with high blood lactate concentration, which is indicated by a significant decrease in the plasma pH detected after exercise, triggers movement of H⁺ and Cl⁻ ions into RBCs (Van Beaumont, 1973). Lactate-H⁺ has been implicated previously in muscular fatigue, but it has been presumed that H⁺ ions rather than lactate anions are responsible (Sahlin, 1992). Our results show that lactate uptake and not pH may be the principal factor involved in the increases in MCV, osmotic fragility and the percentage of low-density RBCs found immediately after maximal exercise. Related increases in osmotic fragility and decreases in density of a significant subpopulation of RBCs may occur primarily as a result of the elevated MCV caused by lactate uptake. Furthermore, swelling of normal RBC in the absence of lactate does not occur unless the pH falls below 7.2 (Van Beaumont et al., 1981). Thus, the lactate anion may also be involved indirectly in muscular fatigue through its morphological effects on RBCs. Maintenance of intra-erythrocyte pH within narrow limits is crucial because it may optimise glycolytic flux through sensitive regulation of phosphofructokinase activity and may prevent the dissociation of pH-sensitive contractile and transport proteins. Swelling of normal RBCs can be induced if the pH falls below 7.2 because this decreases the net negative charges contributed by haemoglobin and 2,3-diphosphoglycerate and results in a compensatory uptake of Cl⁻ ions and water (Brugnara et al., 1989). A compensatory osmotic response to acidosis may explain why, in some published studies, progressive exercise to exhaustion did not alter MCHC or MCV to the same degree as that caused by maximal exercise in this study (see Tables 1 & 2), despite a 7% increase in intraerythrocyte and plasma osmolality (Buono and Faucher, 1985; Van Beaumont and Rochelle, 1974). Although the blood lactate concentration was not measured by these groups, a
threshold rate of lactate uptake by RBCs may have to be exceeded before MCV and MCHC values change significantly.

Acidosis has also been postulated to be involved in the sudden death of some people with sickle cell trait during vigorous exercise (Davis, 1988). Sickle cells patients (HbAS) have an impaired ability, compared with normal people (HbAA), to clear circulating lactate (Freund et al., 1992). The excessive uptake of lactate by HbAS RBCs may trigger the sickling process (independently of decreased pH) and this may, in turn, lead to hypoxia through microvascular obstruction. This mechanism may contribute to the unexplained episodes of sudden death reported in some people with sickle cell trait in response to vigorous exercise (Davis, 1988).

**Conclusion**

In summary, our results show that exercise at maximal effort, which is associated with a high rate of lactate release into the circulation, causes a substantial increase in the percentage of low-density RBCs and an increased susceptibility of the cells to osmotic haemolysis. These exercise-induced responses appear to be due to increased MCV caused by lactate uptake independently of decreased blood pH. Collectively they may have detrimental effects on cell deformability which may, in turn, lead to hypoxia and muscular fatigue in normal people and, possibly, to life-threatening episodes in people with sickle cell trait. Furthermore, this work indicates that the accumulating lactate anion may contribute to fatigue and exhaustion during vigorous exercise.
ACKNOWLEDGMENTS

The authors thank Bon Gray for providing the lactate data for the one min maximal exercise test. We also thank Dr Alan Hahn, David Pyne and Don Campbell (Australian Institute of Sport) for assistance with some parts of the study. We are grateful to the subjects who participated in this study. Financial support was provided by the Faculties Research Fund of the Australian National University. JAS was the recipient of a Australian Postgraduate Research Award.
REFERENCES


CHAPTER 8

GENERAL DISCUSSION
8.1 Introduction

As stated in Chapter 1, the aims of this project were to assess: (i) whether single episodes of submaximal exercise increased the susceptibility of isolated RBCs to oxidative and osmotic stress in vitro, and whether these responses were influenced by regular endurance training and/or antioxidant supplementation; and (ii) whether lactic acid release into the circulation during maximal exercise affected various RBC properties, including their susceptibility to haemolysis by osmotic stress.

