USE OF THESES

This copy is supplied for purposes of private study and research only. Passages from the thesis may not be copied or closely paraphrased without the written consent of the author.
THE EFFECT OF INTENSE INTERVAL EXERCISE ON SELECTED IMMUNOLOGICAL, HAEMATOLOGICAL AND ENDOCRINOLOGICAL PARAMETERS IN TRAINED MALE SUBJECTS.

ATHOL BON GRAY

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

APRIL 1993
STATEMENT:

The experiments associated with production of this thesis were planned, organised and conducted by myself except as indicated below:

1. Catecholamine assays were performed on a commercial basis by the Hormone Assay Service, Garvan Institute of Medical Research, Sydney.

2. Serum iron, transferrin and CRP analyses were performed on a commercial basis by staff of the Biochemistry Department, Woden Valley Hospital, Canberra.

3. Lactoferrin analyses were performed by Ms. Dianna Taylor, Auspharm Institute of Mucosal Immunology, Newcastle.

4. Gel electrophoresis and immunoblotting of neutrophil preparations was conducted in collaboration with Ms. June Hornby and Dr. Gary Buffinton, John Curtin School of Medical Research, Australian National University, Canberra.

5. David B. Pyne and John A. Smith (fellow postgraduate students) occasionally provided technical assistance with certain laboratory procedures, including sample collection, centrifugation, immunological assays and supervision of subjects. This research project was however, greatly facilitated by the extensive discussions conducted with these fellow students.

The paper "Anaerobic exercise causes transient changes in leukocyte subsets and IL-2R expression", (Med.Sci.Sports Exerc. 24:1332-1338, 1992) is presented in the Appendix. The paper was derived from research completed during my enrolment in the Bachelor of Science (Honours) program, and is provided to establish a context for the present research.

Signed:..............

A.B. Gray.

11th April, 1993.
"The known is finite, the unknown infinite; intellectually we stand on an inlet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land."

T.H. Huxley, 1887.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>i</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1-1</td>
</tr>
<tr>
<td>1. Rationale</td>
<td>1-2</td>
</tr>
<tr>
<td>2. Structure of this Investigation</td>
<td>1-5</td>
</tr>
<tr>
<td>3. Types of Exercise</td>
<td>1-7</td>
</tr>
<tr>
<td>4. Energy Systems</td>
<td>1-9</td>
</tr>
<tr>
<td>4.1 Alactic or ATP-PC System</td>
<td>1-9</td>
</tr>
<tr>
<td>4.2 Lactic Acid-Generating System</td>
<td>1-10</td>
</tr>
<tr>
<td>4.3 The Aerobic Energy-Generating System</td>
<td>1-10</td>
</tr>
<tr>
<td>4.4 Relationship of the Three Energy Systems to the Intensity of exercise.</td>
<td>1-11</td>
</tr>
<tr>
<td>5. Interval Exercise</td>
<td>1-12</td>
</tr>
<tr>
<td>6. The Human Immune System</td>
<td>1-15</td>
</tr>
<tr>
<td>7. General Immune Response</td>
<td>1-18</td>
</tr>
<tr>
<td>8. Exercise and the Immune System</td>
<td>1-23</td>
</tr>
<tr>
<td>8.1 Incidence of Infection and Disease</td>
<td>1-23</td>
</tr>
<tr>
<td>8.2 Acute Effects on Leucocytes</td>
<td>1-26</td>
</tr>
<tr>
<td>8.3 Exercise-Induced Leucocytosis: mechanisms</td>
<td>1-29</td>
</tr>
<tr>
<td>9. Granulocytes</td>
<td>1-31</td>
</tr>
<tr>
<td>9.1 Cytoplasmic Granules</td>
<td>1-32</td>
</tr>
<tr>
<td>9.2 Neutrophil Activation: Signal Transduction and NADPH-Oxidase Activation.</td>
<td>1-35</td>
</tr>
<tr>
<td>9.3 Components of the NADPH-Oxidase</td>
<td>1-38</td>
</tr>
<tr>
<td>9.4 Reactive Oxygen Species</td>
<td>1-43</td>
</tr>
<tr>
<td>9.5 Activation States</td>
<td>1-44</td>
</tr>
</tbody>
</table>
9.6 Methods for Assessing the Activation State of the NADPH-Oxidase.

9.6.1 Measurement of ROS production.

9.6.1.1 Chemiluminescence

9.6.1.2 Reduction of Ferricytochrome C

9.6.1.3 Dichlorofluorescin diacetate (DCFH-DA)

9.6.1.4 Dihydrorhodamine (DHR)

9.6.2 The Present Investigation: p47-phox Translocation.

9.7 The Potential for Neutrophil-Dependent Tissue Damage

9.8 Expression of Receptors on Neutrophils

9.9 Extravasation and Interaction with the Endothelium

9.10 Exercise and Neutrophil Function

10. The Complement System

11. Cytokines

12. The Acute Phase Response

13. Iron Status

14. Exercise and the Endocrine System

14.1 Cortisol

14.2 Sex Hormones

14.3 Catecholamines

14.4 Growth Hormone and Prolactin.

15. The Endocrine System and Immune Function

15.1 Catecholamines

15.2 Cortisol (and Other Glucocorticoids)

15.3 Sex Hormones

15.4 Growth Hormone and Prolactin
16. Tissue Damage and Exercise 1-93

17. Summary 1-94

18. References 1-96

19. Papers:

19.1

Gray A.B., Telford R.D., Weidemann M.J. 2-1

Gray A.B., Telford R.D., Weidemann M.J. The 3-1

Gray A.B., Telford R.D., Weidemann M.J. 4-1

Gray A.B., Telford R.D., Collins M., Weidemann M.J. 5-1

Gray A.B., Telford R.D., Collins M., Baker M.S., Weidemann M.J. 6-1

Activation of neutrophils induced by intense interval running: further characterisation. J.Leukoc.Biol. (Submitted).

20. Discussion: 9-1

Appendix: 10-1

Paper written and published whilst enrolled for the PhD:

ACKNOWLEDGEMENTS:

This thesis is the product of three years of research carried out at the Australian Institute of Sport and the Australian National University. However in more personal terms it reflects a life-time of support and encouragement from my family, friends and associates:

I thank my family; my wife Deanie, my mother Thelma Gray, my late father Athol Mackay Gray, my sisters Elaine Barker and Noelene Tietze and their families, for their constant interest and support throughout my studies over the years.

Over the past 5 years I have had the great pleasure of working with Dr Dick Telford, of the Dept. Physiology & Applied Nutrition, Australian Institute of Sport (Canberra). He was instrumental in allowing me to complete my Honours degree at the Australian Institute of Sport (AIS) during 1988. In relation to the PhD he has been a great facilitator of the research, always being available for discussions, has read and reviewed my "draft" papers quickly and applied his sound knowledge of sports science to this task. The PhD has meant much more to me than a mere qualification, and I greatly appreciate the opportunity provided by Dr. Telford to advance my career, and to also develop my skills and confidence across a broad range of areas.

I thank Dr M.J. Weidemann for acting as Chair of the supervisory panel and for his support during both my Honours year and PhD enrolment. His meticulous reading of my manuscripts and enthusiasm for research into the effects of exercise on the immune system has been very much appreciated.

I thank Dr Mark Baker, formerly of the John Curtin School of Medical Research (JCSMR) and now of the University of Wollongong, for his advice, support and encouragement throughout the PhD.

I also thank Mr Geoff Osborne (FACS Unit, JCSMR), Dr Gary Buffinton and Ms June Hornby (Division of Clinical Sciences, JCSMR), Mr Don Campbell (Biochemistry, AIS) and staff of both the Division of Biochemistry and Molecular Biology (ANU) and the Department of Physiology and Applied Nutrition (AIS) for their support and encouragement throughout both my Honours and PhD programs.

In addition the following people made significant contributions to production of this thesis:
Prof W.M. Nauseef, University of Iowa for provision of the anti-p47-phox antibody, Dr Ken Ho, Garvan Institute of Medical Research, Sydney, for provision of
recombinant human growth hormone and Mr Peter Chisholm, Haem Pty Ltd, Melbourne, for provision of the anti-LECAM-1 monoclonal antibody.

I also acknowledge the financial support provided during the period 1991-3 by an Australian Government Post-Graduate Research Award and an ANUTECH supplementary scholarship.

Finally, over the past 4-5 years I have had the great fortune and pleasure to be able to work with "Doctors" David Pyne, John Smith and Ian Gillam. Their involvement in, and sharing of, the "PhD experience" was critical to production of this thesis. I hope that the friendship and mutual support amongst our research group will continue throughout our careers.
ABBREVIATIONS:

ACTH: adrenocorticotropic hormone

ADP: adenosine diphosphate

APP: acute phase protein

APR: acute phase response

ATP: adenosine triphosphate

B cell: bone-marrow derived lymphocyte (differentiates to an antibody producing plasma cell)

C3, C4, C5: complement components 3,4,5.

CD: cluster of differentiation

CGD: chronic granulomatous disease

CK: creatine kinase

CR1: complement receptor 1

CR3: complement receptor 3

CRP: C-reactive protein

DA: dopamine

DCFH-DA: dichlorofluorescin diacetate

DHPG: dihydroxyphenolglycol

DHR: dihydrorhodamine

EDTA: ethylenediaminetetraacetic acid

Ep: epinephrine

FC: flow cytometry

FcγR: Fc receptor γ (receptor for Fc portion of Ig G)

fMLP: N-formyl-methionyl-leucyl-phenylalanine

GH: growth hormone

GM-CSF: granulocyte macrophage colony-stimulating factor

G protein: guanine nucleotide-binding protein

H₂O₂: hydrogen peroxide
ABBREVIATIONS (continued);
HOCl: hypochlorous acid
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IRT: interval running test
LAD: leucocyte adhesion deficiency
LDH: lactate dehydrogenase
LECAM: leucocyte-endothelial cell adhesion molecule
NADH: nicotinamide adenine dinucleotide (reduced form)
NADPH: nicotinamide adenine dinucleotide phosphate (reduced form)
NE: norepinephrine
NK: natural killer
OH⁻: hydroxyl ion
OZ: opsonized zymosan
O₂⁻: superoxide anion
p47-phox: 47kD protein of the NADPH phagocyte oxidase
phox: phagocyte oxidase
PKC: protein kinase C
PMA: phorbol myristate acetate
PRL: prolactin
R: respiratory exchange ratio.
ROS: reactive oxygen species
SOD: superoxide dismutase
T cell: thymus-derived lymphocyte
TNF: tumour necrosis factor

VO_2\text{ max.}: maximal oxygen uptake
ABSTRACT:

Exercise has an established ability to alter human physiological systems. However to this time much of the research in this area, and particularly that in relation to the human immune system, has focused on exercise that has required predominantly aerobic metabolism. Little research has been carried out into the physiological effects of intense interval exercise, commonly used as a training regime by athletes in sports such as track & field, ball sports and swimming. This study investigated the effects of intense interval running on selected physiological parameters, with special reference to the immune system and the function of neutrophils.

Trained male subjects performed intense interval running protocols both in the laboratory and on a 400 metre running track. The protocols required significant anaerobic energy production as indicated by the elevated post-exercise whole blood lactate concentrations of 7.6 mmol.L\(^{-1}\) and 14.0 to 16.0 mmol.L\(^{-1}\) relating to the laboratory and "track" running protocols, respectively.

Intense interval exercise induced a series of biochemical and morphological changes consistent with the multi-faceted activation of neutrophils. Such
activation in vivo may have important implications for both tissue damage induced during, and immunocompetence immediately after, intense interval exercise.

A single session of intense interval exercise produced significant perturbation of other physiological systems known to be capable of altering immune function. This was manifested as a significant elevation in the plasma concentrations of several hormones, minor fluctuations in plasma cytokines concentrations and iron status parameters as well as a characteristic pattern of leucocyte mobilisation.

After exercise neutrophils displayed phenotypic changes characteristic of activation in vivo. The plasma concentration of elastase (derived from the primary granules of neutrophils) was elevated significantly at 1 hr post-exercise (p<0.05), while the expression of complement receptor 3 (CR3;CD11b/CD18), a β2-integrin stored intracellularly in association with the secondary granules, was increased significantly after exercise (p<0.01). These changes imply that degranulation of neutrophils in vivo was induced by intense interval exercise. Neutrophil expression of LECAM-1, a member of the selectin "family" of cell adhesion molecules, was significantly decreased after exercise (p<0.01).
Neutrophils isolated after exercise showed a reduced ability to generate ROS when stimulated in vitro with phorbol myristate acetate (PMA) and opsonized zymosan (OZ), as assessed by both reduction of ferricytochrome c and by luminol-enhanced chemiluminescence, respectively (both p<0.05). A key component of the neutrophil's NADPH-oxidase system, p47-phox, translocated to the plasma membrane during exercise. The reduced ability of neutrophils isolated after intense interval exercise to generate ROS appears to constitute a post-exercise "refractory" period and be secondary to both assembly and activation of the NADPH-oxidase system during exercise. Increased plasma concentrations of immunosuppressive hormones cortisol and epinephrine during and after intense interval exercise may accentuate the post-exercise refractory period induced after this form of exercise.

Our findings indicate that intense interval exercise induced a multi-faceted activation of neutrophils in vivo and imply that both the increased susceptibility of athletes to infection during and after periods of intense competition and training, and tissue damage induced by exercise may be due to activation of neutrophils in vivo during exercise.
INTRODUCTION
1. **RATIONALE:**

Athletes typically employ training programs that involve intense aerobic and anaerobic exercise, often undertaken twice daily. Each athlete must attempt to optimise his/her training and competition by balancing the volume and intensity of physical activity against the ability to adapt to the training load. Unfortunately many athletes are unsuccessful in achieving this balance, succumbing to injury or infection, particularly of the upper respiratory tract (Heath *et al*., 1991; Heath *et al*., 1992; Douglas & Hanson, 1978; Peters & Bateman, 1983).

The majority of the research on the effects of exercise on human physiological systems has focused on exercise protocols that are predominantly aerobic in nature (Table 1.1). This is despite the fact that many training regimes involve a significant anaerobic component that reflects the specific metabolic requirements of the activities being undertaken. In particular, intense interval exercise that involves a significant anaerobic energy contribution, is now a very common training regime (Telford, 1991). This form of exercise is used by athletes in a range of sports including swimming, track & field events and football. Despite the widespread use of this form of training, research into its effects on most physiological systems
is very limited. This situation is clearly unsatisfactory. One cannot assume that the physiological responses induced by various forms of predominantly aerobic exercise are similar to those produced by intense interval exercise. Such exercise is associated with a significant reduction in pH in both muscle tissue and, subsequently the peripheral circulation. This transient chemical disequilibrium distinguishes intense interval exercise from that which employs predominantly the tricarboxylic acid cycle (TCA) and oxidative phosphorylation (ie: aerobic pathways), for generation of adenosine triphosphate (ATP; Fox, 1984).

A lack of knowledge of the specific effects of this form of exercise means that the progress of athletes, undertaking a significant anaerobic training load, is not able to be monitored successfully. In addition, the utility of exercise as a variable parameter with the capacity to perturb physiological systems in a reproducible and quantifiable manner, thus helping to elucidate links between these systems, is compromised if a full range of exercise protocols is not employed.

Human immune function, and specifically the activity of one of the major leucocyte subpopulations, ie: the neutrophils, is regulated by a complex series of interactions involving other leucocyte subsets,
hormones, cytokines, iron and iron-binding proteins. Neutrophils, regarded as a "first line of defence" against infection, participate actively in the recognition and disposal of pathogenic agents. Neutrophil activity is critical to the maintenance of an infection-free status as evidenced by the recurrent infections suffered by patients with compromised neutrophil effector functions as a consequence of inherited chronic granulomatous disease (CGD) or Chediak-Higashi syndrome (Smith & Curnutte, 1991; Ganz et al., 1987; Yang & Hill, 1991).

This investigation will seek to establish the effects of a commonly-employed training regime ie: interval exercise, on the human immune system and related physiological systems. The main focus will be on the immune system. However, both the elucidation of the means by which exercise is able to modulate immune cell activity and distribution, and an appreciation of the possible consequences of such modulation, must begin with an investigation of those systems which interact directly with the immune system. The interrelationship of neutrophils, iron status parameters, hormones, cytokines and various leucocyte subsets will be examined in an attempt to uncover both afferent and efferent pathways associated with modulation of neutrophil activity.
2. STRUCTURE OF THIS INVESTIGATION:

The project will, in its initial stages, profile the physiological changes produced by a "model", laboratory-based protocol, that simulates a relatively intense, interval training session, demanding significant contribution from anaerobic glycolysis for production of ATP. The latter stages will attempt to extend this initial characterisation and to elucidate, where possible, the mechanisms underlying the physiological changes induced by this form of exercise. The final phase will move the investigation out of the laboratory and onto the training track by employing an interval running protocol commonly used by track and field athletes. It will help to establish the changes induced in human neutrophils by a "real" training session.

The first papers comprising this thesis will be concerned with the effects of the exercise protocol employed (see later) on the distribution of circulating leucocyte subsets, the plasma concentrations of hormones and cytokines and indicators of iron status, i.e.: those parameters that may modulate immune cell function. Later papers will deal with functional aspects of neutrophils, with particular emphasis on the putative activating effects of exercise. An attempt will be made to integrate the findings, in order to reach conclusions concerning the possible mechanisms
FIGURE 1: Factors possibly mediating the effects of interval exercise on neutrophil function.
associated with the exercise-induced activation of neutrophils (Figure 1).

3. TYPES OF EXERCISE:
Despite the large range of exercise protocols that have been employed to investigate the effects of exercise on the immune, haematological and endocrine systems, very few relate in any direct way to either the training or competition of athletes involved in events requiring a significant anaerobic energy contribution. The protocols that have been of some relevance to the training of athletes have been predominantly aerobic in nature. Thus the influence of one of the most commonly employed training systems, namely intense interval exercise, has not been investigated. The results of this investigation should contribute to the literature on human exercise biochemistry, physiology and immunology. The findings may have significant implications for athletes and exponents of interval training as well as for community health issues.

The following table (Table 1.1) provides a summary of some (but by no means all) of the studies that have investigated the effects of exercise on the human immune system. Most studies have involved predominantly aerobic metabolism.
<table>
<thead>
<tr>
<th>AUTHOR(S)</th>
<th>EXERCISE PROTOCOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berk et al. (1987)</td>
<td>Balke protocol (run) to exhaustion.</td>
</tr>
<tr>
<td>Berk et al. (1990)</td>
<td>Running; 3 hr.</td>
</tr>
<tr>
<td>Bieger et al. (1980)</td>
<td>Progressive running to exhaustion.</td>
</tr>
<tr>
<td>Bosenberg et al. (1988)</td>
<td>Triathlon (3.2,140,42.2)</td>
</tr>
<tr>
<td>Brahmi et al. (1985)</td>
<td>Progressive cycling to exhaustion.</td>
</tr>
<tr>
<td>Cannon &amp; Kluger (1983)</td>
<td>1 hr, 34-63% VO2 max cycling.</td>
</tr>
<tr>
<td>Cannon et al. (1986)</td>
<td>1 hr, 60% VO2 max cycling.</td>
</tr>
<tr>
<td>Deuster et al. (1988)</td>
<td>Bruce protocol (run) to exhaustion.</td>
</tr>
<tr>
<td>Dufaux &amp; Order (1989a)</td>
<td>2.5 hr running.</td>
</tr>
<tr>
<td>Edwards et al. (1984)</td>
<td>Running; 5 min up/down stairs.</td>
</tr>
<tr>
<td>Eskola et al. (1978)</td>
<td>Running; 35 mins, 2.5 hr.</td>
</tr>
<tr>
<td>Espersen et al. (1990)</td>
<td>5 km run.</td>
</tr>
<tr>
<td>Evans et al. (1986)</td>
<td>45 mins, 250W, cycling</td>
</tr>
<tr>
<td>Ferry et al. (1990)</td>
<td>Running; intermittent (4 min/1 min rest), progressive to exhaustion.</td>
</tr>
<tr>
<td>Field et al. (1991)</td>
<td>80% max. work-load to exhaustion.</td>
</tr>
<tr>
<td>Fry et al. (1992)</td>
<td>25 x 1 minute runs (100% VO2 max.)</td>
</tr>
<tr>
<td>Gabriel et al. (1991)</td>
<td>110% anaerobic threshold, cycling.</td>
</tr>
<tr>
<td>Galun et al. (1987)</td>
<td>40,70,120 km marching.</td>
</tr>
<tr>
<td>Gimenez et al. (1986)</td>
<td>Cycling; 1 min max. every 5 mins.</td>
</tr>
<tr>
<td>Haahr et al. (1991)</td>
<td>1 hr, 75% VO2 max cycling.</td>
</tr>
<tr>
<td>Hansen et al. (1991)</td>
<td>1.7,4.8,10.5 km runs.</td>
</tr>
<tr>
<td>Hanson &amp; Flaherty (1981)</td>
<td>Running; 12.8 km, 75% VO2 max.</td>
</tr>
<tr>
<td>Hedfors et al. (1976)</td>
<td>15 min cycling, heart rate = 150bpm.</td>
</tr>
<tr>
<td>Hedfors et al. (1983)</td>
<td>15 min cycling, heart rate = 150bpm.</td>
</tr>
<tr>
<td>Kendall et al. (1990)</td>
<td>Cycling; 30,65,75% VO2 max.</td>
</tr>
<tr>
<td>Kvernmo et al. (1992)</td>
<td>30km cross-country skiing.</td>
</tr>
<tr>
<td>Landmann et al. (1984)</td>
<td>25,50,75% max.work capacity (5 min,cycling).</td>
</tr>
<tr>
<td>Lewicki et al. (1988)</td>
<td>Progressive cycling to exhaustion.</td>
</tr>
<tr>
<td>Lovlin et al. (1987)</td>
<td>Cycling; 40,70,100% VO2 max.</td>
</tr>
<tr>
<td>Mackinnon et al. (1988)</td>
<td>2 hr, 90% anaerobic threshold.</td>
</tr>
<tr>
<td>MacNeil et al. (1991)</td>
<td>Cycling; 30,65,75% VO2 max.</td>
</tr>
<tr>
<td>Nieman et al. (1989)</td>
<td>Running; 3hr.</td>
</tr>
<tr>
<td>Oshida et al. (1988)</td>
<td>2 hr, 60% VO2 max cycling.</td>
</tr>
<tr>
<td>Osterud et al. (1989)</td>
<td>50 km cross-country skiing.</td>
</tr>
<tr>
<td>Robertson et al. (1981)</td>
<td>Cycling; 2-18 km to exhaustion.</td>
</tr>
<tr>
<td>Smith, Telford et al. (1990)</td>
<td>1 hr, 60% VO2 max.</td>
</tr>
<tr>
<td>Sprenger et al. (1992)</td>
<td>20 km (running) within 2 hr.</td>
</tr>
<tr>
<td>Targan et al. (1981)</td>
<td>5min cycling, 25mph.</td>
</tr>
<tr>
<td>Tomasi et al. (1982)</td>
<td>20,50 km cross-country skiing.</td>
</tr>
<tr>
<td>Verde et al. (1992)</td>
<td>30 minutes treadmill running.</td>
</tr>
<tr>
<td>Viti et al. (1985)</td>
<td>1 hr, 70% VO2 max running.</td>
</tr>
</tbody>
</table>

**TABLE 1.1:** Summary of some of the previous investigations in the area of exercise immunology.
The paper by Fry et al. (1992), published after most of the experimental work for this investigation had been completed, represents one of the very few studies in which the effects of intense interval exercise have been examined, especially as it relates to possible modulation of the immune system.

4. ENERGY SYSTEMS:
Energy for physiological activity is derived from a series of metabolic pathways that function to continuously regenerate the high energy phosphate compound ATP from adenosine diphosphate (ADP). ATP is the primary compound responsible for facilitating metabolic reactions, so that pathways can proceed exothermically. Human physical activity makes use of three linked metabolic pathways or "energy systems" for the production of ATP. These are the aerobic ("with oxygen") energy system, and the anaerobic ("without oxygen") energy system, composed of "alactic" and "lactic" pathways (Fox, 1984).

4.1 ALACTIC OR ATP-PC SYSTEM: Formation of ATP by this system is dependent on the availability of a limited pool of a high energy phosphate compound, phosphocreatine (PC), that can be used to regenerate ATP from its breakdown products ADP and inorganic phosphate (Pi). ATP and PC stored in muscle tissue, provide a readily available, but limited supply of
energy for short term, high intensity physical activity such as sprinting and weight lifting (de Vries, 1974).

4.2 LACTIC ACID GENERATING SYSTEM: This system derives its name from the end product of the glycolytic pathway, lactic acid. The process is also referred to as "anaerobic glycolysis", indicating that energy production that is dependent on the breakdown of glucose (or some equivalent carbohydrate), can occur under "oxygen-free" conditions. Sustained physical activity at high intensity results in the accumulation of lactic acid in both muscle tissue and the circulation. This build-up (experienced in events such as the 400m run and 100/200 metre swims), with its associated decrease in pH, acts both to produce the characteristic symptoms of "lactic" muscular fatigue (eg: "heavy" limbs), and to limit the rate of energy production, via a pH-dependent inhibition of the rate-limiting enzyme, phosphofructokinase. The intensity of exercise must necessarily decrease to allow restoration of a more favourable pH and removal of lactic acid. (Fox, 1984).

4.3 THE AEROBIC ENERGY-GENERATING SYSTEM: This system provides for the complete oxidation of various substrates: carbohydrates, proteins and fats via the sequential pathways of the TCA cycle and the mitochondrial respiratory (electron transport) chain.
The TCA cycle can utilise pyruvic acid formed by anaerobic glycolysis, and acetyl co-enzyme A produced aerobically from fats and proteins, to generate reducing equivalents (hydrogen ions and electrons). The electrons are subsequently transported to oxygen via the respiratory chain, and protons are pumped across the inner mitochondrial membrane to maintain a proton gradient that can be utilised to energise the synthesis of ATP (Fox, 1984). This "Chemiosmotic" theory of ATP synthesis has gained increasing acceptance, and is able to explain most of the phenomena associated with generation of ATP by oxidative phosphorylation (Lehninger, 1982). During this process of aerobic metabolism the majority of the ATP produced is formed by reactions that are coupled with the respiratory chain. Thus in physical activity, with an intensity below the threshold at which the oxygen supply becomes rate-limiting, ATP can be synthesised for prolonged periods provided there is an adequate supply of substrate (Fox, 1984).

4.4 RELATIONSHIP OF THE THREE ENERGY SYSTEMS TO THE INTENSITY OF EXERCISE: If exercise is of relatively low intensity the cardiovascular and respiratory systems are able to supply sufficient oxygen to fully oxidise the available substrates. ATP production is thus accomplished primarily by aerobic metabolism, power production being related linearly to oxygen uptake.
However after the intensity of exercise surpasses a critical power output an increasing proportion of metabolic energy is derived from anaerobic pathways. Finally a level of power output is reached whereby the total body oxygen uptake reaches a plateau (representing the maximal oxygen uptake; VO$_2$ max) so that further energy requirements beyond this power output must be met exclusively by anaerobic pathways. At the cessation of exercise the accumulated lactic acid (produced as a consequence of pyruvate, rather than oxygen, acting as an acceptor of the reducing equivalents produced during substrate oxidation) is removed by a combination of oxidation through aerobic pathways and conversion to liver glycogen, blood glucose or protein (Fox, 1984).

5. INTERVAL EXERCISE:
The term "interval exercise" can be applied to any form of intermittent exercise in which periods of physical activity are interspersed with periods or "intervals" of recovery. Interval exercise can take many forms, from very intense maximal efforts of short duration with long recovery periods (designed to train anaerobic energy pathways) to extensive efforts of longer duration and lower intensity (designed to train aerobic pathways), interspersed by relatively short recovery periods. Such exercise forms a continuum from predominantly anaerobic to predominantly aerobic energy
metabolism. It is the adaptability of such training to meet a variety of energy system requirements that has been one factor in its popularity. Another key factor in this regard is the opportunity it affords to perform repeated high intensity efforts that are often faster than normal "race-pace", without the fatigue that would result if the training session were to be attempted on a continuous, rather than an intermittent, basis (de Vries, 1974; Fox, 1984).

The investigation reported here has employed an intense interval running protocol that was modelled on those used routinely by track & field athletes. Two comparable forms of this protocol were employed. In the laboratory subjects were asked to exercise until voluntary exhaustion. This protocol required subjects to perform 1 minute periods of running at the level of VO\textsubscript{2} max (ie: speed and gradient at which VO\textsubscript{2} max was attained using a protocol employing 1 minute increments in speed or gradient) alternating with 1 minute active (walk) recovery periods. In the field (or on the track) subjects were asked to perform 10 x 400 metres runs with 200 metres jogging in between, on a 3 minute cycle. The latter protocol is more than a mere "model", as it is commonly employed by track & field athletes as an interval training session (Refer to TABLE 1.2).
TABLE 1.2: Extract from the training diary of a nationally ranked athlete (1500m Personal Best: 3min 39.5s).

Monday 20th December:

(am): 10km easy run.

(pm): 3 km warmup
      5 x 1000m/400m jog recovery
      2 km warm-down

Tuesday 21st December:

(am): 8km run

(pm): 3km warmup
      10 x 200m sprint (27-28 seconds)/200m jog recovery.
      2km warm-down

Wednesday 22nd December:

(am): 10km run

(pm): 3km warmup
      10 x 400m (60 seconds)/400m jog recovery.
      2km warm-down

Thursday 23rd December:

(am): 8km easy run

(pm): 3km warmup
      3 x 300m (40 seconds)/100m walk recovery.
      2km warm-down

Friday 24th December:

(am): no training

(pm): 3km warmup
      2000m time trial (5m25s)
      2km warm-down

Saturday 25th December:

10km easy

Sunday 26th December:

Race.
Exercise at the level of maximal oxygen uptake was chosen for three reasons. Firstly, it provides an appropriate "model" of training regimes employed by athletes (de Vries, 1974; Telford, 1991). Secondly, it provides an exercise "stress" standardised according to each individual's physical capacity. Thirdly, exercise at the designated intensity is known to be associated with a progressive accumulation of lactic acid, which differentiates this exercise protocol from other more aerobic protocols, that have been employed previously. Telford (1991), in a chapter summarizing various forms of training, has classified interval running sessions, in part, as "maximal lactic anaerobic", "lactic tolerance A", or "lactic tolerance B" depending on both the intensity of exercise (representing the following ranges of maximum speed: 95-98%, 95% and 90-95%, respectively), the rest period between runs, and the resulting blood lactate concentrations (8-16, 8-12 and 6-8 mmol.L\(^{-1}\), respectively). According to this classification scheme the "field" protocol employed in this study, 10 x 400 metres, on a 3 minute cycle, would be classified as either "lactic tolerance A or B".

6. THE HUMAN IMMUNE SYSTEM:

The human immune system is a multi-faceted, dispersed collection of organs and tissues with four main inter-related functions:
1. Detection and removal of foreign organisms or material.
2. Discrimination between "self" and "non-self".
3. Inflammation and tissue repair.
4. Communication with other physiological systems.

These functions are accomplished by the co-ordinated action of a diverse range of organs, cells and biochemical mediators. Organs include the primary immune system organs, the bone marrow and thymus, and those of a secondary nature including the spleen, lymph nodes and both mucosal and gut associated lymphoid tissue. Cellular components include those that are mobile (lymphocytes, polymorphonuclear leucocytes, and monocytes), and those that are tissue-localised including the Kupffer cells of the liver, dendritic cells, and resident tissue macrophages (Lydyard & Grossi, 1989).

The human immune system operates continuously, largely without our conscious knowledge, to recognise and selectively remove foreign agents from the circulation and body tissues. These agents, including potential pathogens such as viruses, bacteria, fungi and parasites, all of which find the human body a suitable location for growth and reproduction, can take a multitude of forms. The system has a remarkable ability to respond to a vast array of infective agents, many
not encountered previously, whilst allowing for the selective removal of foreign agents without injuring our own tissues (Katz, 1982).

The "surveillance" role of the immune system is well illustrated by the consequences of infection by the human immunodeficiency virus (HIV), manifested as a complex of symptoms collectively termed the "acquired immune deficiency syndrome" (AIDS). One, but by no means the only, consequence of HIV infection is a gradual, but apparently inevitable, deterioration in the regulation of the immune system, largely through elimination of the critical T helper (CD4+) subset of lymphocytes. The body then falls prey to a host of "opportunistic" infections, many entirely foreign to humans, and most normally eliminated by the immune system (Crowe & Mills, 1991).

The immune system should be regarded as being constantly active, rather than being simply turned "on" to fight infection and "off" following recovery. Normal physiological function requires that the immune system, like other body systems, be constrained to operate within a narrow band of activities through the operation of homeostatic mechanisms. The consequences of too little immune system activity (immunosuppression) are just as serious as those brought about by too much activity as exemplified by
auto-immune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE), in which the immune system is directed against components of the body's own tissues (Wells, 1982).

7. GENERAL IMMUNE RESPONSE:
The precise nature of the immune response will depend on many factors, including the nature of the infective agent (eg: virus, bacteria), the point of entry of the agent into the body, which components of the immune system are encountered and the physiological status of the individual (eg: hormonal environment, nutritional status). However some generalisations can be made. The body's first line of defence can be considered to be the skin and mucosal surfaces. Both function as a physical barrier, with the latter providing additional protection in the form of secreted antibodies [principally immunoglobulin (Ig) A] and bactericidal/bacteriostatic enzymes and proteins such as lysozyme and lactoferrin (Selsted, 1988).

The next line of defence is contributed by the granulocytes, a subset of the leucocytes with a polymorphonuclear form consisting of neutrophils (>90%), basophils (1%) and eosinophils (5%) (Lydyard & Grossi, 1989). These cells are capable of ingesting (phagocytosing) and destroying potential pathogens via two effector mechanisms. Firstly by the generation of a
range of reactive oxygen species (ROS) produced by a membrane-bound NADPH-oxidase (Morel et al., 1991). Secondly by release of enzymes and anti-microbial proteins from a system of cytoplasmic granules (Selsted, 1988). Phagocytosis, mediated by specific cell-surface receptors, usually occurs after the invading agent has been "opsonised" or coated with antibodies or components of the complement cascade (a system of blood proteins; see Section 10) (Werb, 1982; Cooper, 1982). These cells are the most numerous in the peripheral circulation (50-80% of total leucocytes), where they spend some 6-10 hours, after leaving the bone marrow, and before migrating to the tissues.

Operating in concert with granulocytes are the monocytes (present in blood) and macrophages (resident in tissues) of the mononuclear phagocytic lineage. Both of these cell types phagocytose invaders, digest them and display portions of antigenic structures derived from them on their surface, in association with Type II major histocompatibility complex (MHC) antigens. Subsequently lymphocytes, small mononuclear cells normally constituting 25-40% of peripheral blood leucocytes, recognise antigen presented in conjunction with MHC gene products (Male & Roitt, 1989).

Lymphocytes themselves can be further subdivided into subsets based on surface structures and function.
Broadly lymphocytes can be divided into three groups: T lymphocytes, B lymphocytes, and natural killer (NK) cells. T lymphocytes (T representing thymus-derived, which describes their site of differentiation) all display a surface structure termed CD3 [CD: cluster of differentiation]). They can be subdivided further into T helper (\(T_H\)) lymphocytes, displaying the CD4 surface structure, and T cytotoxic/suppressor lymphocytes (CD8\(^+\)).

The second group of lymphocytes comprise B lymphocytes (CD19\(^+\)), which display, as an antigen receptor, the single type of antibody molecule which they are programmed to synthesise when they are stimulated to differentiate into antibody-secreting plasma cells. The third group comprises NK cells which do not express CD3, but typically express CD16 and CD56. These cells, as the name implies, are able to recognise and kill aberrant cells including those affected by cancer and viruses (Katz, 1982).

\(T_H\) lymphocytes provide "help" in relation to antibody production by B cells/plasma cells and the cytotoxic activities of lymphocytes and NK cells (Rook, 1989). \(T_H\) lymphocytes recognise the foreign proteins displayed by monocytes/macrophages in association with the macrophage's own MHC Type II gene products (Owen, 1989). Cytotoxic (\(T_C\)) lymphocytes recognize antigen in
conjunction with MHC Type I gene products present on nucleated cells, thus acting against virally infected cells. Antigen recognition by NK cells and B lymphocytes does not need to occur in association with MHC gene products (Rook, 1989).

Each lymphocyte subset or "clone" displays antigen receptors with a very limited (possibly single) range of specificities. A particular antigen will be bound by only a small number of lymphocytes, thus "selecting" a clone (or clones). These cell will then be stimulated to proliferate, generating more cells of the same antigenic specificity, in a process termed "clonal expansion" (Male & Roitt, 1989). Clonal expansion is initiated by \( T_H \) lymphocytes recognizing antigen (in association with MHC gene products) displayed on the surface of antigen presenting cells (APC) such as macrophages. This initial interaction induces release of the cytokine interleukin-1 (IL-1) from the macrophage. IL-1 stimulates \( T_H \) lymphocytes to secrete interleukin-2 (IL-2) and to increase their expression of IL-2 receptors (IL-2R) (Waldmann, 1986). This "autocrine" stage in the proliferative sequence continues until IL-2R expression reaches a critical level. At this stage the expression on the cell-surface of receptors for transferrin (TrfR) is increased (Neckers & Cossman, 1983), allowing for cellular uptake of iron that is critical to replication of DNA, via its
role in nucleotide reductase enzymes (Hoffbrand et al., 1976). This cycle of the proliferative sequence concludes with cell division and production of two new cells with the same antigenic specificity as the progenitor. The immune response is amplified by repetition of this process over a number of "cycles".

This expanded clone of $T_H$ lymphocytes orchestrates the nature and the magnitude of the subsequent immune response via the elaboration of a range of polypeptide factors collectively termed "cytokines", for example the interleukins, interferons and tumour necrosis factors. A dichotomy in human $T_H$ lymphocytes, similar to that established previously in mice (Mosmann & Coffman, 1989), has been identified recently (Romagnani, 1991). This dichotomy is manifested as a distinct cytokine secretion phenotype being associated with particular subsets of $T_H$ lymphocytes ($T_{H1}$ and $T_{H2}$; Romagnani, 1991). The specific cytokines secreted will depend on the nature of the invading agent and the $T_H$ subset so stimulated. Cytokines such as tumour necrosis factor-α (TNF-α) may be released to increase the activity of NK cells (Reynolds & Ortaldo, 1990) or other cytotoxic cells (Henkart, 1990). IL-4 together with $T_H$ lymphocytes may stimulate B lymphocytes to differentiate into antibody producing plasma cells. TNF-α, interferon-γ (IFN-γ) and IL-8 may cause neutrophils to increase their capacity and readiness to
release both proteolytic enzymes and reactive oxygen species (ROS) (Kowanko & Ferrante, 1987; Smith, Sam et al., 1992; Test, 1991).

With time, following the elimination of the particular invading agent, the immune system will return to a state of lower activity via the action of specific suppressor T lymphocytes (CD8\(^+\); primarily directed against \(T_H\) and B lymphocytes) and elaboration of the immuno-suppressive hormone cortisol from the adrenal cortex in a "negative feed-back" process induced by the initial elevation of plasma cytokine concentrations (Sternberg, 1992). While the immune system's state of activation is returned to pre-infection levels, the system itself is no longer the same. Specific, long-lived "memory" lymphocytes (both T and B cells) have developed that will continue to circulate for a considerable time in readiness for future exposure to the same agent. Such an encounter will produce a much faster and a generally more effective immune response. eg: measles virus (Lydyard & Grossi, 1989).

8. EXERCISE AND THE IMMUNE SYSTEM:

8.1 INCIDENCE OF INFECTION AND DISEASE: Despite anecdotal reports concerning the susceptibility of athletes in training to infection (Simon, 1984; Roberts, 1986; and Nash, 1986), there is a dearth of epidemiological evidence concerning the possibility
that there are exercise-induced changes in immune function. Of the few studies that have compared directly the incidence and severity of upper respiratory tract infections (URTI's) in trained and untrained subjects, Douglas and Hanson (1978) came to the conclusion that "...the training group........ experienced selected URI symptoms more frequently and with greater severity than the control group". Peters and Bateman in their 1983 study of ultra-marathon runners stated that their results suggested "......a relationship between acute stress (eg: ultra-marathon running) and susceptibility to upper respiratory tract infections".