8.2 Overview of results

During exercise, red blood cells (RBC) are subjected to a differential range of osmotic and oxidative stresses which may increase the rate of cellular senescence. Oxygen uptake (and potentially the generation of reactive oxygen species) increases at least 10-fold above resting levels during exercise, and considerable osmotic changes may also occur (see Chapter 1).

The exercise-induced changes in RBC density and related osmotic responses are clearly intensity-dependent. Exercise sustained for a brief period at maximum effort - through the large increase in the blood concentration of lactate - induced a significant increase in mean cell volume and a two- to three-fold increase in the percentage of low-density cells. These responses may have increased the susceptibility of the cells to osmotic haemolysis in vitro. The physiological consequences of these changes are that the ability of the cells to pass through the microcirculation may be impaired (Shand, 1986; Brun et al., 1991). In contrast, submaximal (alactic) exercise induced a significant increase in the percentage of high-density cells and an associated decrease in osmotic fragility, presumably because of loss of intracellular water which increases mean cell haemoglobin concentration (MCHC) and may, in turn, decrease RBC filterability. Thus, changes in MCHC in either direction may impair oxygen transport to working muscle during exercise. Submaximal exercise also imposes considerable oxidative stress on the erythrocyte because these cells are more vulnerable to peroxidation when exposed to various free radical generating systems in vitro post exercise. However, supplementation of the diet with vitamins C & E attenuates this to some extent. Thus, depletion of endogenous antioxidant protection and/or uncoupling of antioxidant interactions are the most likely explanation for the increase in RBC susceptibility to oxidative stress in vitro because the very small amount of haem iron associated with the membrane - which might have had the potential to "short-circuit" α-tocopherol protection - did not change
significantly after exercise. Because the membrane concentration of α-tocopherol and plasma ascorbate did not change after exercise, loss of intracellular glutathione (GSH) due to a fall in the GSH/GSSG ratio is the most likely explanation (Chapter 6.2). It is also possible that exercise may uncouple the co-operative interactions between membrane and cytosolic antioxidants. This may explain the beneficial effects of vitamin-C and -E supplementation on the exercise-induced responses of RBCs to oxidative and osmotic stress despite the lack of measurable changes in the concentration of RBC α-tocopherol and plasma ascorbate.

The cumulative effects of oxidative damage may contribute, along with mechanical and osmotic stress, to an increased rate of RBC destruction during intensive training periods when individuals undertake repeated sessions of high-intensity and/or endurance exercise each day. The training-associated emergence of a younger RBC population, which may increase endurance capacity, could explain why RBCs from trained subjects were more resistant to peroxidative, but not osmotic, stress compared to cells isolated from their untrained colleagues. The acute responses to submaximal and maximal exercise and the chronic effects of training will now be discussed separately.

8.2.1 Submaximal exercise
The submaximal exercise experiments show clearly that the percentage of high-density cells increases significantly during exercise, which correlates with increased MCHC and decreased osmotic fragility; the RBCs also become more vulnerable to oxidative stress. Furthermore, the vitamin-C and -E supplementation experiments demonstrate conclusively that the increases percentage of high-density RBCs and the vulnerability of the RBC population as a whole to peroxidation in vitro are related. There was no evidence that the met-haemoglobin concentration increased either acutely or chronically, or that iron originating from haemoglobin was incorporated into the membrane after exercise. However, my results do not rule out the possibility that the increase in O₂ flux during, and for some hours after, exercise may have caused reversible haemoglobin auto-oxidation. For example, met-haemoglobin produced during high O₂ flux may have been reduced to haemoglobin at a similar rate by met-haemoglobin reductase, but superoxide release concomitantly during exercise may have initiated some free radical reactions that led to an increase in the percentage of high-density cells, perhaps through the loss of damaged membrane and/or protein crosslinking. This may have increased the vulnerability of the cells to oxidative stress in vitro immediately after exercise.
In addition to the acute effects of submaximal exercise, I have shown that RBCs isolated from trained subjects are more resistant to peroxidative stress by $\text{H}_2\text{O}_2$, but not by organic hydroperoxides, both before and after exercise. The reason for this discrepancy is not clear but it may involve the different solubilities of these compounds, the nature of the secondary radicals generated, and their different molecular targets in the cell. Furthermore, exercise-induced changes in RBC antioxidant capacity and their susceptibility to oxidative stress may be transient and show considerable intra-individual variability in their kinetics. Thus, these parameters need to be monitored several times daily in both exercised and non-exercised subjects.