Weinstein (1973) has proposed a role for exercise, late in the incubation period of poliomyelitis, in increasing the extent and degree of paralysis associated with the disease. Heath et al. (1991), in a 12 month longitudinal study of 530 male and female runners, reported that training mileage is a risk factor for upper respiratory tract infections. Nieman et al. (1990b) reported a reduction in the number of days per incident, but not the number of individual incidents, of upper respiratory tract infections in untrained individuals, following a 15 week training program (5 x 45 minute sessions of walking per week). Nehlsen-Cannarella et al. (1991) reported, that a 15 week training program (as employed by Nieman et al.,
1990b) using 36 sedentary, mildly obese women, was associated with an increase in serum immunoglobulin concentration (Ig G,A,M) but no detectable change in lymphocyte function. The changes were evident by week 6 of the study, with some attenuation noted by week 15. Nieman et al. (1990a) showed that runners in heavy training or following a marathon race increased their probability of becoming ill with infectious episodes. After controlling for important variables, runners logging more than 97 km.wk$^{-1}$ experienced double the odds of contracting infectious episodes compared with runners training less than 32km.wk$^{-1}$.

Blair et al. (1989) found, in an 8 year prospective study involving 13,000 subjects, a negative association between physical fitness (and presumably physical activity) and the incidence of cancer. Whether this relationship can be attributed to a direct effect of physical activity on the immune system or to changes in quite different parameters, such as intestinal transit times, has yet to be determined.

Heath et al. (1992), in a review of the relationship between exercise and the incidence of URTI's, concluded that this is "J-shaped" ie: with the most sedentary subjects and those most vigorously active being at the greatest risk of infection, while those undertaking moderate levels of physical activity have a reduced
incidence of infection. The means by which these changes are mediated, particularly in relation to high intensity exercise, has been investigated only minimally.

8.2 ACUTE EFFECTS ON LEUCOCYTES: In contrast to the paucity of information on the role of exercise in altering the incidence and severity of disease, the modulatory effect of exercise on the dynamics of circulating leucocytes have been of interest for over 50 years (Martin, 1932). A survey of the literature reveals that, subject to acknowledgement of the range of exercise protocols employed (most of which are predominantly aerobic) and the parameters measured, short-term physical exercise causes a transient perturbation of the haemodynamics of circulating leucocytes, with normalisation usually occurring within 24 hours. Generally such exercise evokes a transient biphasic leucocytosis, involving an initial catecholamine-dependent lymphocytosis and a later cortisol-dependent granulocytosis (see review by McCarthy & Dale, 1988).

The lymphocytosis is generally manifested as an increase in the circulating concentrations of all the main lymphocyte subsets, including CD3⁺ (T lymphocyte), CD4⁺ (T helper/inducer), CD8⁺ (T cytotoxic/suppressor), CD19⁺ (B cells), and CD3⁻CD16/56⁺ (Natural
Killer (NK) cells. There are changes in the proportions of these lymphocyte subsets, typically involving a fall in CD4+, and an increase in CD8+, which causes the CD4+:CD8+ ratio to fall, and increases in the CD3−CD16/56+, and CD19 (B cell) subpopulations (Mackinnon & Tomasi, 1988). These changes are usually transient, with normalisation, or even a slight "rebound" effect, apparent at 1 hour post-exercise (Gray et al., 1992).

An exercise-induced monocytosis has also been reported (Bieger et al., 1980), as has a post-exercise reduction in the ability of lymphocytes to proliferate in response to mitogenic stimulation in vitro (Tharp & Preuss, 1991; Gray et al., 1992). It is likely that this decreased proliferative ability is due to the effects of elevated plasma concentrations of both cortisol (Heilman et al., 1973) and epinephrine (Crary et al., 1983a) seen during and after exercise (Viru, 1985). Mucosal immunity appears to be compromised after exercise with salivary IgA concentrations having been found to decrease significantly after cross-country skiing (Tomasi et al., 1982).

Mackinnon et al. (1988) reported that NK cell cytotoxicity, expressed on a per-cell basis, increased after extended aerobic exercise. The biphasic changes reported in NK after exercise (Kappel et al., 1991;
initial increase, later decrease) appear to have epinephrine- (Brahmi et al., 1985) and prostaglandin-dependent (Kappel et al., 1991) components.

The duration and intensity of exercise, together with the times after exercise at which the blood is sampled, will affect the concentrations of leucocytes measured. Short duration, high intensity maximal cycling has been found to produce very significant increases in most lymphocyte subsets immediately post-test, including a minor elevation of granulocytes (Gray et al., 1992). However, lymphocyte concentrations at 6 hours post-test were not significantly different from rest while granulocyte concentrations were elevated significantly (Gray et al., 1992). Extended protocols of greater than 30 minutes duration, during which the initial lymphocytosis may not be apparent unless blood is sampled whilst the exercise is in progress, produce significant increases in granulocyte concentration (Moorthy & Zimmerman, 1978). However extremely long aerobic protocols (eg: 120km marching; Galun et al., 1987) cause leucocyte concentrations to return to pre-exercise levels before cessation of exercise, possibly reflecting the migration of some leucocyte subsets to sites of tissue/muscle damage.
8.3 EXERCISE-INDUCED LEUCOCYTOSIS: MECHANISMS: It is commonly held that the various leucocyte subsets are distributed between circulating, marginated (attached to endothelial cells on blood vessel walls) and organ/tissue specific "pools" (Peters et al., 1985; Lien et al., 1987). [Pabst (1988) makes the point that only some 2% of the total population of lymphocytes are in the peripheral circulation at any one time]. At rest these pools are in dynamic equilibrium with circulating leucocytes (lymphocytes), controlled in a directed fashion by specific cell-surface structures (addressins, selectins, integrins), that maintain the association of leucocytes with particular organs and tissues (Butcher, 1986). Exercise, with its associated effects on the circulating concentrations of some hormones, and the inevitable increase in "shear" stress exerted on marginated leucocytes (Gallik et al., 1989; Foster et al., 1986), acts to alter the dynamic equilibrium existing between these pools (Muir et al., 1984).

McCarthy & Dale (1988), in their comprehensive review of exercise-induced leucocytosis, draw upon a large number of studies to propose that the initial lymphocytosis is due to the effect of the hormone epinephrine (adrenaline) from the adrenal medulla, which releases cells from storage sites (eg: in the spleen and lungs; van Tits et al., 1990) and "low-flow"
areas, possibly via a cyclic adenosine monophosphate [cAMP]-dependent reduction in the adherence of lymphocytes to endothelial cells (Boxer et al., 1980). The early studies of Ahlborg & Ahlborg (1970) clearly support a β-adrenergic-dependent mechanism to explain exercise-induced leucocytosis. The granulocytosis that occurs later is thought to relate to the effects of cortisol, from the adrenal cortex, and epinephrine, from the adrenal medulla, in releasing these cells from their site of production in the bone marrow. The proposed actions of epinephrine in producing an initial increase in peripheral lymphocytes with a later increase in neutrophils and decrease in lymphocytes (lymphopenia), have been confirmed by the work of Steel et al. (1971).

The proposed role of cortisol in modulating both granulocyte and lymphocyte concentrations (positively and negatively, respectively) is based largely on clinical investigations of granulocyte kinetics following the infusion of either corticosteroids (Bishop et al., 1968; Fauci & Dale, 1975) or radioactive/fluorescent labelled granulocytes (Athens et al., 1961). Corticosteroids appear to induce release of granulocytes from the bone marrow whilst, at the same time, causing a redistribution of lymphocytes from the peripheral circulation, possibly to the bone marrow (Fauci, 1979). Moorthy & Zimmerman (1978) have reported
a significant positive correlation between post-exercise circulating concentrations of cortisol and granulocytes following a 20 mile race.

9. **GRANULOCYTES:**
Granulocytes are phagocytic cells that normally constitute approximately 50-80% of peripheral blood leucocytes, although this may increase markedly during infection or, indeed, following exercise. These cells, so named because of the presence of prominent cytoplasmic granules that are visible under the microscope, can be subdivided further into neutrophils (constituting over 90% of granulocytes), basophils (1%) and eosinophils (5%) (Lydyard & Grossi, 1989). Most research into the effects of exercise on granulocytes has dealt primarily with neutrophils. Neutrophils have a very limited half-life in plasma (approximately 6-10 hours) before migrating to the tissues (Yang & Hill, 1991) (see Section 9.8). Neutrophils not only ingest (phagocytose) foreign agents and tissue fragments, but also exert bactericidal and bacteriostatic effects via the interaction of two effector mechanisms: a membrane bound NADPH oxidase (which generates ROS) and a system of cytoplasmic granules (primary, secondary and tertiary) that contain proteolytic and bacteriostatic proteins (Selsted, 1988). These oxidative and non-oxidative effector mechanisms are of vital importance in the maintenance of disease-free status
(Yang & Hill, 1991). The effector mechanisms are "coupled" via processes that occur in either extracellular or phagolysosomal spaces subsequent to phagocytosis (Fantone, 1991).

9.1 CYTOPLASMIC GRANULES: Neutrophils possess a system of cytoplasmic granules the contents of which are released, during phagocytosis, into either phagolysosomal or extracellular spaces. This process is referred to as degranulation. The integrity of the granules is critical, either directly or indirectly (to the extent that they modify the production of ROS), in maintaining normal immunocompetence. The granules have been classified as primary (azurophilic), secondary (specific) and tertiary. They contain a range of bactericidal and bacteriostatic substances, which constitute the "non-oxidative" arm of the neutrophil's effector mechanisms. The contribution of these components is of great importance in maintaining a disease-free state as exemplified by the recurrent infections suffered by patients with Chediak-Higashi syndrome, which is associated with a deficiency of the primary granule enzymes elastase and cathepsin G (Ganz et al., 1987).

Generally the "granule proteins" are localised differentially within the three types of granule. Primary granules contain elastase and cathepsin G
(serine proteases), lysozyme (a cationic enzyme), defensins (arginine and cysteine-rich antimicrobial peptides) and the haem-containing enzyme, myeloperoxidase (Selsted, 1988), important in the generation of chlorinated ROS. As an example of the degradative potential of these proteases, elastase has an established capacity to degrade most of the components of the extracellular matrix, as well as clotting factors, immunoglobulins and complement proteins (Weiss, 1988). This powerful potential for tissue destruction is balanced by the presence, in plasma and interstitial fluids, of a system of antiproteases, such as $\alpha_1$-antiprotease inhibitor and $\alpha_2$-macroglobulin (Weiss, 1989).

Secondary granules contain lysozyme and lactoferrin [(Lf; an iron-binding glycoprotein, present in the iron-free "apo" form (van Snick et al., 1974)]. The importance of Lf in the modulation of both immune cell function (Brock & de Sousa, 1986) and in limiting the ability of bacteria to replicate (Chandra, 1991), because of its ability to chelate free iron, has not been fully determined. However Lf has been found to be capable of modulating release of cytokines from mononuclear cells (Crouch et al., 1992), to be involved in the killing of gram-negative bacteria through an interaction with lysozyme (Ellison & Giehl, 1991), and to affect bacterial replication differentially
depending on its iron saturation (Byrd & Horwitz, 1991). In addition Lf may participate in preventing the peroxidation of tissues due to its ability to sequester free iron (Gutteridge et al., 1981). Secondary granules have also been found to be associated with pre-formed "pools" of receptors that are normally expressed on the cell surface such as complement receptor 3 (O'Shea et al., 1985) (expressing CD11b/CD18) and CD69 (Gavioli et al., 1992) (see Section: 9.8 "Receptor Expression").

Tertiary granules, although somewhat elusive to isolate, have been reported to contain gelatinase (Dewald et al., 1982), and to degranulate selectively (ie: in the absence of degranulation of either primary or secondary granules) under the influence of fMLP (Porteu & Nathan, 1992).

Flow cytometry (FC) has proved to be a powerful tool in the investigation of the effects of exercise on the immune system. In particular, and without reviewing the general area of FC, measurement of 90° scattered light has been found to be particularly useful, not only in the discrimination of leucocyte subsets (Ritchie et al., 1983) but also as an indicator of neutrophil degranulation. This process is reflected in changes in the 90° light-scatter profile. Investigations by Sklar et al. (1984), Fletcher & Seligmann (1985) and Abrams et al. (1983) have shown that the degranulation of
primary granules is associated with a reduction in 90° light-scatter whilst degranulation of secondary granules has only a minimal effect on this parameter (Fletcher & Seligmann, 1985). The information on degranulation and changes in 90° light-scatter obtained to date has been derived from studies using non-physiological agents such as phorbol myristate acetate (PMA) and the effects of physiological stimuli such as exercise have not been evaluated.

9.2 NEUTROPHIL ACTIVATION: SIGNAL TRANSDUCTION and NADPH-OXIDASE ACTIVATION: Binding of ligands to specific cell-surface receptors is, in physiological situations, the first step in a complex, and as yet incompletely defined, series of biochemical pathways that result in a range of possible manifestations of neutrophil activation, including activation of the NADPH oxidase and degranulation. The effector mechanisms appear to be under differential regulation in that ROS production and degranulation may occur simultaneously or separately, depending on the nature of the activating stimulus (Manara, 1991; Baggiolini & Kernen, 1992). Consideration is given here to signal transduction pathways that are generally applicable to a number of manifestations of neutrophil activation.

Stimulation of neutrophils results in a series of characteristic biochemical changes. These include an
increase in non-mitochondrial oxygen consumption, increase in adhesiveness, the appearance of superoxide in the extracellular medium and an increase in hexose-monophosphate shunt activity (Yang & Hill, 1991; Baggioolini & Wymann, 1990).

The membrane-bound NADPH oxidase can be activated by a number of stimuli, including those acting via different membrane receptors [complement component C5a, formyl met-leu-phe (fMLP), opsonized zymosan (OZ), IgG] and those acting via stimulation of intracellular components of signal transduction pathways (PMA, calcium ionophores, diacylglycerol: Morel et al., 1991). Under physiological conditions activation of the NADPH-oxidase system is initiated by binding of a ligand to specific cell-surface receptors (as above). It has been established that ROS production is critically dependent on the maintenance of the ligand-receptor interaction (Baggioolini & Wymann, 1990). It appears that individual components of the NADPH oxidase system become deactivated rapidly, so that sustained ROS production is dependent on continuous replacement of previously activated "units" by newly-activated enzyme (Akard et al., 1988). Oxidase assembly and activation can occur within 2 seconds of ligand binding (Baggioolini & Kernan, 1992). The "onset" times for production of ROS in response to stimuli such as fMLP, C5a, platelet activating factor (PAF) and
leukotriene B4 (LTB4) are identical, which implies that signals from these different receptors may share a common transduction pathway (Baggiolini & Kernen, 1992).

The NADPH-oxidase is a transmembrane electron transport chain which uses cytosolic NADPH as an electron donor and molecular oxygen a single electron acceptor (Morel et al., 1991). The $K_m$ for NADPH is 0.015–0.08mM, and for NADH 0.5–1.0mM, indicating that the former is the physiologically relevant substrate (Rossi, 1986).

Many components of the signal transduction pathway that leads to NADPH oxidase activation have been determined. The signal generated by ligand binding appears to be transmitted via a pertussis toxin sensitive guanine nucleotide binding protein (G protein) to phospholipase C (PLC) (Morel et al., 1991). PLC induces hydrolysis of phosphoinositol 4,5-bisphosphate to form inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG). These compounds (IP$_3$ and DAG) are capable of activating protein kinase C (PKC). The former compound activates PKC indirectly, by mobilisation of Ca$^{2+}$ from either intracellular stores (calciosomes) or extracellular pools, whilst the latter compound lowers the $K_m$ for Ca$^{2+}$ and allows PKC to be stimulated by a much lower concentration of Ca$^{2+}$ (Morel et al., 1991). Activation of PKC results in its translocation to the plasma
membrane where it brings about further phosphorylation of regulatory proteins, particularly p47-phox (Rossi, 1986; Babior, 1991). The mobilisation of calcium and subsequent rise in cytosolic Ca\(^{2+}\) were once regarded as critical to initiation of the respiratory burst. However it now appears that Ca\(^{2+}\)-independent pathways also exist, that are also possibly independent of PKC (Morel et al., 1991). In this regard Maridoneau-Parini et al. (1986) have reported that activation of the oxidase induced by OZ differs from that induced by PMA in that the former is mediated by PLA\(_2\), and independent of PKC. Sha'afi et al. (1988) demonstrated that phosphorylation of p47-phox (see below), as a consequence of stimulation of neutrophils with either PMA or fMLP, is not a prerequisite for the process of degranulation. Nevertheless PKC does play a critical role in some signal transduction pathways. Staurosporine, a PKC inhibitor, has been found to inhibit multiple manifestations of NADPH-oxidase activation, including phosphorylation and translocation of p47-phox, and PMA-induced generation of O\(_2^-\) (Nauseef et al., 1991).

9.3 COMPONENTS OF THE NADPH OXIDASE: The NADPH-oxidase complex appears to be composed of multiple components, which are, prior to stimulation, localised in both cytosolic and membrane sites (Figure 2). The NADPH oxidase system, once assembled and activated, generates
the superoxide anion \((O_2^-)\) as its primary product, and this is a precursor of other highly-reactive oxygen-based species generated by subsequent chemical reactions (see below). Assembly and activation of the oxidase system may be synonymous processes, as Tyagi et al. (1992) have reported a close correspondence between these two events. NADPH, derived from the hexose monophosphate shunt, is used as an electron donor. Single electrons are transferred from the cytosolic side of the membrane to molecular oxygen on either the extra-cytoplasmic or phagolysosomal side. The transfer of electrons is accompanied by the transfer of cytosolic \(H^+\) ions across the membrane in the same direction, thereby ensuring maintenance of the cytosolic pH and acidification of the phagolysosomal or extracellular space (Henderson et al., 1988). The increased consumption of oxygen (as an electron acceptor giving rise to \(O_2^-\)) associated with neutrophil activation has been termed the "respiratory burst".

Cells of the myeloid lineage (i.e.: granulocytes and monocytes/macrophages) have been found to contain a membrane-bound cytochrome b as a component of the NADPH oxidase system. This cytochrome has been given two designations: cytochrome b\(_{558}\), due to the characteristic absorption peak exhibited in its reduced form; and cytochrome b\(_{-245}\), due to its mid-point redox potential (Clark, 1990). The former terminology will be
Figure 2: Components of the NADPH-oxidase of Neutrophils (From Clark, 1990).
used here. Woodman et al. (1991) have isolated respiratory burst oxidase activity confined to a cytoskeletal-containing neutrophil fraction. In unstimulated cells a minor part (15%) of total neutrophil cytochrome b₅₅₈ has been reported to be located in this fraction (Woodman et al., 1991). A majority of the cellular content of this cytochrome (approximately 85%) is thought to be associated with the secondary granules, and this has been confirmed by the use of immuno-electron microscopy (Ginsel et al., 1990). [There are, however, conflicting reports, with Mollinedo & Schneider (1984) finding that cytochrome b₅₅₈ is distributed between the tertiary and secondary granules. This discrepancy is unexplained]. There are reports that a fraction of the cytochrome b₅₅₈ translocates from the secondary granules to the plasma or lysosomal membrane upon activation (Borregaard et al., 1983), although there is some evidence to the contrary (Woodman et al., 1991).

The cytochrome consists of at least two subunits: a glycoprotein β-subunit of approximately 91kD (91-phox; phagocyte oxidase), and a 22kD peptide α-subunit (22-phox) which has been reported to bind the haem component of the complex (Nugent et al., 1989). A 22kD GTP-binding protein (rap-1) has been found in association with the cytochrome (Quinn et al., 1992). There are reports of storage "pools" of G proteins
being transferred from the specific granules of resting neutrophils to the membrane upon stimulation (Rotrosen et al., 1988). This implies that the process of degranulation is an integral component of NADPH oxidase activation. There are in addition other possible components: a flavoprotein (p45-phox), ubiquinone and at least three cytosolic factors (Babior, 1991 & 1992; Clark, 1990).

The cytosolic phagocyte oxidase components, all proteins of molecular weight 32kD, 47kD, 67kD, termed p32-phox, p47-phox and p67-phox respectively, have been found to translocate from cytosolic to membrane fractions upon stimulation of the intact neutrophil with PMA (Nauseef et al., 1991; Clark et al., 1990; Babior, 1992). Translocation appears to be directed primarily to the plasma membrane, rather than to membranes associated with either primary or secondary granules (Clark et al., 1990). It has been suggested that cytochrome b$_{558}$ forms a "docking site", allowing translocation of the p47-phox and p67-phox cytosolic components to the membrane, and that the presence of p47-phox is critical to this process (Heyworth et al., 1991). p47-Phox appears to undergo multiple phosphorylations in association with the translocation process (Clark, 1990). This may begin with partial phosphorylation in the cytosol, and be completed at the plasma membrane in a p91-phox-dependent process.
(Babior, 1992). However, a dissociation between p47-phox phosphorylation and induction of $O_2^-$ production (and degranulation) has been reported (Sha'afi et al, 1988). There is evidence to suggest that the transition of the NADPH oxidase from an inactive to active state is related to the phosphorylation of some endogenous proteins (Rossi, 1986; Babior, 1988). The exact relationship between phosphorylation, translocation and oxidase activation remains to be fully elucidated. p32-Phox has been identified as the NADPH-binding subunit (Babior, 1992).

9.4 **REACTIVE OXYGEN SPECIES (ROS):** An impaired ability of neutrophils to produce ROS, as in individuals suffering from chronic granulomatous disease (CGD), which is produced by a genetic deficiency in the NADPH oxidase system, results in recurrent infections, which until recent times culminated in early mortality (Smith & Curnutte, 1991). The "respiratory burst" of neutrophils normally results in the formation of a range of ROS. The initial product of the NADPH oxidase system is the superoxide anion ($O_2^-$) (Morel et al., 1991). Either spontaneously, or in the presence of the enzyme superoxide dismutase (SOD), superoxide gives rise to hydrogen peroxide ($H_2O_2$). There has been much conjecture as to the possible formation of highly reactive hydroxyl radicals ($OH^-$) by neutrophils. While it is has been demonstrated that $OH^-$ can be formed in
vitro in the presence of $O_2^-$, $H_2O_2$ and a transition metal catalyst (particularly iron), the place of this reaction in physiological systems has not been established (Halliwell & Gutteridge, 1986).

Myeloperoxidase (MPO) released from primary (or azurophilic) granules catalyses the conversion of hydrogen peroxide to hypochlorous acid (HOCl). Formation of a variety of hypohalous acids (eg: with other halides such as Br$^-$ or I$^-$) can occur in a similar manner, although formation of the chlorinated form predominates physiologically due to the high plasma concentration of chloride (Weiss, 1989). HOCl can react, in turn, with $H_2O_2$ to form highly reactive singlet oxygen (Klebanoff et al., 1988) or with amines to form a range of bactericidal chloramines (Weiss, 1989).

9.5 ACTIVATION STATES: The NADPH oxidase system has a diversity of responses to differing combinations of stimuli. Sequential stimulation with homologous stimuli, for example, leads to a marked depression in the response to the second stimulus. In contrast, heterologous stimulation sometimes produces an enhanced production of ROS (Rossi, 1986). Neutrophils with an enhanced ability to produce ROS are said to be "primed" (Rossi, 1986). Thus, neutrophils can exist in four states of activation: inactive, primed, activated or
refractory. The magnitude of the stimulus required to induce a primed state is approximately only 10% of that required for activation. Agents such as IL-1 (Balkwill & Burke, 1989), TNF-α (McColl et al., 1990), TNF-β (Ferrante et al., 1988), IFN-γ (Shalaby et al., 1985; Kowanko & Ferrante, 1987; Cassatella et al., 1988), GM-CSF (McColl et al., 1990), IL-8 (Yuo et al., 1991) and growth hormone (Spadoni et al., 1991; Weidermann et al., 1991) have all been shown to be capable of priming neutrophils. Using bone-marrow derived macrophages, which contain an NADPH oxidase system similar to that of neutrophils, Green et al. (1992) have shown that the known priming agents TNF-α and lipopolysaccharide (LPS) increase the level of both p47-phox and p67-phox proteins in these cells. This observation, which may provide a molecular explanation for the phenomenon of priming, is consistent with the conclusion of McColl et al. (1990) that priming is regulated at the post cell-surface level. Priming has also been attributed to changes in cytosolic calcium and PKC that influence protein phosphorylation (Babior, 1992). Primed neutrophils have also been found in the peripheral circulation of humans during infectious episodes (Bass et al., 1986).

While activation of the NADPH oxidase by a primary stimulus has been well investigated, the ability of activated granulocytes to produce ROS upon
re-stimulation has been studied only recently. Prasad et al. (1991) have shown that the induction of an initial activation of neutrophils by zymosan produces a "refractory" state during which the ability of the cells to produce ROS (as assessed by chemiluminescence) in response to further stimulation is severely impaired. The activity recovers with time, in approximately 90 minutes. These findings are consistent with those of Seifert & Schultz (1987), who demonstrated a reversible activation of NADPH oxidase by exogenous guanine nucleotides (presumably binding to G-proteins), in the HL-60 cell-line. These proteins appear to be vitally involved in activation, deactivation and reactivation of the NADPH oxidase. The study by Prasad et al. (1991) supports the contention that activated cells are not destroyed immediately but continue to remain as a viable component of the immune system for some time.

9.6 METHODS FOR ASSESSING THE ACTIVATION STATE OF THE NADPH OXIDASE.

9.6.1 MEASUREMENT OF ROS PRODUCTION: A number of very sensitive methods have been developed to measure the production of ROS, in their various forms, by activated neutrophils. The majority of the methods employed in the present study rely on the detection of particular species following stimulation of neutrophils with a
range of activating agents. All of these methods assess
the potential of the cells to respond to an \textit{in vitro}
challenge, rather than providing direct information on
the activity of the cells \textit{in vivo}.

The following methods have been employed in this study:

9.6.1.1 \textbf{Chemiluminescence (CL)}: CL uses photon (light)
emission associated with relaxation of an excited
chemiluminogenic indicator back to its "ground" state
as a basis for detection of ROS derived from stimulated
neutrophils. Luminol (5-amino-2,3-dihydrophthal-
zindion-1,4) is used to amplify the signal derived from
its oxidation by a number of ROS (including hydrogen
peroxide, hypochlorous acid and hydroxyl radicals). The
generation of CL is dependent on the haem-containing
enzyme, myeloperoxidase (MPO), located in the primary
granules (Weber, 1990). Luminol provides information on
both the intra- and extracellular production of ROS
(Hosker \textit{et al.}, 1989).

Recent studies (Takahashi \textit{et al.}, 1991) indicate that a
different spectrum of ROS is produced depending on the
neutrophil-activating agent employed. Using
luminol-enhanced CL, for example, the response to fMLP
was found to reflect primarily OH\textsuperscript{•} production, whilst
that to PMA and OZ reflected formation of HOCl and/or
O_{2}^{-}. Iron (in the ferric form, Fe\textsuperscript{3+}) has also been
shown to increase luminol-enhanced CL in response to stimulation by fMLP (Takahashi et al., 1991) and S. aureus (van Asbeck et al., 1984). Thus alterations in iron status and iron binding proteins may be another means by which exercise is capable of influencing ROS generation (see Section 13: Iron Status). These findings reflect the underlying complexity of the neutrophil signal transduction pathways that are associated with the generation of ROS.

9.6.1.2 Reduction of Ferricytochrome C: The production of superoxide can be assessed by quantifying SOD-inhibitable, ferricytochrome-C reduction (assessed at 550nm) induced by neutrophils in response to exogenous stimulation (Weber, 1990). This reaction measures the extra-cellular production of superoxide over the time period of the experiment, and has the advantage of measuring production of the primary product of the NADPH-oxidase system ie: superoxide.

9.6.1.3 Dichlorofluorescin Diacetate (DCFH-DA): The coupling of the non-fluorescent dye DCFH-DA and flow cytometry has provided a relatively simple and reliable means of assessing ROS (hydrogen peroxide) production by individual neutrophils (Bass et al., 1986). Incubation of neutrophils with DCFH-DA leads to cellular uptake and chemical modification (deacetylation of the dye) by esterases to produce
non-fluorescent 2,7-dichlorofluorescin (DCFH), which is then effectively "trapped" inside the cell. The basis of the assay is the rapid oxidation of DCFH to fluorescent 2,7-dichlorofluorescein (DCF) by intracellular hydrogen peroxide (Bass et al., 1983) upon cell activation. Flow cytometry allows the rapid counting of individual cells, and provides data that reflects cumulative individual responses rather than group dynamics.

9.6.1.4 Dihydrorhodamine (DHR): This dye, which appears to be more sensitive to ROS production than DCFH-DA, (Smith & Weidemann, 1993) has been applied in a similar manner to DCFH-DA to determine the intracellular production of ROS by isolated phagocytes (Emmendorffer et al., 1990). The non-fluorescent dye is taken up by the neutrophils and converted to a green fluorescent compound during the respiratory burst.

9.6.2 The Present Investigation; translocation of p47-phox: The present study has attempted to overcome the deficiency inherent in all of these assays (that is, their propensity to measure the "potential" of neutrophils to respond to stimulation, rather than the actual response during exercise) by investigating the activation state of the NADPH oxidase system during exercise. Assembly of the oxidase components is almost certainly indicative of NADPH-oxidase activation (Tyagi
et al., 1992). Thus, the possible translocation of a critical oxidase component, p47-phox, from the cytosol to the membrane during exercise, was investigated by use of a polyclonal antibody to p47-phox and polyacrylamide gel electrophoresis (PAGE), as described previously by Clark et al. (1990).

9.7 THE POTENTIAL FOR NEUTROPHIL-DEPENDENT TISSUE DAMAGE: Recent research indicates that the oxidative and non-oxidative effector mechanisms of neutrophils may interact to produce tissue pathology in certain disease conditions (Weiss, 1989).

In particular it has been hypothesised that neutrophil activation acts as a causal agent in pathological conditions such as the adult respiratory distress syndrome (ARDS; Martin et al., 1991), re-perfusion injury (Fisher et al., 1991) and inflammation (Fantone, 1991). Weiss (1989) has concluded, in a review of neutrophil-mediated tissue destruction, that few studies have yet been performed that demonstrate a direct cytotoxic role for either the superoxide anion or hydrogen peroxide alone. This has led, in turn, to conjecture regarding the possible formation of highly reactive, and potentially tissue-damaging, hydroxyl radicals (OH·) by transition-metal (eg: copper or iron) catalysis. Human tissues are normally protected from non-oxidative degradation by the presence of a large
excess of protease inhibitors such as α-1 proteinase inhibitor (Weiss, 1988). In addition, cells have a range of protective enzymes including SOD, catalase (acting on H₂O₂) and a range of chemical antioxidants including vitamins E (α-tocopherol), C (ascorbate) and reduced glutathione, which protect tissues from direct damage by ROS (Jenkins, 1988).

Excessive production of ROS by activated neutrophils can lead to the oxidative destruction of this anti-protease "shield", leaving tissues or, more accurately, particular tissue micro-environments, open to non-oxidative degradation via a system of powerful neutrophilic proteases derived from the cytoplasmic granules (Weiss, 1989). Serine proteases, such as elastase and cathepsin G, and metallo-proteinases such as collagenase and gelatinase, attack human tissues through their selective destruction of components of the extra-cellular matrix (Weiss, 1989).

Exercise, particularly in those forms which are of long duration, high intensity or with a large eccentric component, has a well established role in producing tissue (muscle) damage, as manifested by elevations in the plasma activities of enzymes [such as creatine kinase (CK)] that are normally intra-muscular (Noakes, 1987). Smith (1991), in a recent review of the relationship between exercise-induced muscle damage and
activation of neutrophils, raises the possibility that these cells may be responsible for, rather than merely being recruited in response to, such tissue damage. Athletes have been reported to show an increased rate of erythrocyte "turnover" as indicated by an elevated percentage of reticulocytes (or immature erythrocytes) in the peripheral circulation (Schmidt et al., 1987). Increased erythrocyte turnover is also characteristic of inflammatory diseases (Weiss et al., 1983). Weiss et al. (1992) have postulated that neutrophil effector mechanisms, both proteases and ROS, may be involved in mediating this increase in erythrocyte destruction.

Cognizance must also be taken of other possible sources of ROS during exercise (see review by Jenkins, 1988). Increased flux through the electron transport chain of muscle mitochondria during exercise, for example, may be responsible for an increased production of ROS in muscle (Davies et al., 1982). The enzymatic xanthine-xanthine oxidase system (Sjodin et al., 1990) and the reoxygenation of tissues involved in ischaemic reperfusion (Fisher et al., 1991) may be responsible, in some situations, for generation of ROS. Certainly ROS-induced tissue damage cannot be ascribed solely to neutrophil activation. However, demonstration of a neutrophil-activating role for exercise, particularly were it to include both oxidative and non-oxidative effector mechanisms, would provide insight into the
broader physiological effects of exercise.

9.8 EXPRESSION OF RECEPTORS ON NEUTROPHILS:
There are few data on the ability of exercise to modulate the expression of neutrophil receptors for either the Fc portion of immunoglobulin G (FcγR) or specific complement components, despite the well established changes in these surface molecules during granulocyte activation (Huizinga et al., 1990a/b; Crockard et al., 1992). These receptors mediate clearance of antibody and complement-opsonised (coated) particles respectively from the circulation during the phagocytic process (Fantone, 1991). Neutrophils have the capacity to express three types of FcγR (Unkeless, 1989). FcγRI (CD64; 72kD) is not normally expressed on neutrophils but can be induced by interferon-γ (IFN-γ). Activation of the NADPH-oxidase can be induced by ligands that act via FcγRI (Akerley et al., 1991; Pferfferkorn & Fanger, 1989), but its involvement in degranulation has yet to be determined. Both FcγRII (CD32; 40kD) and FcγRIII (CD16; 50-80kD) are expressed constitutively on neutrophils. The former has the capacity to mediate both activation of the NADPH-oxidase and degranulation (Huizinga et al., 1990b), whilst the latter can apparently induce only degranulation (Unkeless, 1989; Huizinga et al., 1990b).
In a physiological context, both soluble and insoluble immune complexes can activate the NADPH oxidase, apparently by distinct FcγR-dependent mechanisms (Crockett-Torabi & Fantone, 1990). Dufaux et al. (1985) have reported that a 3 hour running test induced formation of circulating immune complexes. Neutrophil activation via PMA or fMLP has been reported to cause shedding and decreased cell surface expression of CD16 (Huizinga et al., 1990a). This phenomenon has also been found in association with inflammation (Fleit et al., 1992). Gavioli et al. (1991) have found, in contrast, that the expression of CD16 increases following stimulation of neutrophils by PMA, a difference that is difficult to reconcile with the other observations cited given that human neutrophils were employed in each case.

Leucocytes possess a variety of cell surface proteins capable of reacting with activated complement components (reviewed by Becherer et al., 1989). Complement receptor 3 (CR3), which binds complement fragment C3bi (Arnaout, 1990), belongs to a family of leucocyte adhesion molecules termed β2-integrins. Members of this family are heterodimeric, with a common β-subunit (CD18; 95kD) and differing α-subunits: LFA-1 (CD11a; 180kD), Mo1 or Mac-1 (CR3) (CD11b; 155kD) and p150,95 (CR4) (CD11c; 155kD) (Arnaout, 1990). All of the subunits are transmembrane proteins (Arnaout, 1990)
which by virtue of their ability to bind complement components, may participate in the process of neutrophil activation.

Increased expression of complement receptors by neutrophils (both CR1 and CR3) has been found in association with thermal injury, which is indicative of their possible participation in cell activation (Davis et al., 1987; Moore et al., 1986; Bjerkenes et al., 1990). A number of agents, including cytokines such as TNF-α (Berger et al., 1988) and GM-CSF (Edwards, 1991), are capable of upregulating the expression of neutrophil cell-surface complement receptors. Whether these agents are relevant in the context of exercise-induced neutrophil activation remains to be determined.

The β2-integrins have been found to have an essential role in both complement-mediated and ROS-dependent injury of the lung (Mulligan et al., 1992a). Soluble forms of the complement receptors are being used in clinical situations to reduce the severity of tissue damage that occurs secondary to activation of the immune system (soluble CR1; Mulligan et al., 1992b), and specifically that which follows reperfusion of myocardial tissue (Ma et al., 1991).

Despite this emerging clinical use of soluble receptors, it is apparent that a mere increase in the
expression of complement receptors is not sufficient to explain the increase in adhesion induced in neutrophil during activation with agents such as PMA or fMLP (Buyon et al., 1988; Vedder & Hartan, 1988; Merrill et al., 1990). It seems likely that receptors undergo a conformational change during which new structures with a significant role in cell adhesion are expressed. Even though neutrophils have been reported to show altered adhesion following exercise, the mechanism of this has not been determined (Lewicki et al., 1987).

Both CR1 (receptor for complement fragment C3b) and CR3 (C3bi) are able to mediate, and indeed play an important part in, phagocytosis but not the release of ROS from human neutrophils (Brown, 1991; Wright & Silverstein, 1983). It has been found that CR3 and other receptors (including CD69; Gavioli et al., 1992) are stored, pre-formed, in intracellular pools. In the case of CR3 this pool is associated with the secondary cytoplasmic granules (O'Shea et al., 1985).

Up-regulation of the cell-surface expression of such receptors is consequently not dependent on protein synthesis, but it can be used as both a "marker" of translocation from the pre-formed pools and an indicator of cell activation (degranulation). A number of agents, including PMA and fMLP (Gavioli et al., 1991), have been shown to be capable of inducing increased expression of CR3. Neutrophil aggregation,
which involves the CD11b/CD18 complex, has been shown to be mediated by at least two independent signal transduction pathways that differ in their involvement of PKC (Merrill et al., 1990). Neutrophil adhesion, and expression of complement receptors (Leino & Lilius, 1992), may also be influenced by the presence of divalent cations such as magnesium (Mg$^{2+}$) and manganese (Mn$^{2+}$) (Lundgren-Akerlund et al., 1992).

The $\beta_2$-integrins play an essential role in maintenance of "normal" immune function. Patients with an inherited disorder (eg: leucocyte adhesion deficiency; LAD), that manifests as either a partial or complete deficiency of the $\beta_2$-integrins, present with frequent bacterial infections of soft tissues, gingivitis, periodontitis and poor wound healing. Their leucocytes also show a reduced ability to phagocytose complement-coated particles, to adhere to surfaces and to migrate in response to a chemotactic gradient (Anderson et al., 1985).

The fact that neutrophil activation, as discussed previously, leads to differential regulation of LECAM-1 (see Section 9.9) and CR3 (von Andrian et al., 1991; Kishimoto et al., 1989) raises the possibility that changes in receptor expression can be used as an index of activation of these cells in vivo.
9.9 EXTRAVASATION AND INTERACTION WITH THE ENDOTHELIUM:
Neutrophils are attracted to sites of infection and inflammation by chemotactic (Male & Roitt, 1989) or haptotactic (Rot, 1992) agonists. In order to reach tissue sites beyond the circulation, neutrophils must first interact with the vessel wall endothelium. Two families of molecules normally expressed on the neutrophil surface appear to be involved in these interactions. "Selectins" [eg: LECAM-1 (leucocyte-endothelial cell adhesion molecule)] have been implicated in initiating the first of two main steps in the extravasation process. The final step involves the heterodimeric $\beta_2$-integrins (CD11/CD18) (Rot, 1992).

Extravasation across the endothelium appears to depend upon an interaction of neutrophil surface molecules, such as the selectins and $\beta_2$-integrins, with receptor sites on the endothelium (Lawrence & Springer, 1991). These interactions are mediated by specific endothelial cell molecules such as sialyl-Lewis$^X$ antigens (for E-selectin) and ICAM-1 and ICAM-2 (for the $\beta_2$-integrins) (Pardi et al., 1992).

The initial interaction involving LECAM-1, in which neutrophils "roll" along the endothelium (Lawrence & Springer, 1991), is a pre-requisite for subsequent, firmer interactions with the endothelium, involving
β2-integrins such as complement receptor 3 (CR3:CD11b/CD18), that are necessary for completion of the extravasation process (Lawrence & Springer, 1991). The later interaction, which appears to follow and be dependent on neutrophil activation, appears to affect the cell surface expression of LECAM-1 (down-regulated) and CR3 (CD11b/CD18; up-regulated) differentially (von Andrian et al., 1991).

9.10 EXERCISE AND NEUTROPHIL FUNCTION: Early studies of the effects of exercise on neutrophils were concerned principally with the induction of a relatively sustained neutrophilia, that persisted for hours. Typically, values were normalised by 24 hours post-exercise (McCarthy & Dale, 1988). Important questions concerning the origin of these cells, particularly their phenotypic characterisation, and the relationship of the leucocytosis to neutrophil "turnover", are still largely unexamined.