8.2.2 Oxidative challenge assays
In this project, I have used oxidative challenge assays because exercise-induced changes in the major antioxidants in RBCs have been reported by several groups (see Chapter 5) and because examination of individual antioxidants may produce equivocal results because of the considerable redundancy involved in antioxidant protection. For example, a decrease in the activity of one antioxidant in response to exercise may be compensated by an increase in another. Therefore, the antioxidant network needs to be examined in its entirety. The luminol-amplified chemiluminescence (LCL) assay was developed for this purpose (Chapter 6.1). While our vitamin-E and -C enrichment experiments in *vitro* showed that the induction (lag) time of LCL increased and peak-LCL decreased compared to untreated cells, the assay was unable to detect differences in these parameters in RBCs isolated from the placebo or vitamin-E and -C supplemented groups, despite the two- to three-fold increase in RBC membrane $\alpha$-tocopherol concentration (Chapter 6.3). There are several possible explanations for these discrepancies. While control cells and those enriched with vitamin-E *in vitro* were always prepared and assayed on the same day, there was a six week gap between commencing and completing the supplementation program which gave rise to the control and vitamin-C and -E enriched cells *in vivo*. The other possibility is that haemoglobin present in intact cells quenches a substantial proportion of the light generated by luminol excitation. MCHC may have increased over the supplementation period. Further work is needed to optimize this method and to counter haemoglobin quenching.

The clearest differences between vitamin-E-enriched RBCs and untreated cells *in vitro* was obtained with haemoglobin-depleted RBC membranes suspended in a medium containing a small ($\mu$g) concentration
of haemoglobin sufficient to initiate homolytic scission of the organic hydroperoxide (Chapter 6.1). These assays were not carried out in the vitamin supplementation experiments. Use of a non-haem source of iron (or some other catalytically-active transition metal) may overcome quenching so that all luminol-reactive oxidants are detected and quenching is confined to antioxidant activity. Alternatively, other chemiluminogenic probes that emit light at wavelengths outside the haemoglobin-absorption range may be used. Finally, the cellular targets of oxidative attack during exercise should be examined. This could be assessed by measuring markers of lipid peroxidation (e.g. malonyldialdehyde, conjugated dienes) and protein damage (thiol groups, protein carbonyls). The oxidative stress assays used in this project do not enable any discrimination to be made between lipid and protein damage because free radicals generated from H$_2$O$_2$ and organic hydroperoxides attack both of these species.

8.2.3 Maximal exercise
While submaximal (alactic) exercise caused a small increase in the percentage of high-density RBCs and reduced RBC osmotic fragility, the opposite responses occurred in response to maximal exercise. The large increase in the circulating concentration of lactate was possibly responsible for the two- to three-fold increase in the percentage of low-density RBCs and the large increase in osmotic fragility detected in the cell suspension immediately after exercise at maximum effort (Chapter 7.1). There are several reasons why RBCs may take up and/or generate lactate during intensive exercise. RBCs under mechanical stress have an increased requirement for glucose, which may increase RBC lactate through accelerated glycolysis (Kodickek, 1986) to supply ATP for contractile processes and to support ATPases involved in ionic transport. Lactate uptake by RBCs during strenuous exercise may prevent oxidative damage because the relative concentrations of intracellular exogenous lactate and pyruvate may influence the activity of met-haemoglobin reductase (Sullivan and Stern, 1983), perhaps through increasing the NADH/NAD$^+$ ratio. We did not determine whether maximal exercise caused oxidative damage to RBCs because the duration of the workloads was fairly short (~ 10 mins). However oxidative damage may occur in the early post-exercise period when reactive oxygen species are generated during reperfusion of hypoxic tissues - the so-called ischaemia/reperfusion phenomenon (Witt et al., 1992). This may explain why, although GSH levels in the blood do not fall during progressive exercise to exhaustion despite a two-fold increase in
GSSG, a two-to three fold decrease in GSH occurred during the one hour recovery period (Sastre et al., 1992).