Although relatively few studies have been carried out on the effects of exercise on the functional status of neutrophils, a number of recent studies have started to explore this area. Prolonged aerobic exercise appears to induce at least a partial activation of neutrophils. Schaefer et al. (1987), Dufaux & Order (1989a), Hansen et al. (1991), Kokot et al. (1988), after runs of 2000m, 2.5hr, 10.5 km and 10km respectively, have all
reported a significant post-exercise increase in the plasma concentration of the elastase-inhibitor complex. Additionally Pincemail et al. (1990) have shown that the plasma concentration of myeloperoxidase increases following a graded exercise protocol. These findings are consistent with a significant degranulation of neutrophil primary granules in vivo. Whether this degranulation extends to the secondary and tertiary granules has not been determined. The findings of Kokot et al. (1988) and Hack et al. (1992) indicate that granulocytes have a reduced ability to produce ROS via the NADPH oxidase system when they are stimulated in the post-exercise period. This may indicate that exercise had already induced activation of the NADPH-oxidase system of a significant proportion of neutrophils, resulting in the appearance of a refractory period immediately post-exercise (Prasad et al., 1991). In another series of experiments Smith, Telford et al. (1990), using a relatively mild aerobic protocol (cycling for 1 hr at 60% VO₂ max), found significant priming, as opposed to activation, of granulocytes in the post-exercise period. These results are in contrast to those of Kokot et al. (1988) and Hack et al. (1992); "priming" clearly does not equate with activation and the existence of a post-exercise refractory period. This discrepancy remains to be resolved. The nature of the exercise undertaken (ie: type, intensity, duration) and the training status of
the subjects employed may be factors that influence the effects of exercise on the immune system. Rodriguez et al. (1991) have reported that aerobic running of relatively short duration had no significant effect on the adherence capacity, spontaneous mobility, chemotaxis or ingestion of Candida albicans by neutrophils. However, opsonization of Candida albicans as well as candidicide power both increased after exercise. In contrast to some of the findings of Rodriguez et al. (1991), Ortega et al. (1993) reported that chemotaxis, phagocytosis and spontaneous mobility of neutrophils were all increased significantly following 1 hr cycling at 50% VO₂ max. Espersen et al. (1991) have reported a similar increase in chemotaxis following a 5 km run.

Lewicki et al. (1987) have reported differential effects of progressive bicycle ergometry to exhaustion on human neutrophil function, such effects being dependent on the "training-status" of the subjects tested. In trained subjects both neutrophil adherence and bactericidal activity decreased significantly, whilst phagocytic activity did not change, in response to the exercise protocol. On the other hand, a similar exercise protocol undertaken by untrained subjects did not induce significant changes in adherence and bactericidal activity, but increased phagocytic activity.
These results add support to the idea that exercise may influence functionally important components of neutrophil effector mechanisms. However, the effects of such factors as the duration, nature and intensity of exercise, and the fitness level of the subjects tested, remain to be investigated further.

10. THE COMPLEMENT SYSTEM:
The complement system of approximately 30 blood proteins plays a key role in the inflammatory process in general, via generation of anaphylatoxins such as C3a, C4a, C5a, and via modulation of neutrophil functions such as phagocytosis and degranulation in particular (Frank & Fries, 1991). The complement system can be regarded as having three distinct activities: (1) activation of phagocytes; (2) lysis of target cells; and (3) opsonisation (coating) of microorganisms and immune complexes (Walport, 1989).

There are two initially independent pathways that lead to the final, biologically significant, steps in the complement activation sequence. The "classic" and "alternative" pathways are activated differentially, the former by antigen-antibody complexes, and the latter by microorganisms (Walport, 1989). The pathways converge at the mid-point of the sequence, so that the reactions of C5 to C9 are common to both pathways (Cooper, 1982). In addition, epinephrine can cause
activation of C5 via an interaction with platelets (Boros & Leonard, 1990). Neutrophils can also generate C5a in a feed-forward pathway (Boros & Leonard, 1990), which may have implications for both the exercise-induced mobilisation (McCarthy & Dale, 1988) and activation (Kokot et al., 1988) of neutrophils.

Generation of the anaphylatoxins C3a, C4a, and C5a gives rise to a spectrum of events typically associated with the inflammatory process which include smooth muscle contraction, increased vascular permeability, the release of vasoactive substances such as histamine from basophils and mast cells, and the release of granules from neutrophils (Cooper, 1982). These anaphylatoxins are degraded rapidly in vivo to their much less biologically active "des arg" forms (e.g.: C3a des arg) through removal of the C-terminal arginine by a serum carboxypeptidase (Cooper, 1982). Measurement of the plasma concentrations of these "des arg" fragments can be used as an index of complement activation.

Cleavage of C3 by either the classical or alternative pathway C3 convertases leads to generation of C3b and C3a fragments (Lambris, 1988), with subsequent formation of fragments such as C3bi (due to cleavage of C3b by the serine protease Factor I; Lambris, 1988), C3f, C3c and C3dg (Becherer et al., 1989). Opsonisation of surfaces with C3bi leads to phagocytosis of the coated particles, mediated primarily by the C3bi
receptor (CR3) present on neutrophils and monocytes (Brown, 1991; Arnaout, 1990).

There are conflicting reports of the effects of aerobic exercise on complement activation. Dufaux & Order (1989b) have reported that 2.5 hours of running by "moderately trained" subjects induced a significant increase in the plasma concentration of C3a and C4a. Smith, Chi et al. (1990) found that "short-term" aerobic activity leads to significant increases in the plasma concentrations of the same components. In contrast, Thomsen et al. (1992) found no change in the plasma concentration of either C3d or C3c following 60 minutes of exercise at 75% VO₂ max by untrained subjects.

11. CYTOKINES:
Cytokines are a diverse, heterogeneous group of protein and glycoprotein factors produced by cells of the immune and other physiological systems, that serve as both inter- and intra- system "communication" molecules. Cytokines participate in the regulation of a variety of immune functions but also regulate a number of physiological parameters that relate to iron status (Maury & Lahdevirta, 1990), thermoregulation (fever) (Vogel & Hogan, 1990), and muscle proteolysis (Baracos et al., 1983). Cytokines, typically, are of low
molecular weight (less than 80kD). They are primary (and transient) agents in the modulation of the immune response, have a site-specific (paracrine or autocrine) rather than systemic (endocrine) action, are present in plasma at very low (picomolar) concentrations and bind to specific cell-surface receptors (Balkwill & Burke, 1989; Arai et al., 1990). Cytokines can be largely grouped into "families" such as the interleukins (IL), ranging through IL-1α and IL-1β to IL-11, colony stimulating factors (CSF) [eg: granulocyte-CSF (G-CSF), granulocyte-macrophage-CSF (GM-CSF)], tumour necrosis factors (TNF) [eg: TNF-α and TNF-β] and interferons [IFN-α,β,γ] (Kelso, 1989).

Cytokines display both pleiotropy and redundancy. Typically, a particular cytokine will be capable of interacting with a wide range of target tissues, yet its action may be replaced by other cytokines that act either singly or in combination (Kelso, 1989; Arai et al., 1990). Cytokines as diverse as IFN-γ (Klein et al., 1992; Kowanko & Ferrante, 1988; Cassatella et al., 1988), IL-8 (Smith, Sam et al., 1992; Westlin et al., 1992), TNF-α, TNF-β, IL-1, granulocyte-CSF and GM-CSF (Balkwill & Burke, 1989) are capable of altering the activation state of neutrophils.

Exercise, as a stimulus capable of altering most physiological systems, has also been reported to
increase the plasma concentrations of many cytokines. However, interpretation of the data in this area of research has been complicated by the use of different assay methodologies. Many investigators have used bioassays that are in many cases, relatively non-specific (Northoff & Berg, 1991) to quantitate plasma cytokine activity (Cannon et al., 1986). Others have assessed plasma "immunoreactivity" via use of either radioimmunoassay (RIA), enzyme-linked immunosorbent (ELISA) or immunoradiometric (IRMA) assay techniques (Smith, Telford et al., 1992). While these latter assays are specific for particular epitopes, the relationship between "immunoreactivity" and biological activity is, in most cases, not well established. Still other investigators have employed in vitro stimulation of cells collected pre/post exercise to assess "cytokine activity" (Haahr et al., 1991). The relationship between "cytokine-activity", particularly as assessed by in vitro assays involving mitogenic, and usually non-physiological, stimulation and in vivo activity, has not been established. Furthermore, the interpretation of results has been complicated by a diversity of what have been largely aerobic exercise protocols, using subjects of widely-differing physiological characteristics.

Plasma IL-1 immunoreactivity appears not to be increased by exercise protocols that are largely
aerobic (Smith, Telford et al., 1992; Simpson & Hoffman-Goetz, 1991; Sprenger et al. 1992; Northoff & Berg, 1991). However Cannon et al. (1986), using a bioassay, have reported an increase in plasma "IL-1 activity" following aerobic exercise. Similarly, Haahr et al. (1991), Lewicki et al. (1988) and Weight et al. (1991), using mitogenic stimulation in vitro, have found increases in what they term "IL-1 production" or "IL-1-type activity" after aerobic exercise. Trained subjects have been found to have higher plasma IL-1 activity pre-exercise compared to resting untrained subjects (Evans et al., 1986).

Plasma IL-6 immunoreactivity has generally been difficult to detect. Sprenger et al. (1992) report a pre-exercise concentration of 0 pg.mL$^{-1}$, with a post-exercise increase to detectable levels, while Smith, Telford et al. (1992) failed to observe consistent changes in plasma IL-6 concentrations following 1 hr of cycling. Northoff & Berg (1991), using an ELISA technique, found an increase in plasma IL-6 following a marathon run.

Neopterin, produced by macrophages following stimulation by T lymphocyte-derived IFN-γ (Huber et al., 1984), and often used as a "marker" of cell-mediated immunity (Fuchs et al., 1988), has been found to be increased in plasma following extended
aerobic exercise (Sprenger et al., 1992; Rokos et al., 1987), although there are reports to the contrary (Smith, Telford et al., 1992). Viti et al. (1985) could find no increase in plasma IFN-γ after exercise, despite demonstrating an increase in IFN-α. TNF-α immunoreactivity has been reported to increase in plasma following aerobic exercise (Espersen et al., 1990; Dufaux & Order (1989a), although Sprenger et al. (1992) detected an increase only in urine, and not plasma, after exercise. Similarly Smith, Telford et al. (1992) failed to detect a significant post-exercise increase in plasma TNF-α immunoreactivity greater than that seen in an unexercised control group studied over the same period of time.

A recent report by Sprenger et al. (1992) has greatly assisted our understanding of the effects of exercise on cytokine production in vivo, and helps to explain some of the conflicting reports in the literature. Sprenger et al. (1992) reported that, following extended aerobic exercise (running 20km in 2 hrs), plasma neopterin and urinary IFN-γ, IL-1β, IL-6 and TNF-α were increased significantly. These findings suggest, overall, that exercise increases cytokine production. In addition the small changes in plasma cytokine concentration may indicate that the production of particular cytokines is restricted to particular tissue micro-environments and may not be detectable
systemically. This contention is supported by the finding of increased immunoreactive IL-1β in skeletal muscle tissue after exercise (Cannon et al., 1989). The significance of the small changes in plasma cytokine concentrations induced by exercise, also needs to be considered in the light of the extremely high plasma concentrations (100–1000 pg.mL⁻¹) associated with pathological conditions (Kwiatkowski et al., 1990).

Taken together the findings discussed above indicate that aerobic exercise induces an activation of the immune system, specifically of macrophages and T lymphocytes. This interpretation is consistent with reports by Fehr et al. (1989), Bieger et al. (1980) and Dufaux & Order (1989a) that aerobic exercise induces activation of both macrophages, monocytes and T lymphocytes, respectively.

12. THE ACUTE PHASE RESPONSE:
The term "acute phase response" (APR) refers to a characteristic pattern of metabolic changes that occur following challenge by infective agents, inflammation or tissue damage (Pepys and Baltz, 1983) and extended aerobic exercise (Liesen et al., 1977). The principal features of this phenomenon are an elevation of body temperature, leucocytosis, negative nitrogen balance, hypoferraemia, hypozincaemia and increases in the plasma concentrations of hormones such as cortisol and
the catecholamines (Pepys and Baltz, 1983, and Kushner, 1982). There are associated alterations in the profile of proteins produced by the liver, which result in increases in the plasma concentrations of "acute phase proteins" (APP) such as C-reactive protein (CRP), serum amyloid protein, ceruloplasmin, complement components and protease inhibitors and decreases in the plasma concentrations of proteins that have been termed "negative acute phase proteins" [eg: albumin and transferrin (Sipe, 1990)]. The roles of exercise and direct tissue injury in initiation of the APR may differ. Weight et al. (1991) have concluded that, although the physiological changes induced by strenuous exercise and the APR are similar, they are not analogous. Transferrin, for example, has been identified as a "negative acute phase reactant", but Liesen et al. (1977) have found an increase in its plasma concentration following aerobic exercise that occurs in conjunction with increases in other "positive" APPs.

Pepys & Baltz (1983) have argued that "...from a teleological point of view, one must assume that the systemic acute phase response is of value in helping to permit survival during the period immediately following injury". The APR can be induced by a number of factors, but all of them include the involvement of cellular or tissue death or injury (Pepys and Baltz, 1983). The APR
is thought to be mediated by at least three cytokines: IL-1 (Gauldie et al., 1987a, Pepys and Baltz, 1983, and Rushner, 1982), IL-6 (Gauldie et al., 1987b; Gauldie et al., 1990), and TNF (Bertini et al., 1989). The actual contributions which each of these cytokines makes to specific elements of the APR is still the subject of some debate. CRP has been reported to inhibit the respiratory burst induced in human neutrophils by PMA (Dobrinich & Spagnuolo, 1991), to inhibit intracellular calcium mobilisation and superoxide production in pig alveolar macrophages (Foldes-Filep et al., 1992), to inhibit enzyme secretion (Buchta et al., 1987) and to act as an opsonin (Kilpatrick & Volanakis, 1985). Glucocorticoids, which are generally immunosuppressive, have been found to augment the APR (Arai et al., 1990).

Given the potential immunomodulatory role of the APR (Sipe, 1990; Li et al., 1982) and the associated hypoferraemia (see below), the question of whether such a response is elicited by intense, interval exercise, which gives rise to a quantitatively different hormonal milieu characterised by a marked elevation in epinephrine and cortisol (Viru, 1985), both of which are known to suppress IL-1 production, is of considerable interest.

The APR is also characterised by increases in the plasma concentrations of protease inhibitors and
complement components (Sipe, 1990). It would appear to be capable of modulating neutrophil activation, either positively or negatively, so that the net effect is dependent on the precise nature of the changes in these plasma factors.

13. IRON STATUS:
Normal functioning of the physiological mechanisms which act to regulate the concentrations of both iron and iron-binding proteins such as transferrin, ferritin and lactoferrin are of critical importance in maintaining normal immune function (Chandra et al., 1977). Iron, via its catalytic role in the production of ROS as antimicrobial agents (eg: hydroxyl radicals), has also been implicated in both the functional capacity of neutrophils to kill infective microorganisms (van Asbeck et al., 1984) and the tissue damage which results from the activation of such leucocytes (Gutteridge et al., 1981).

While endurance training has been shown to have a strong association with low iron status (Clement & Sawchuk, 1984), there has been comparatively little research into the acute effects of interval exercise on iron status. However Gimenez et al. (1988) have reported that interval exercise is associated with a significant increase in the plasma concentrations of both serum iron and transferrin, the latter effect
probably due to haemoconcentration. Roberts & Smith (1989) have reported a differential effect of intense anaerobic cycling on serum iron, which increased in trained subjects, an effect being dependent upon the training status of the subjects tested. Thus acute high-intensity exercise may increase the potential for exercise-induced tissue damage via increasing the availability of iron to act as a catalyst in ROS generating reactions.

The hypoferraemia associated with the APR (Kushner, 1982), which is manifested as a decrease in the concentration of serum iron and a reduction in transferrin saturation, as well as an increase in the plasma concentration of the iron-binding proteins lactoferrin and ferritin, has been postulated to have a bacteriostatic effect (Gordeuk et al., 1986; Wienberg, 1974 & 1992).

Chronic hypoferraemia, that is, a combination of low plasma ferritin values, indicating pre-latent iron deficiency, and sub-optimal haemoglobin, indicating iron deficiency, is a characteristic of many endurance-trained athletes (Roalstad et al., 1986). The origin of this potentially immunomodulatory (Brock & de Sousa, 1986; Weinberg, 1992) and indeed performance-reducing hypoferraemia (due to the role of iron-containing enzymes in the respiratory chain; Lehninger, 1982) is
open to debate; it may be a consequence of either the chronically elevated IL-1 seen in some trained athletes (Evans et al., 1986) or possibly, to repeated activation of the immune system.

Iron-binding proteins in human plasma, such as lactoferrin, transferrin and ferritin are all potential antioxidants that prevent the oxidation and peroxidation of body tissues (Halliwell and Gutteridge, 1986; Gutteridge et al., 1981) by reducing the ready availability of free iron for the catalysis of ROS generating reactions. Indeed, one may postulate that the lower specific activity of ROS production by stimulated granulocytes in trained, relative to untrained, subjects when assessed at rest (Smith, Telford et al., 1990), may be a consequence of the lower iron-status of the trained subjects, especially as the investigation involved the use of luminol-enhanced chemiluminescence, which is sensitive to iron status (van Asbeck et al., 1984). In contrast, the readier mobilisation of iron from iron-binding proteins (eg: ferritin) at low pH (Aruoma and Halliwell, 1987) raises the possibility that intense, interval exercise (with its associated decrease in plasma pH) may provide a suitable environment for just such a mobilisation of iron stores.

As discussed in Section 9.4, stimulation of
granulocytes by infective challenge leads to the production of the superoxide anion \( (O_2^-) \), hydrogen peroxide \( (H_2O_2) \) and, possibly via the iron-catalysed Haber-Weiss reaction, the hydroxyl radical \( (OH^-) \) (Halliwell and Gutteridge, 1986). These ROS have an antimicrobial action that makes them key participants in the phagocytic process. The hydroxyl ion has been implicated in the peroxidation of membrane lipids (Aruoma and Halliwell, 1987). Iron-binding proteins, for instance, may influence the rate and intensity of ROS production. Apo-lactoferrin and apo-transferrin (ie: in their iron-free states) have been shown to have a protective, and ferritin (iron plus apo-ferritin) an enhancing, effect on the generation of hydroxyl radicals in the presence of a superoxide-generating system (Aruoma and Halliwell, 1987). The lower iron status displayed by endurance athletes (Roalstad et al., 1986) may have its origin in the action of hydrogen peroxide on erythrocytes in, firstly, releasing iron from haemoglobin (Gutteridge, 1986) and, secondly, in giving rise to the formation of met-haemoglobin and peroxidised cytoskeletal proteins, and subsequently the increased phagocytosis of the aberrant erythrocytes so produced (Snyder et al., 1985). This contention is supported by the work of Weiss et al. (1992) which suggests that activated neutrophils, acting via release of proteases and ROS,
may participate in the destruction of erythrocytes during the course of inflammatory disease.

14. EXERCISE AND THE ENDOCRINE SYSTEM:

One of the primary avenues of communication between physiological systems is the endocrine system. This collection of ductless glands, connected by the circulatory system, is controlled by what has been termed the principal endocrine gland, the pituitary, and a part of the brain with which it is intimately associated, the hypothalamus. The hypothalamus is responsible, in many cases, for the elaboration of specific hormone-releasing and -inhibitory factors which act on the pituitary. Pituitary hormones, including those produced by the anterior pituitary [prolactin, growth hormone, luteinizing hormone, adrenocorticotropic hormone (ACTH)] and posterior pituitary (vasopressin), may have direct effects on target tissues via the systemic circulation or may cause the elaboration of "secondary" hormones from other endocrine glands such as the ovaries (estrogens), testes (testosterone) or adrenal glands (cortisol, catecholamines) (Ganong, 1983).

The endocrine system plays an important role during both long and short-term exercise and training in allowing the body to adjust to the associated metabolic demands (eg: increased blood flow, increased energy
utilisation etc). Cortisol acts, for instance, to induce gluconeogenesis and increased glucose-6-phosphatase activity in the liver (Ganong, 1983). It is firmly established that exercise, at different intensities, produces significant alterations in the plasma concentrations of most of the endocrine hormones (Viru 1985). While individual responses vary depending on the fitness level, nature, intensity and duration of exercise, exercise results generally in an intensity and duration-dependent activation of both the sympatho-adrenal and pituitary-adrenocortical systems, which produce increases in the plasma concentrations of catecholamines (eg: epinephrine and norepinephrine) and glucocorticoids (eg: cortisol), respectively (Viru, 1985). It appears, in particular, that regular training enhances the secretory capacity of many of these hormone systems (Viru, 1985). Thus trained subjects exercising at their maximum capacities may subject their various physiological systems to very high concentrations of particular hormones. The following sections will consider some of the more relevant endocrine hormones.

14.1 CORTISOL: The glucocorticoid cortisol, which is one of a number of corticosteroids, is produced by the cells of the adrenal cortex in response to adrenocorticotropic hormone (ACTH) released from the pituitary. Cortisol has a relatively long plasma
half-life of 60-90 minutes and is present in both free (unbound) and bound forms, the former representing only some 4% of total plasma cortisol (Galbo, 1983). Cortisol is normally transported in plasma bound to its specific binding protein (cortisol binding globulin; CBG; Westphal, 1983). It plays an important part in mobilisation of energy reserves (carbohydrates, fat, protein), especially during periods of "stress", whether this be physical or psychological (Ganong, 1983; Guyton, 1982; Harbuz & Lightman, 1992). The interpretation of many investigations in this area is complicated by the fact that the plasma cortisol concentration displays significant diurnal variation: peak values occur in the early morning and minimum values late in the afternoon and evening (Walmsley & White, 1983).

Plasma cortisol levels have been shown to be increased significantly almost immediately after short-term, high-intensity and relatively intense aerobic exercise (Kuoppasalmi et al., 1980; Kindermann et al., 1982) with the highest levels being attained following short-term bicycle ergometry (Viru, 1985). These findings indicate that it is exercise intensity rather than duration (see below) that is the critical factor in generating elevated cortisol concentrations. This supports the view that the pituitary–adrenocortical axis has an activation threshold of
approximately 60-70% of each individual subject's maximal oxygen uptake (Viru, 1985). Duration may play a role in elevation of the plasma cortisol concentration if exercise of lower intensity is continued for 2-3 hours (Viru, 1992), or at least exceeds 1.5 hrs (Schwarz & Kindermann, 1989). Exercise-induced elevation of plasma cortisol is secondary, and probably related causally, to increases in both corticotropin releasing hormone (CRH) and ACTH (Farrell et al., 1983; Buono et al., 1986). Peak plasma cortisol levels occur 30 minutes after the cessation of exercise, following the peak CRH and ACTH concentrations that are achieved immediately post-exercise (Elias et al., 1991).

Many of the previous investigations of the effects of exercise on the immune system (Brahmi et al., 1985 and Eskola et al., 1978) which have commonly employed exercise protocols that were largely aerobic in nature, and of relatively short duration, may have failed to sufficiently stress subjects and hence have not exposed their subject's immune systems to the concentrations of corticosteroids that are, at least potentially, immunosuppressive. The changes in both leucocyte numbers and function produced by such protocols may not reflect those produced during a typical intense interval training session.
14.2 SEX HORMONES: The plasma concentrations of sex steroids (ie: testosterone and estradiol) have generally both been found to increase following exercise (Viru, 1985), although there are reports to the contrary (Wilkerson et al., 1980; Galbo et al., 1977). Each appears to be influenced by the intensity and duration of the exercise undertaken.

The plasma testosterone concentrations achieved depend more on the exercise intensity rather than on either total work-output or work duration (Viru, 1985; Jezova et al. (1985). In particular, intense exercise [running 3 x 300 metres (Aldercrutz et al., 1976)] has been reported to elevate plasma estradiol by 160%. Kuoppasalmi et al. (1980) found that the plasma testosterone concentration was not increased by repeated short term running (over 20-60 metres), but was increased by runs of 21km (approximately 90 minutes; 4.2-4.3 min.km^{-1}) and 13-14km (45 minutes; 3.2-3.3 min.km^{-1}). The results achieved under these conditions, which raised the plasma testosterone concentration by 7% and 21% respectively, suggest that exercise intensity rather than duration is the critical factor in determining the testosterone response to aerobic exercise. These investigators also reported a significant post-exercise decrease in the plasma testosterone level, which was more pronounced following the intense run.
It must be emphasized that a consideration of the effects of exercise on hormone concentrations measured in plasma must take into account the effects of both haemoconcentration (van Beaumont, 1972) and altered metabolic clearance rates (MCR). Sutton et al. (1976) have been able to account for post-exercise testosterone concentrations as high as 50% above resting values simply on the basis of a reduction in MCR.

Plasma estrogen concentrations increase in females in response to exercise over a range of intensities (30–35%, 60–66% and 85–95%; Jurkowski et al., 1978). In males both a single bout of high intensity cycling (156% maximal capacity, 1.5 minutes) and 50 minutes of cycling at the anaerobic threshold (plasma lactate of approximately 4 mM.L⁻¹) failed to produce increases significantly greater than those that could be accounted for by the effects of haemoconcentration alone (Kindermann et al., 1982). However Brown et al. (1980) have reported that 2 hours cycling at 55% VO₂ max. by trained and untrained subjects of both sexes produced significant increases in plasma estradiol concentrations. As a partial explanation of this finding, Keizer et al. (1980) have shown a strong decrease in the MCR for estradiol following submaximal bicycle ergometry in female subjects. In women, a role for estradiol in alteration of substrate utilisation
and perception of effort is indicated by the findings of Hackney et al. (1990).

14.3 CATECHOLAMINES: The catecholamines are a heterogeneous group of compounds formed by the hydroxylation and decarboxylation of the amino acids tyrosine and phenylalanine (Ganong, 1983). The principal catecholamines are epinephrine (Ep), norepinephrine (NE) and dopamine (DA). Dopamine (DA) is an immediate precursor for the production of both Ep and NE (Ganong, 1983). Ep and NE are both critically important for the mobilisation of energy reserves particularly during relatively high stress, "fight-or-flight" situations (Ganong, 1983). The availability of dietary energy reserves (eg: simple or complex carbohydrates) during exercise can actually blunt the secretion of hormones related to mobilisation of endogenous energy reserves (ie: catecholamines, cortisol; Viru, 1992). Catecholamines are being recognized increasingly as potential regulators of the immune system (McCarthy & Dale, 1988; Roff et al., 1986).

Plasma catecholamines are derived from two main sources: adrenal medullary secretion and autonomic neurons (Ganong, 1983). The adrenal medulla produces mainly Ep, some NE and little DA (Callingham & Barrand, 1979). The adrenals represent the only source of plasma
Ep, whilst most of the NE found in the circulation is derived from the sympathetic nervous system (Duncan & Smythe, 1985). NE is the chemical transmitter utilised at the majority of sympathetic (postganglionic) synapses. During exercise active skeletal muscle is the major source of plasma NE (Kjaer, 1989), although both Ep and NE are secreted by the adrenal medulla (Ganong, 1983). Measurement of a specific neuronal metabolite of NE, dihydroxyphenol glycol (DHPG), allows the relative contributions of neuronal and extra-neuronal sources to be determined when examining the origin of plasma NE (Ganong, 1983; Kopin, 1985).

Plasma concentrations of catecholamines, which have a very short half-life (2-4 minutes; Kjaer et al., 1985), have been found to depend directly on exercise intensity (Farrell et al., 1987; Haggendal et al., 1970; McMurray et al., 1987). An intensity greater than approximately 60% VO2 max has been identified as a "threshold" above which plasma catecholamine concentrations increase markedly (Viru, 1985). It appears that an individual's secretory capacity is increased by training, so that when trained subjects, exercise to maximal capacity they may be exposing their physiological systems routinely to relatively high concentrations of catecholamines (Kjaer et al., 1985; Kjaer, 1989).
Both Ep and NE increase in response to anaerobic exercise (approximately 15 fold) and aerobic treadmill exercise (50 minutes at the anaerobic threshold) 3–4 fold and 6–9 fold, respectively (Kindermann et al., 1982). Schwarz & Kindermann (1989) report 3–4 fold increases in both Ep and NE following 90 minutes of cycling, whilst Macdonald et al. (1983) have shown 4–5 fold and 2–10 fold increases in NE and Ep respectively, following 30 seconds of maximal cycling. Jezova et al. (1985) concluded that plasma catecholamine concentrations measured in response to exercise relate more to its intensity than its duration. Urinary excretion of catecholamines appears to be decreased in "overtrained" athletes (Lehmann et al., 1992), possibly indicating a depletion of one or more of the key components of the synthetic pathway.

14.4 GROWTH HORMONE AND PROLACTIN: Aerobic exercise has been found to induce increases in the plasma concentrations of the anterior pituitary hormones prolactin (PRL) and growth hormone (GH). Both hormones are regulated by specific hypothalamic-derived releasing and inhibitory factors (Ganong, 1983).

GH, a polypeptide hormone of 191 amino acids (Macintyre, 1987), has a half-life in plasma that has been reported as 17–45 minutes (Shephard & Sidney, 1975) and 16–23 minutes (Lassarre et al., 1974). It has
a well-established role in promoting protein synthesis (anabolism), acromegaly (when in excess) (Ganong, 1983) and an emerging role as an agent capable of modulating certain cellular processes in the immune system (Spadoni et al., 1991; Weidemann et al., 1991). Its anabolic actions are thought to be mediated by a range of small proteins called somatomedins that are synthesised in the liver (Ganong, 1983). Both Kindermann et al. (1982) and Farrell et al. (1983) have reported increases in plasma GH concentrations following aerobic exercise. Farrell et al. (1983) detected increases of 6, 20 and 11 times resting levels at 65%, 80% and 100%, respectively of VO$_2$ max. Viru (1985), in an extensive review of the literature, concluded that GH release has an activation threshold in excess of 50% VO$_2$ max. Galbo (1983), again in an extensive review, cites studies in which exercise of a much lower intensity (10-15% VO$_2$ max) has been shown to be capable of evoking increases in plasma GH concentration, provided such exercise is continued for 30-60 minutes.

PRL has a well defined role of promoting milk production in the female, but its function in males is unknown. It does, however, have an emerging role as an agent capable of influencing the activity of neutrophils and other immune cells (Gala, 1991). In terms of exercise-induced modulation concentrations of
PRL in plasma have been shown frequently to increase following acute exercise, with changes that relate apparently to the intensity of exercise (Sowers et al., 1977; Brisson et al., 1981; Galbo, 1983), and possibly to elevated body temperature (Brisson et al., 1986).

An investigation of the influence of intense, interval exercise on plasma hormone levels is of high priority if the mechanism of changes in other physiological systems, as a consequence of exercise and training, are to be understood.

15. THE ENDOCRINE SYSTEM AND IMMUNE FUNCTION:
Hormones are known to affect various elements of, as well as in some cases, to produce functional changes in, the immune system. It would appear that such changes are mediated by specific receptors displayed in a differential fashion on leucocyte subsets (Plaut, 1983).

15.1 CATECHOLAMINES: Catecholamines have been implicated in an exercise-induced leucocytosis, mediated by interactions with β-adrenergic receptors on both endothelial cells (Boxer et al., 1980) and leucocytes (Harkness et al., 1983). Exercise has also been shown to influence the level of expression of β-adrenergic receptors on mononuclear leucocytes (Butler et al., 1982 and 1983). The blunted
leucocytosis seen following training (Soppi et al., 1982) may be the result of chronic exposure to high concentrations of plasma catecholamines (known to affect β-adrenoceptor expression, Tohmeh and Cryer, 1980) that occur during exercise. This may influence, in turn, the β-adrenoceptor dependent interactions of leucocytes and endothelial cells and, ultimately, the joint processes of margination and extravasation.

Crary et al. (1983b) have shown that elevated epinephrine not only increases the number of mononuclear cells in the peripheral blood, but also decreases the proportion of T helper/inducer (CD4⁺) cells. It has no effect on the proportion of T suppressor/cytotoxic (CD8⁺) cells, and produces biphasic (and opposite) changes in both the CD4⁺:CD8⁺ ratio (an initial decrease followed by an increase) and the proportion of NK cells in the peripheral blood. It also decreases the mitogen- responsiveness of mononuclear cells isolated from peripheral blood. Landmann et al. (1984) have also noted a negative relationship between plasma Ep concentration and the CD4⁺:CD8⁺ ratio. These changes in leucocyte number and function virtually parallel the changes produced by exercise, which implicates the elevated plasma Ep concentration, acting via a β-adrenoceptor dependent differential mobilisation of lymphocytes (Crary et al., 1983) in the leucocytosis produced by exercise. Ep is
also capable of affecting the function of leucocytes. Kappel et al. (1991) using Ep at concentrations that can be attained physiologically, and Brahmi et al. (1985) using Ep at higher than physiologic concentrations, have shown that Ep inhibits natural killer cell activity, while Helstrand et al. (1985) have reported a β-adrenoceptor dependent modulation of human NK cells by Ep. Koff et al. (1986) have noted a catecholamine induced suppression of macrophage IL-1 production.

15.2 CORTISOL (AND OTHER GLUCOCORTICOIDS): The clinical use of glucocorticoids (eg: prednisolone) as anti-inflammatory and anti-tissue rejection agents has led to the discovery that they are able to influence both the dynamic equilibrium between leucocyte recirculation pathways (eg: blood stream versus the lymphatic system) (Yu et al., 1977) and functional immunity. Patients undergoing prolonged courses of treatment with corticosteroids often exhibit "more morbidity than that produced by the underlying disease" (Webb & Winkelstein, 1982).

Glucocorticoids given intravenously (often in pharmacological doses rather than at physiological concentrations) have been shown to induce a selective lymphocytopenia (Fauci and Dale, 1974 and 1975) which can be explained by a redistribution of recirculating
lymphocytes to other body compartments, particularly the bone marrow (Fauci and Dale, 1975). They also induce granulocytosis [through mobilisation of neutrophils from the bone marrow (Fauci, 1979)], alteration of serum complement components (Atkinson and Frank, 1973), decreased serum IgG concentration (Butler and Rosen, 1973), variable effects on the incorporation of tritiated thymidine by lymphocytes in response to various mitogens (Heilman et al., 1973) and reduced interleukin-2 production in lymphocyte cultures stimulated ex vivo (Frey et al., 1984). Dexamethasone, a synthetic corticosteroid, has been reported to inhibit macrophage production of IL-1 and TNF (Bertini et al., 1989) both of which induce IL-6 production (Zilberstein et al., 1986). Cortisol and synthetic corticosteroids both inhibit the production of the cytokines IL-1 and TNF by leucocytes (Staruch & Wood, 1985; Ferran et al., 1990). It might be expected, therefore, that high concentrations of cortisol, released into the plasma during exercise in an intensity-dependent fashion (Viru, 1985) may act to inhibit the elevations in plasma cytokine concentrations that are commonly seen following relatively low intensity aerobic exercise. Conversely, interval exercise of high intensity may lead to significant tissue damage (relative to aerobic exercise), which may stimulate cytokine production at the affected sites. The net result of these two
opposing influences has still to be established.

Corticosteroids have been reported to produce a decrease in neutrophil oxidative function (Webb & Roth, 1987), so that elevation in plasma cortisol induced by exercise may contribute to the fall in neutrophil ROS production by the NADPH oxidase system, seen following exercise (Kokot et al., 1988).

15.3 SEX HORMONES: Evidence for a sexual dimorphism in the immune response (Grossman, 1984) has led to an interest in the role of sex hormones in the maintenance of immune homeostasis. A number of findings are relevant here, since, as discussed above, exercise has been reported to induce chronic and acute changes in circulating concentrations of plasma estrogen [see Viru (1985) for review]. Estrogens have been reported to stimulate antibody production in a two-stage process that involves an initial inhibition of T suppressor cells followed by enhanced B cell maturation (Grossman, 1984). Oral contraceptives have been found to reduce mitogen-stimulated blastogenesis (Morishima and Heinrich, 1974), and Grossman (1984) has concluded that estrogens depress many of the major functions attributed to the cell mediated immune system, possibly via inhibition of the synthesis of thymic serum factors. In contrast there is a relatively recent report that certain estrogens can modulate neutrophil
function (either positively or negatively) (Jansson, 1991).

Exercise-induced changes in the concentration of plasma testosterone may have important implications, given that androgens have been reported to depress cell-mediated immunity (Grossman, 1984). Evidence for the role of testosterone in the regulation of antibody production is conflicting with opposite effects being noted in animal studies on chick embryos and rabbits (Grossman, 1984). However, Sullivan and Allansmith (1987) have reported that testosterone induces a significant increase in the concentration of immunoglobulin A (IgA) in the tears of rats when compared with saline controls.

15.4 GROWTH HORMONE AND PROLACTIN: GH has an emerging role as an agent capable of modulating immune system, and particularly neutrophil, activity (Gala, 1991). GH, at concentrations in the range 100–500 ng.mL⁻¹ and higher, is capable of priming human neutrophils for enhanced ROS production in vitro (Fu et al., 1991). This finding is supported by the work of both Spadoni et al. (1991) and Weidermann et al. (1991). PRL has been reported to have similar activity (Fu et al., 1991), but at an even lower concentration range, beginning at 50ng.mL⁻¹. Neither of these hormones have any known direct (eg: activating) effects on ROS
production by human neutrophils. However Weidermann et al. (1991) noted that neutrophils were more adhesive after incubation with GH. Since GM-CSF has been reported to be capable of priming neutrophils for increased ROS production as well as inducing up-regulation of CR3 (Edwards, 1991) it remains to be seen whether the change in adhesiveness induced in neutrophils by GH (noted by Weidermann et al., 1991) involves modulation of the expression of receptors such as CR3 on the cell surface.

The contrasting propensities of corticosteroids and GH/PRL respectively, to suppress and potentiate neutrophil function led Smith & Weidemann (1990) to propose a model concerning what they term the "exercise-immunity paradox". This "paradox" centres on the differential effects of exercise intensity on the immune system, with intense exercise increasing the incidence (Heath et al., 1991; Douglas & Hanson, 1978) and moderate exercise reducing the days per incident of upper respiratory tract infection. The authors contend that at low exercise intensity plasma concentrations of immunopotentiating hormones such as GH and PRL (regarded as being released at relatively low exercise intensity) will predominate over concentrations of immunosuppressive hormones such as the catecholamines and cortisol. However, at high exercise intensity, the situation will be reversed,
thus "tipping the balance" toward immunosuppression. The hypothesis is soundly based but awaits experimental verification.

Overall, changes in the concentration of plasma hormones may be one means, even the primary means, by which exercise is able to alter immune system function.

16. TISSUE DAMAGE AND EXERCISE:
Eccentric exercise results in increases in the plasma activities of the intracellular enzymes creatine kinase (CK) (Pilis et al., 1988 and Evans et al., 1986) and lactate dehydrogenase (LDH) (Pilis et al., 1988), events which are usually attributed to muscle damage. According to different investigators trained subjects have a resting activity of CK that is greater than (Evans et al., 1986) or within the normal range (Taylor et al., 1987), and a post-exercise plasma activity that is significantly lower than that observed in untrained subjects (Evans et al., 1986). The lower post-exercise activity is apparently attributable to a significant elevation in the plasma concentration of protease inhibitors, which act to limit the degree of tissue damage sustained during exercise (Liesen et al., 1977). Allessio and Goldfarb (1987) have shown in a study of exercise intensity using rats, that higher exercise intensities directly affect the formation of lipid peroxidation by-products (which presumably reflect
tissue damage) during exercise. Thus intense, interval exercise may lead to higher activities and concentrations of circulating tissue enzymes than are normally found in the plasma following less intense exercise.

17. SUMMARY:
While aerobic exercise affects most physiological systems in ways that are well researched, intense interval exercise, with its significant anaerobic component, has been subject to relatively little investigation. In particular, the association between periods of intense competition and training with an increased incidence of upper respiratory tract infections is largely unexplained, except in terms of being a general response to "stress". This study seeks to investigate the effects of intense exercise, of relevance to the training of athletes, on the immune system. However in an attempt to elucidate both the means by which the putative changes are produced, and the implications of such changes, a number of physiological systems directly relevant to the immune system will be examined. An attempt will be made to relate any changes in the immune system resulting from the exercise protocol employed to changes in iron status parameters, plasma hormone and cytokine concentrations. Knowledge of the changes induced in these physiological systems by interval exercise may
provide a basis for the establishment of "markers" of immune status that can be applied routinely to both the training of athletes and participation in recreational activities by members of the general public.
18. REFERENCES:


THE END
19. PAPERS
ENDOCRINE RESPONSE TO INTENSE INTERVAL EXERCISE.

A.B.GRAY\textsuperscript{1}, R.D.TELFORD\textsuperscript{2}, and M.J.WEIDEMANN\textsuperscript{1}

\textsuperscript{1} DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, SCHOOL OF LIFE SCIENCES FACULTY OF SCIENCE THE AUSTRALIAN NATIONAL UNIVERSITY GPO BOX 4 CANBERRA ACT 2601 AUSTRALIA.

\textsuperscript{2} DEPARTMENT OF PHYSIOLOGY AND APPLIED NUTRITION, AUSTRALIAN INSTITUTE OF SPORT, PO BOX 176 BELCONNEN ACT 2616 AUSTRALIA.