8.3 Physiological implications and future studies

The results reported in the experimental manuscripts are consistent with the hypothesis that the oxidative and osmotic stress that occurs during exercise may contribute to almost a doubling of the rate of RBC turnover in some athletes (Weight et al., 1991). However, providing the rate of RBC destruction is in equilibrium with that of replacement, no detrimental effects on athletic performance should occur. It is obvious from my work that more sensitive assays need to be developed to detect oxidative and osmotic damage which may occur in only a small subpopulation of vulnerable cells. All of the assays used in this work measured the aggregate response of a fixed and constant number of cells. However, as reported in Chapter 2.2, flow cytometric assays that examine cells individually offer considerable advantages over batch assays. There have been very few studies that have utilised this technique to study erythrocyte function (Rolfes-Curl et al., 1991). While RBC size and density are distributed over a wide range, these parameters are unlikely to be sufficiently sensitive to separate young and old cells unequivocally and, furthermore, there is considerable debate as to whether these parameters change irreversibly with advancing age of the cells (Clark, 1988; Corbett and Golan, 1993). Studies on the effect of long-term endurance training on RBC turnover and ageing are likely to remain equivocal until a cellular marker that changes progressively and irreversibly with advancing age is identified. Recent reports suggest that aggregated glycoporphin and band III are likely candidates for "footprints" or markers of senescence (Rolfes-Curl et al., 1991; Corbett and Golan, 1993). The distribution patterns of cell populations isolated from trained and untrained subjects could be compared. The "abnormal" cells could then be sorted and isolated for further studies.

Some hormones such as catecholamines released during exercise may influence the osmotic properties of RBCs by modulating cation transport (Staubli and Roessler, 1986; Bodemann et al., 1987). Furthermore, atrial natriuretic peptide, which is released into the circulation during vigorous exercise (Follenius and Brandenberger, 1988), has been reported to increase RBC filterability (Zamir et al., 1992), which may translate into decreased transit times through the microcirculation. To my knowledge, the effect of neuroendocrine hormones on lactate uptake and metabolism in RBCs has not been investigated but warrants attention in light of the intensity-dependent effects of exercise on various RBC properties.
Future projects should include a longitudinal study of the various "footprints" of RBC osmotic and oxidative damage, documenting how these parameters change during the different phases of a training program. An age- and sex-matched non-exercising control group should be included to rule out variability in these parameters. The differences between low-impact sports such as swimming and cycling and high-impact activities such as running, and the possible variability in RBC turnover between endurance and power sports, should be investigated to determine whether RBC turnover is amplified by mechanical stress. These studies could involve the radio-labelling of isolated RBCs and re-injection of the labelled cells into the original donor (Weight et al., 1991). This type of experiment could also be conducted on antioxidant-supplemented subjects and a matched placebo group. Age is another variable worthy of investigation because the rate of RBC turnover increases substantially in the elderly (Kosower, 1993).

8.4 Conclusions
The results presented in this part of the thesis show clearly that exercise has intensity-dependent effects on the osmotic properties of RBCs. While submaximal exercise increases MCHC and the percentage of high density cells in the circulation by, presumably, cellular dehydration, maximal exercise causes exactly the opposite changes in these cellular parameters which are mediated through the uptake of lactate anions and not H⁺ ions by RBCs. Furthermore, submaximal exercise for 45 to 60 min imposes considerable oxidative stress on RBCs which can be virtually abolished by supplementation of a normal diet with ascorbate and α-tocopherol. At present the physiological implications of these results are not known but they provide further evidence that oxidative and osmotic stress to RBCs during exercise may accelerate the onset of fatigue in the short-term, while the cumulative effects of osmotic and oxidative "wear-and-tear" on RBCs during athletic training may be a substantial contributor in the long-term to a rapid rate of RBC turnover.
REFERENCES