Correspondence to:

A.B.Gray (B.Sc(Hons), Dip.T.(PE), Dip.Ed.) Department of Physiology and Applied Nutrition, Australian Institute of Sport, PO Box 176, Belconnen, ACT, 2616 Australia.

ph: 011 616 252 1253
fax: 011 616 252 1603
ABSTRACT:
GRAY A.B., R.D.TELFORD, and M.J.WEIDEMANN.
Endocrine response to intense interval exercise.
This investigation provides an insight into the physiological changes produced, and processes operating, during and after, a typical interval exercise training regime. The role of interval exercise in modulation of the plasma concentration of sex hormone binding globulin (SHBG) and the hormones ß-estradiol, testosterone, prolactin and growth hormone was assessed. Eight trained male athletes (VO₂ max (mean (SD) = 64.3 (3.8) ml.kg⁻¹min⁻¹, mean age = 31.5 (4.5) years) undertook an intense interval exercise (treadmill running) protocol to exhaustion. Subjects completed an average of 15.6 one minute runs. This interval protocol produced significant increases in the plasma concentration of SHBG and all four endocrine hormones (all p<0.01) in the immediate post-test period. The plasma concentration of the hormones increased as indicated: ß-estradiol (45%), testosterone (38%), prolactin (230%), growth hormone (2000%). These hormones have an established capacity to interact with components of many physiological systems and as such may provide a mechanism for the changes induced by intense exercise in many of these systems.
Key Words: Anaerobic, growth hormone, estradiol, testosterone, prolactin, SHBG.
INTRODUCCION:
The hormonal response to exercise has been of interest for many years and has been shown to depend on the intensity, duration and nature of the exercise undertaken (Viru, 1985; Galbo, 1983). However, despite the abundance of data concerning exercise-induced changes in peripheral hormone concentrations, little effort has been directed toward the study of exercise regimes that are directly relevant to the training of athletes. Particularly lacking have been studies investigating training regimes requiring a significant anaerobic ("lactic") energy contribution. One of the most commonly used training regimes involves interval exercise, that is, repeated intense bouts of exercise interspersed with only partial recovery periods. Such regimes result in a significant accumulation of lactic acid in the blood and tissues (Telford, 1991).

This investigation employs an intense interval exercise protocol modelled on those frequently used by athletes involved in swimming, middle/long distance running and football (Telford, 1991). The effects of such a protocol on peripheral hormone concentrations are largely undetermined. Many of the previous investigations into the effects of interval exercise on the endocrine system have unfortunately employed protocols of little relevance to the training of athletes.
Previous research indicates that moderate aerobic exercise, above a threshold intensity, is capable of elevating plasma concentrations of most hormones (Viru, 1985). Given the repetitively stressful nature of intense interval exercise, the protocol employed in this study may lead to both quantitative and qualitative differences in the hormonal milieu from that produced by other forms of exercise eg: aerobic or a single supra-maximal effort.

These hormones are derived from different endocrine organs. Growth hormone and prolactin are anterior pituitary hormones whose release is regulated by specific hypothalamic derived releasing and release-inhibiting factors. In males testosterone is produced principally by the testes, with minor amounts by the adrenal medulla. Minor amounts of \( \beta \)-estradiol, in males, are produced by adreno-cortical and testicular pathways associated with testosterone synthesis (Ganong, 1983). A proportion of both total \( \beta \)-estradiol and total testosterone present in plasma are transported by SHBG (Anderson, 1974). Knowledge of the plasma concentrations of these sex hormones and SHBG provides an index of the amount of unbound hormone readily available for target tissues.

The four hormones under investigation have a range of physiological effects including modulation of
lipoprotein profiles (β-estradiol), protein anabolism (testosterone and growth hormone) and breast milk secretion in females (prolactin) (Ganong, 1983). In addition each has an ability to modulate elements of the immune system (Gala, 1991; Grossman, 1984; Jansson, 1991; Kelley, 1989).

However while exercise-induced modulation of many physiological systems may be mediated by the action of these hormones the aim of this study was to establish the effects of an interval exercise regime on the peripheral blood concentrations of the hormones β-estradiol, testosterone, prolactin and growth hormone.

MATERIALS AND METHODS:
Subjects: Eight trained males, mean age (SD) = 31.5 (4.5) years were employed as exercise subjects. All were, and had been for at least one year, engaged in a regular training program. All were free of symptoms of infection and none were taking medication or drugs of any kind. All refrained from food overnight and none had exercised within the previous 24 hours.

Exercise Tests: Subjects were familiarized with testing procedures for both the determination of maximal oxygen uptake (VO₂ max) and interval running tests and all participants signed a consent form that detailed the
scope of the study and its attendant risks. Testing commenced between 08.00 and 09.00 hours. Procedures were approved by the Ethics Committee of the Australian Institute of Sport.

a) Maximal Oxygen Uptake Test (Test #1): All subjects underwent a progressive running test employing increases in speed and gradient to determine their maximal oxygen uptake \( (V_O_2\max) \). The initial treadmill speed was 11 km.hr\(^{-1}\) with an increase of 1 km.hr\(^{-1}\) each minute for the first 7 minutes. At this stage the treadmill gradient was increased at a rate of 2% per minute. All subjects exercised until subjective estimation of exhaustion brought about voluntary termination of the test. The respiratory analysis followed procedures described previously (Hahn et al., 1988). Criteria for attainment of \( V_O_2\max \) were: a "plateau" in oxygen consumption (an increment of less than 0.15 L.min\(^{-1}\)), heart rate equal to predicted maximum or an R value in excess of 1.10.

b) Interval Running Test (IRT) (Test #2): This test was performed at least one week following Test #1. All subjects underwent a standard warm-up procedure involving 5 minutes running at both 7 km.hr\(^{-1}\) and 11 km.hr\(^{-1}\) and 3 minutes at 15 km.hr\(^{-1}\). All warmup procedures were performed at 0% treadmill gradient. Subjects then performed the IRT ie: alternating one
minute periods of treadmill running and active (walk) recovery. Treadmill speed and gradient for the IRT were individually selected using the speed and gradient at which VO$_2$ max was achieved. Subjects were informed of the elapsed time every 15 seconds and all received encouragement during the course of each exercise period. Subjects were asked to perform as many exercise periods as possible, and continued until they could no longer maintain the required speed.

**Blood Collection:** Blood samples, obtained with subjects in a supine position, were collected by venipuncture of an antecubital vein. Six samples were collected; before exercise (after the subject had been sitting quietly for 20 minutes), post warm-up, post-test, 1, 6, 24 hours post-test. Samples were placed immediately into EDTA Vacutainers (Becton Dickinson, Mountain View, Ca.) prior to centrifugation and separation at 4°C. Samples were stored at -20°C before analysis.

**Full Blood Counts:** A Coulter S550 cell counter (Coulter Electronics, Hialeah, Fl.) was employed to obtain a full blood count, including haematocrit, for each sample.

**Whole Blood Lactate:** All blood samples were analysed for whole blood L-lactate with a YSI Model 23L L-lactate analyser (Yellow Springs Instrument Company, Ohio).
Hormone Assays: All hormone assays (for total β-estradiol, growth hormone, total testosterone, prolactin) were performed using commercially available radioimmunoassay (RIA) kits (Biodata/Serono, Australian Diagnostics, Sydney). SHBG was also assayed by RIA (Farmos, Australian Laboratory Services, Sydney).

Statistics: Data were analysed by a repeated measures one-way ANOVA and Fisher's PLSD was used for post-hoc comparisons. Significance was indicated at the p<0.05 level. Data are presented as mean (standard deviation).
RESULTS:

1. Physical and Physiological Characteristics of Subjects:
Table 1 provides details of the subjects employed in this study. The intensity of the exercise protocol is illustrated by the high post-test whole blood lactate level (7.6mmol.L⁻¹), such accumulation of lactic acid indicating considerable involvement of anaerobic energy pathways.

2. Variation of Hormones with Exercise.
The resting concentrations of all four hormones in all subjects were within the appropriate reference ranges (as specified by the manufacturers of the RIA kits employed).

Plasma β-estradiol concentrations were elevated significantly post-warmup (p<0.05), immediately after (45% increase), and at 1 (38%), 6 (26%) and 24 (25%) hours after the exercise test (all p<0.01) (Table 2). Evidence of a haemoconcentration occurred only at the time of the immediate post-test sample where, according to the estimation outlined by van Beaumont (1972), the plasma volume decreased by approximately 10%. At post-test sampling times plasma volume was estimated to undergo either a minor reduction (-2.0% at 1 hour post-test) or was increased slightly in relation to resting values (+3.0% and +0.5% at 6 and 24 hours.)
post-test respectively; data not shown). SHBG differed significantly (p<0.01) from rest only immediately post-test where it increased by approximately 8%, which might well be explained by the estimated haemoconcentration (Table 2).

Testosterone concentration increased significantly immediately after completion of the exercise test (38% elevation; p<0.01), but had decreased significantly by 6 hours post-test (16% decrease; p<0.05).

An elevation in prolactin occurred immediately post-test where the plasma concentration increased 230% (p<0.01) but at no other time did values differ from those obtained at rest, prior to exercise.

Growth hormone was elevated significantly both immediately after exercise (approximately 20 times resting value), and at 1 hour later (six times) (p<0.01). At no other times were growth hormone concentrations significantly different from the resting values.
DISCUSSION:
This study has demonstrated that interval exercise, involving considerable anaerobic energy production, is associated with significant elevation of plasma SHBG, $\beta$-estradiol, testosterone, prolactin and growth hormone concentrations.

The limited exercise-induced modulation of SHBG concentration (aside from that due to haemoconcentration), indicates that the interval exercise-induced changes in total $\beta$-estradiol and total testosterone are largely due to increases in the concentration of the specific unbound hormones.

Despite this protocol producing increases in the plasma concentration of all four hormones, the kinetics of this process differed between hormones. The significant increase in $\beta$-estradiol and growth hormone following warmup indicates that plasma concentrations of these hormones are elevated at relatively low exercise intensities. The absence of significant increases in the plasma concentration of prolactin and testosterone suggests, given constant metabolic clearance rates (MCR), that the activation thresholds for these hormones were not exceeded during the warmup procedure.
Data are limited on the effect of exercise on male estradiol levels. Nevertheless, Aldercreutz et al. (1976) have reported a 160% elevation of estradiol in males following running 3x300 metres, while Brown et al. (1980), in a study employing 2 hours of exercise at 55% VO\(_2\) max, reported a post-exercise elevation of plasma estradiol. There thus appears to be both exercise duration and intensity dependent components to exercise induced elevation of plasma β-estradiol concentrations.

The sustained elevation in β-estradiol, apparent at 24 hours post-exercise with this interval training protocol, indicates alteration of the dynamics of β-estradiol production and/or elimination. A post-exercise decrease observed in the MCR for β-estradiol (Keizer et al., 1980) implies that increased secretion may not be the sole cause of the sustained elevation of plasma β-estradiol. The mechanism of this altered MCR has not been fully established but a decrease in hepatic blood flow (Galbo, 1983) appears to be only one of a number of components capable of contributing to this phenomenon (Keizer et al., 1980). The sustained elevation of estradiol may have beneficial effects in relation to the incidence of coronary heart disease, being associated with favourable changes in high density lipoproteins (Godsland et al., 1987).
The increase in plasma testosterone concentration observed immediately post-test does not agree with the findings of Kuoppasalmi et al. (1980), who reported no increase in plasma testosterone following repeated 20-40 metre runs at maximal speed. However the 38% increase in testosterone concentration observed with this protocol immediately post-test does agree with Kindermann et al. (1982), who reported an increase of 14% in plasma testosterone following a maximal anaerobic running test to exhaustion. Despite the differences in subject characteristics and exercise conditions relating to these studies this discrepancy supports a role for exercise duration in modulation of plasma testosterone levels. These findings also appear to indicate an interaction of exercise intensity and duration in elevation of plasma testosterone levels, and may be related to the anaerobic energy contribution. The protocol employed by Kuoppasalmi et al. (1980) appears to be predominantly "alactic" whilst that of Kindermann et al. (1982) and the current authors was largely "lactic" in nature.

Our findings conflict with those of both Wilkerson et al. (1980) and Galbo et al. (1977). Wilkerson et al. (1980) reported no change in plasma testosterone concentrations (above that due to haemoconcentration) following 20 minutes running at various exercise intensities below that corresponding to VO$_2$ max. Galbo
et al. (1977) have proposed that increases in plasma testosterone following maximal exercise can be explained by changes in plasma volume. Our findings do not support this hypothesis and indicate that both the intensity, and nature of the exercise undertaken (ie: intermittent or continuous) are important factors affecting plasma testosterone levels.

The preceding point clearly illustrates the need to very carefully examine the effects of interval exercise protocols on physiological parameters and not assume that such exercise will induce similar changes to those produced by other protocols, as evidenced by the work of Karagiorgos et al. (1979). The relatively high post-exercise whole blood lactate (7.6 mmol.L\(^{-1}\)), probably sustained throughout much of the exercise protocol due to the repeated high intensity efforts (Telford, 1991), is indicative of the anaerobic energy contribution required during this form of exercise. Moreover Karagiorgos et al. (1979), in a comparison of intermittent and continuous physical activity, noted that the former resulted in significantly higher post-exercise blood lactate levels. These findings serve to illustrate the unique physiological demands associated with intense interval exercise.

The post-exercise elevation in plasma testosterone appears to have been mediated by a reduction in MCR.
Sutton et al. (1976a) have shown that exercise-induced increases of up to 50% (above resting levels) in testosterone could be explained by a reduction in MCR. A role for luteinizing hormone (LH) in this increase appears unlikely given the repeated finding of either no change or a reduction in plasma LH levels following a range of exercise protocols (Jurkowski et al., 1978; Sutton et al., 1973; Galbo, 1983). In particular Elias et al. (1991) have found a significant post-exercise decrease in plasma LH concentration which reached its nadir 90 minutes post-test. Kuoppasalmi et al. (1980) also report a delayed decrease (30 mins post-test) in plasma LH levels following aerobic exercise. Such changes may play a role in the decreased testosterone concentration reported in this study at 6 hours post-test.

The limited time-course of post-exercise testosterone elevation suggests that the anabolic activities of testosterone would appear to be of limited importance in relation to this interval exercise protocol. Greater importance could be attached to the significant decrease in testosterone at 6 hours post-exercise should this change be sustained for an extended period.

The 20 fold increase observed in plasma growth hormone concentration (to approximately 30 ng.mL$^{-1}$) corresponds closely to values reported by Kindermann et al. (1982)
following aerobic exercise. Farrell et al. (1983) has reported increases in peripheral growth hormone concentrations following exercise at 65% (6 fold increase), 80% (20 fold) and 100% (11 fold) VO₂ max. Bunt et al. (1986) reported an increase to only 16 ng.mL⁻¹ following 60 minutes running at 60% VO₂ max. Viru (1985), in an extensive review of exercise induced modulation of peripheral hormone concentrations, concludes that growth hormone displays an activation threshold in excess of 50% maximal oxygen uptake level. The growth hormone concentration observed in this study, immediately post-exercise, while being in excess of those produced by moderate aerobic exercise, at approximately 60% VO₂ max (Bunt et al., 1986), is in agreement with those obtained by relatively intense exercise, at 80-100% VO₂ max (Farrell et al., 1983). These findings support an exercise intensity dependent modulation of plasma growth hormone concentration, although the work of Kindermann et al. (1982) may indicate a durational effect as well.

The similar changes in plasma growth hormone levels produced by aerobic (Farrell et al., 1983; Kindermann et al., 1982) and interval exercise (this study) imply that post-exercise plasma growth hormone concentrations may be influenced by either the intensity or duration of exercise rather than by the nature of the exercise undertaken.
As indicated in Table 2, plasma growth hormone exhibited a sustained (1 hour) post-exercise elevation. Given that the half-life of growth hormone has been reported as being approximately 16 minutes (Lassare et al., 1974) and between 17 and 45 minutes (Shephard & Sidney, 1975) the significantly elevated concentration at 1 hour post-test is consistent with normal plasma clearance rates. However other factors such as low blood glucose (Ganong, 1983) (not measured here) or elevated lactic acid/reduced pH may be involved. However Sutton et al. (1976b) have reported an independence of plasma lactate and growth hormone levels. This may indicate that exercise-induced increases in growth hormone and prolactin, relate to alteration of either hypothalamic regulation or plasma clearance. Measurement of specific releasing or inhibitory factors during and after the exercise period, may better define the mechanism of these exercise-induced changes.

The physiological role of prolactin in males is unclear. However the effect of this protocol on the kinetics of plasma prolactin concentration appear to differ from those displayed by growth hormone. Despite the sequence homology shared by growth hormone and prolactin (Ganong, 1983) and similar immune system activities (Gala, 1991), exercise-induced elevation of prolactin concentration appears to have a threshold
higher than that of growth hormone, given that the former was not elevated by the warmup procedure. The increase in growth hormone is more sustained, still being significantly elevated at 1 hour post-test.

Prolactin has been frequently reported to increase following acute exercise, a change apparently related to the intensity of exercise (Sowers et al., 1977; Brisson et al., 1981, Galbo, 1983). Intense interval exercise clearly provides a potent stimulus for increase in peripheral prolactin concentration, this protocol producing an increase of 230%. Prolactin secretion from the anterior pituitary is inhibited by dopamine (Ben-Jonathan, 1985). An exercise-induced decrease in peripheral plasma dopamine (DA) concentration reported by Gray et al. (unpublished observations) may reflect a role for DA in elevation of prolactin in the post-exercise period.

Brisson et al. (1986) implicate thermal stress as an agent mediating exercise-induced elevations in plasma prolactin concentrations. The relative contributions of thermal, psychological and physical stressors provided by this form of exercise, to elevation of plasma prolactin levels, remain to be determined. The physiological effects, and in-particular those relating to the immune system, of the hormonal changes induced by this commonly used form of extended interval
exercise await further investigation.

In conclusion, this study has demonstrated that interval training involving considerable anaerobic energy production, is a stimulus to elevation of peripheral concentrations of β-estradiol, testosterone, prolactin and growth hormone.
ACKNOWLEDGEMENTS

The authors wish to thank Don Campbell for excellent technical assistance, David Pyne for helpful discussions and members of the Canberra Triathlon Club for acting as subjects, during the course of this work.
REFERENCES:


TABLE 1. Physical and Physiological Characteristics of Subjects:

<table>
<thead>
<tr>
<th>Maximal Oxygen Uptake (mL.kg(^{-1}).min(^{-1}))</th>
<th>Age (years)</th>
<th>Mass (kg)</th>
<th>Repetitions Completed</th>
<th>La(mmol.L(^{-1})) post-IRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.3 (3.8)</td>
<td>31.5 (4.5)</td>
<td>74.0 (8.8)</td>
<td>15.6 (6.6)</td>
<td>7.6 (1.6)</td>
</tr>
</tbody>
</table>
TABLE 2: Hormone Concentrations

Sampling time

<table>
<thead>
<tr>
<th></th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Post-test</th>
<th>1 hour post</th>
<th>6 hours post</th>
<th>24 hours post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Estradiol (pg.mL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34.2</td>
<td>40.5*</td>
<td>49.8**</td>
<td>47.2**</td>
<td>43.2**</td>
<td>42.8**</td>
<td></td>
</tr>
<tr>
<td>(7.2)</td>
<td>(6.7)</td>
<td>(7.1)</td>
<td>(6.3)</td>
<td>(5.3)</td>
<td>(10.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Testosterone (ng.mL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>8.6</td>
<td>10.4**</td>
<td>7.2</td>
<td>6.3*</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>(2.5)</td>
<td>(2.8)</td>
<td>(3.9)</td>
<td>(2.6)</td>
<td>(2.3)</td>
<td>(3.1)</td>
<td></td>
</tr>
<tr>
<td><strong>Prolactin (ng.mL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.7</td>
<td>10.1</td>
<td>32.6**</td>
<td>11.2</td>
<td>8.4</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>(2.6)</td>
<td>(2.0)</td>
<td>(15.1)</td>
<td>(2.7)</td>
<td>(2.3)</td>
<td>(3.4)</td>
<td></td>
</tr>
<tr>
<td><strong>Growth Hormone (ng.mL⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>6.7*</td>
<td>26.8**</td>
<td>8.7**</td>
<td>0.9</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>(0.7)</td>
<td>(6.7)</td>
<td>(6.2)</td>
<td>(9.5)</td>
<td>(0.2)</td>
<td>(0.1)</td>
<td></td>
</tr>
<tr>
<td><strong>SHBG (n.mol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30.7</td>
<td>32.3</td>
<td>33.1**</td>
<td>32.0</td>
<td>30.9</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>(9.2)</td>
<td>(8.2)</td>
<td>(8.8)</td>
<td>(8.2)</td>
<td>(8.2)</td>
<td>(9.3)</td>
<td></td>
</tr>
</tbody>
</table>

Significantly different from rest: * = p<0.05
** = p<0.01
THE EFFECT OF INTENSE INTERVAL EXERCISE ON IRON STATUS PARAMETERS IN TRAINED MALE SUBJECTS.

AUTHORS: A.B.Gray\textsuperscript{1}, R.D.Telford\textsuperscript{2}, M.J.Weidemann\textsuperscript{1}.

\textsuperscript{1} DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY SCHOOL OF LIFE SCIENCES FACULTY OF SCIENCE THE AUSTRALIAN NATIONAL UNIVERSITY, GPO BOX 4 CANBERRA ACT 2601 AUSTRALIA.

\textsuperscript{2} DEPARTMENT OF PHYSIOLOGY AND APPLIED NUTRITION AUSTRALIAN INSTITUTE OF SPORT, PO BOX 176 BELCONNEN ACT 2616 AUSTRALIA.

RUNNING TITLE: IRON STATUS AND INTERVAL EXERCISE.
Iron and iron binding proteins play a critical role in the physiology of many human systems, including the immune system. Intense interval exercise in trained male subjects (mean (SD) age = 31.5 (4.5) years; VO₂ max = 64.3 (3.8) ml.kg⁻¹.min⁻¹) is associated with significant modulation of iron status parameters. The concentration of red blood cells, haemoglobin, and transferrin all increased significantly immediately post-test (p<0.01), increases which can largely be attributed to haemoconcentration. Serum iron was elevated by approximately 25% both immediately, and 1 hour post-test (p<0.08). Maximum post-test serum iron values (either immediately, or 1 hr post) were significantly elevated with respect to rest (p<0.01). Transferrin concentration was also significantly elevated at 24 hours post-test (p<0.05). Transferrin saturation was not significantly altered by this protocol (p>0.10). Despite a trend toward elevation at 24 hours post-test ferritin concentration was not significantly different from the resting value at any sampling point. Intense interval exercise appears to be associated with significant modulation of iron status, the biological importance of which remains to be determined.
Keywords: Anaerobic, iron status, serum iron, transferrin, ferritin.
INTRODUCTION:
This study focused on the effects of interval exercise on selected iron status parameters in trained male subjects. Interval exercise, that is alternating periods of activity and recovery, was first popularised among track & field athletes by Waldemar Gerschler in the 1930’s. Interval exercise is now widely used by athletes involved in numerous other sports including swimming, rowing, canoeing, skiing and ball-sports. Until now very little of the research into the relationship between exercise and iron status has been concerned specifically with the effects of interval exercise.

Iron is an essential element for normal physiological functioning. It is involved in DNA synthesis (5), high energy phosphate production via the respiratory chain (21), and generation of bacteriocidal reactive oxygen species (ROS) (16).

Iron status has typically been assessed by measurement of a number of parameters including the peripheral blood concentration of red blood cells, their content of the major oxygen carrying component of the blood, haemoglobin, the concentration of iron binding proteins transferrin and ferritin and total iron (both free and bound) in serum (32).
There is a strong association between participation in endurance exercise (e.g., long-distance running, triathlons) and low iron status (8). Endurance athletes have been found to have lower indices of iron stores than sedentary control subjects (23), and in some cases low haematocrits, a condition which has been termed "sports anaemia". The existence and cause of this phenomenon has been the subject of considerable debate (18).

Both frank anaemia and iron deficiency without anaemia may have important implications for the performance of athletes. The former produces significant reductions in the maximal oxygen uptake capacity (9), whilst evidence relating to the latter is conflicting. Iron therapy in non-anaemic iron deficient athletes has been shown to lead to both improved endurance performance (25), and no change in performance measures (29).

Exercise-induced modulation of iron stores has additional implications for immune function. Lymphocyte proliferation in response to mitogenic stimulation is decreased under conditions of reduced iron status (7). Polymorphonuclear leucocyte oxidative and phagocytic function are impaired under conditions of hyperferremia (12,30). This effect is thought to be due to excessive ROS production as a consequence of iron catalysis. Hyperferremia has also been found to both increase in
vitro bacterial growth following ingestion of iron salts in healthy control subjects (14) and to decrease natural killer (NK) cell activity in β-thalassemia patients (1).

Modulation of iron availability, either free or in combination with iron binding proteins, has a significant role in catalysis of ROS species generating reactions (16). Putative hydroxyl radical generation following the involvement of both the granulocyte NADPH oxidase system (2) and iron catalysts, may have important implications for exercise-induced tissue damage (26).

Despite the evidence that chronic endurance exercise reduces iron stores few investigations have been carried out into the acute effects of high intensity exercise (13,24). The purpose of this investigation was to assess the effects of a commonly used component of modern endurance training, that is high intensity interval running, on the iron status of trained male subjects.
MATERIALS AND METHODS:

Subjects: Eight trained male triathletes, mean (SD) age = 31.5 (4.5) years were employed as subjects. All were, and had been for at least one year, engaged in a regular (daily) training program. This typically involved between 10 and 25 hours per week of various combinations of swimming, cycling and running (including interval training). All were free of symptoms of infection and none were taking medication or drugs of any kind. All refrained from food overnight and none had exercised within the prior 24 hours.

Exercise Tests:

Subjects were familiarised with the testing procedures for both VO₂ max and interval running tests, and all participants signed a consent form that detailed the scope of the study and its attendant risks. Procedures complied with human subject guidelines of the American College of Sports Medicine and were approved by the Ethics Committee of the Australian Institute of Sport.

a) Maximal Oxygen Uptake Test (Test #1):

Subjects underwent a progressive treadmill running test employing increases in speed and gradient to determine their maximal oxygen uptake (VO₂ max). The initial treadmill speed was 11km.hr⁻¹ with an increase of 1km.hr⁻¹ each minute for the first seven minutes. At this stage the treadmill gradient was increased at a
rate of 2% per minute. All subjects were exercised until a subjective estimation of exhaustion brought about voluntary termination of the test. Criteria for attainment of VO\textsubscript{2} max were: an R value in excess of 1.10, a "plateau" in oxygen consumption (an increment of less than 0.15 L.min\(^{-1}\)), or a heart rate equal to the predicted maximum. Procedures for respiratory analysis have been described previously (15).

b) **Treadmill Interval Running Test (IRT) (Test #2):**
This test was performed at least one week following test #1. Beginning at approximately 9.00 hours all subjects underwent a standard warm-up procedure involving 5 minutes running at both 7 and 11 km.hr\(^{-1}\) and 3 minutes at 15 km.hr\(^{-1}\). All warmup procedures were performed at 0% treadmill gradient. Subjects then performed the IRT i.e.: alternating one minute periods of treadmill running and active (walk) recovery. Treadmill speed and gradient for the IRT were individually selected using the speed and gradient at VO\textsubscript{2} max was achieved. Subjects were informed of the elapsed time every 15 seconds and all received encouragement during the course of each exercise period. Subjects were asked to perform as many exercise repetitions as possible, and continued until they could not maintain the required speed.
Blood Sampling: Blood samples, obtained with subjects in the supine position, were collected from venipuncture of an antecubital vein. Six samples were collected from each exercise subject; before exercise (after the subject had been sitting quietly in the laboratory for 20 minutes), post-warmup, post-test, 1, 6, 24 hours post-test. Samples for iron parameter analyses were allowed to clot at room temperature for 30 minutes prior to centrifugation at 4°C, separation, and storage of serum at -20°C. Samples for full blood counts were placed into EDTA vacutainers (Becton Dickinson, Mountain View, Ca.).

Full Blood Counts: These were obtained from a Coulter S550 Cell Counter (Coulter Electronics, Hialeah, Fl.) and provided information on the concentration of red blood cells, haemoglobin and haematocrit. Quality control was evaluated via daily analysis of a standard blood preparation (4C, Coulter Electronics, Hialeah, Fl.) and was in all cases within acceptable limits.

Blood Lactate Analyses: All blood samples were analysed for whole blood L-lactate by use of a YSI L-lactate analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio).
**Iron Parameter Analyses:** These were performed according to the following methods:—

**Ferritin:** Microparticle enzyme immunoassay (Abbott IMX, Abbott Laboratories, Sydney); (27).

**Serum Iron (SI):** Guanidine/Ferrozine method using a Roche Cobas bio-centrifugal analyser (Roche, Sydney); (33).

**Transferrin (Trf):** Rate Nephelometry (Beckman Assay Systems, Beckman Instruments, Sydney); (27).

**Transferrin Saturation (Trf Sat) (%):** This was calculated from the serum iron and transferrin values.

**Statistics:** Data were analysed by a repeated measures one-way ANOVA. Fisher's PLSD was used for post-hoc comparisons. Student’s t-test was employed as indicated. Significance was indicated at the p<0.05 level. Data are presented as mean (standard deviation).
RESULTS:

1. Physical and Physiological Characteristics of Subjects. Details of the subjects appear in Table 1. The intensity of exercise is indicated by the elevated whole blood lactate measured immediately post-test (La = 7.6mmol.L\(^{-1}\)). Subjects completed an average of 15.6 one minute efforts.

2. Iron status Prior to exercise:
When assessed at rest, prior to exercise, the mean value of all parameters was within the appropriate reference range (Table 2). However haematocrit, red blood cells (RBC), haemoglobin and transferrin saturation were all toward the lower end of these ranges.

On an individual basis, many subjects when assessed at rest prior to exercise, displayed a low iron status. Three subjects had serum iron concentrations either below or equal to the lower limit of the reference range ie: below 10 \( \mu \text{mol.L}^{-1} \). Three subjects had a ferritin concentration of 30 ng.mL\(^{-1}\) or below, whilst five subjects were below 40 ng.mL\(^{-1}\).

Intense interval exercise resulted in significant changes in the peripheral concentration of red blood cells (RBC), haemoglobin (Hb) and transferrin (Trf)
The increase in both RBC (4.5%) and Hb (5.4%) concentrations show similar kinetics to the pattern of haemoconcentration observed. According to the methods of van Beaumont (31) and Dill & Costill (11) respectively, plasma volume varied in relation to resting values, as follows: post warm-up (-6%;-4%), post-test (-10%;-9%), 1 hour (-2%,-1%), 6 hours (+3%;+2%) and 24 hours (+1%;0%).

Transferrin concentration was significantly elevated post warmup, post-test, 1 hour (p<0.01) and 24 hours post-test (p<0.05) by approximately 6-10%.

Despite all subjects showing an increase in serum iron concentration (producing a mean elevation of approximately 25%) immediately post-, and 1 hour post-test, this latter change was significant only at the p<0.08 level. However using Student's t-test to assess the significance of the difference between pre-exercise and maximum values obtained either immediately, or 1 hour post-test, revealed a significant increase (p<0.01). Serum iron decreased non-significantly to 14% below the resting value at 6 hours post-test. Ferritin showed no significant change (p>0.05) across the sampling times despite a trend toward elevation at 24 hours post-test (7% above resting level).
Percentage transferrin saturation did not vary significantly (p>0.05) from resting values at any sampling point despite an elevation of 9% post-test, 14% 1 hour post and a 17% decrease below the resting value at 6 hours post-test. On an individual basis at 6 hours post-test six subjects had a transferrin saturation below 16.5%, with four below 16%.
DISCUSSION:
This study, investigating the effects of high intensity interval running, has clearly required the subjects to derive a considerable proportion of their metabolic energy from anaerobic pathways, as indicated by the elevated post-test whole blood lactate concentration (Table 1).

When assessed at rest the mean values of many of the iron status parameters were toward the lower end of the reference ranges, a finding consistent with reports of the low iron status of endurance athletes (23).

Intense interval exercise produced significant modulation of iron status parameters in trained male subjects. Both transferrin and serum iron increased in the post-exercise period. Significant changes in the concentration of RBC, haemoglobin and transferrin, (except for transferrin at 24 hours post-test) can largely be attributed to haemoconcentration. These parameters increased by between 5% and 10%, thus allowing the changes to be accounted for by the approximate 10% decrease in plasma volume noted immediately post-test. However the elevation of serum iron, both immediately and 1 hour post-test, and transferrin at 24 hours, cannot be attributed to this cause.
The exercise-induced elevation of both transferrin and serum iron is in agreement with that of previous investigators who, although employing interval protocols, chose regimes of little relevance to the training of athletes (13,24). Significant increases have been reported in both serum transferrin (largely attributed to haemoconcentration) and serum iron in response to a protracted interval exercise protocol (13). Roberts & Smith (24) report a differential effect of training on serum iron changes following 3 x 20 second high intensity efforts. Trained subjects showed a significant increase, and untrained subjects a significant decrease, in serum iron following repeated supra-maximal exercise. Our results confirm an exercise-induced increase in serum iron in trained subjects. While it has been postulated that post-exercise increases in serum iron may be associated with increased iron elimination (13), this hypothesis requires further research.

The significant elevation of transferrin at 24 hours post-test (P<0.05) is in agreement with the findings of Liesen et al. (20) in an investigation of prolonged exercise. Such post-exercise elevations in transferrin are in direct opposition to expectation given the putative acute phase response (APR) inducing role of exercise (28) although there have been reports to the contrary (Gray; unpublished observations). The APR is a
characteristic set of metabolic changes involving altered hepatic protein synthesis and hypoferremia. Transferrin has been termed a "negative acute phase reactant" as its plasma concentration normally decreases during the APR (19). The mechanism of the exercise-induced elevation in transferrin concentration requires further investigation.

This increase in transferrin is not associated with a corresponding decrease in transferrin saturation (see Table 2). This parameter is of greater physiological importance given the influence of transferrin saturation on erythropoiesis (17), lymphocyte proliferation (3) and bacterial growth (7).

Plasma ferritin concentrations have been found to have a direct positive relationship with body iron stores, and thus provide a ready index of this parameter (32). The evidence relating to the effects of exercise on plasma ferritin concentrations is conflicting. Pattini et al. (22) report an acute elevation after endurance skiing, Taylor et al. (27) no significant change after a 160km triathlon, whilst Clement & Sawchuk (8) report low concentrations among endurance athletes when assessed at rest. Interestingly Dickson et al. (10) report a "false" elevation of ferritin in endurance runners after heavy training. Thus the already low levels measured in endurance athletes may in-fact not
provide a true indication of iron stores. Our findings are in agreement with those of Taylor et al. (28), in that even though there was a trend to an increase in plasma ferritin concentration at 24 hours post-test, this did not reach significance. Nevertheless our findings also support the concept of a chronic reduction in ferritin level amongst endurance athletes, with a majority of the subjects exhibiting low ferritin concentrations when assessed at rest.

The approximate 25% elevation of serum iron in the post-test period cannot be explained solely by a decrease in plasma volume. Any modulation of either iron or iron binding proteins (and consequent availability of iron) may have implications in terms of both immunomodulation (4) and production of reactive oxygen species (16). An increase in iron availability, given its role in catalyzing the formation of hydroxyl radicals via the Haber-Weiss reaction (2), may have important implications for exercise-induced tissue damage. A transient increase in serum iron may potentiate bacterial infections (6,7). However the concurrent elevation of transferrin, resulting in a relatively constant transferrin saturation, may offset any deleterious consequences associated with increases in serum iron.

The non-significant reduction, below resting levels, at
6 hours post-test in relation to both serum iron and transferrin saturation may relate to the finding that almost two-thirds of untrained subjects generally show a diurnal variation (afternoon decrease) in these parameters (17). Erythropoiesis is affected if transferrin saturation drops below approximately 16% (17). This indicates that irrespective of whether this level resulted from diurnal variation or was exercise-induced, the ability to maintain optimal erythropoiesis in some subjects was threatened.

This study has investigated the effects of a single interval training session. However, given that most, but not all, of the iron status parameters had returned to pre-exercise values by 24 hours post-test, and chronic exercise appears to be associated with low iron status (13), further research is required to assess the effects of repeated bouts of intense interval exercise on iron status.

In summary intense interval exercise in trained male subjects resulted in significant modulation of iron status parameters. Both transferrin and serum iron concentrations (using the maximum value either immediately or 1 hour post-test) were significantly elevated in the post-exercise period, whilst transferrin saturation reached near critical minimum values 6 hours post-test. Many subjects also exhibited
low iron status when assessed at rest and 6 hours post-exercise. Ferritin concentration was not significantly altered by this protocol. The biological significance of these changes awaits further investigation.
ACKNOWLEDGEMENTS: The authors thank members of the Canberra Triathlon club, staff of the Biochemistry Department Woden Valley Hospital, D.Pyne and D.A.Carbon for assistance with this work.

A.B.Gray was the recipient of an Australian Government Post-Graduate Award and an ANUTECH supplementary scholarship.

Correspondence to:–

A.B.GRAY
Department of Physiology and Applied Nutrition,
Sports Science/Sports Medicine Centre,
Australian Institute of Sport,
PO Box 176 Belconnen ACT 2616 AUSTRALIA.

Ph: 011 616 252 1253
Fx: 011 616 252 1603

-------
REFERENCES:


Table 1: Physical and Physiological Characteristics of Subjects:

<table>
<thead>
<tr>
<th>Maximal Oxygen Uptake (mL.kg(^{-1})min(^{-1}))</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Intervals</th>
<th>La post-test (mmol.L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.3 (3.8)</td>
<td>31.5 (4.5)</td>
<td>74.0 (8.8)</td>
<td>15.6 (6.6)</td>
<td>7.6 (1.6)</td>
</tr>
</tbody>
</table>

La = lactic acid
Table 2: Iron Parameters and Interval Exercise

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Post-test</th>
<th>1 hour post</th>
<th>6 hour post</th>
<th>24 hour post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematocrit: (%)</td>
<td>43.9</td>
<td>45.6*</td>
<td>46.6**</td>
<td>44.4</td>
<td>43.1</td>
<td>43.8</td>
</tr>
<tr>
<td>(2.5)</td>
<td>(2.4)</td>
<td>(2.4)</td>
<td>(3.0)</td>
<td>(2.6)</td>
<td>(3.5)</td>
<td></td>
</tr>
<tr>
<td>Red Blood cells: (x 10^6 µL^-1)</td>
<td>4.88</td>
<td>5.07**</td>
<td>5.10**</td>
<td>4.94</td>
<td>4.82</td>
<td>4.90</td>
</tr>
<tr>
<td>(0.36)</td>
<td>(0.34)</td>
<td>(0.37)</td>
<td>(0.38)</td>
<td>(0.35)</td>
<td>(0.39)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin: (g.dL^-1)</td>
<td>14.9</td>
<td>15.6**</td>
<td>15.7**</td>
<td>15.2</td>
<td>14.8</td>
<td>15.0</td>
</tr>
<tr>
<td>(1.1)</td>
<td>(0.9)</td>
<td>(1.0)</td>
<td>(1.1)</td>
<td>(1.1)</td>
<td>(1.3)</td>
<td></td>
</tr>
<tr>
<td>Serum Iron: (µmol.L^-1)</td>
<td>15.1</td>
<td>17.0</td>
<td>18.9</td>
<td>19.0</td>
<td>13.0</td>
<td>14.6</td>
</tr>
<tr>
<td>(7.7)</td>
<td>(7.1)</td>
<td>(8.1)</td>
<td>(7.1)</td>
<td>(2.9)</td>
<td>(6.4)</td>
<td></td>
</tr>
<tr>
<td>Transferrin: (g.L^-1)</td>
<td>3.0</td>
<td>3.2**</td>
<td>3.3**</td>
<td>3.2**</td>
<td>3.1</td>
<td>3.2*</td>
</tr>
<tr>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.3)</td>
<td>(0.4)</td>
<td>(0.3)</td>
<td>(0.4)</td>
<td></td>
</tr>
<tr>
<td>Transferrin Saturation: (%)</td>
<td>20.4</td>
<td>20.6</td>
<td>22.3</td>
<td>23.2</td>
<td>16.9</td>
<td>20.1</td>
</tr>
<tr>
<td>(8.7)</td>
<td>(8.8)</td>
<td>(9.1)</td>
<td>(7.7)</td>
<td>(4.6)</td>
<td>(10.4)</td>
<td></td>
</tr>
<tr>
<td>Ferritin: (ng.mL^-1)</td>
<td>78.4</td>
<td>78.6</td>
<td>81.8</td>
<td>79.1</td>
<td>79.6</td>
<td>84.1</td>
</tr>
<tr>
<td>(69.3)</td>
<td>(69.5)</td>
<td>(72.4)</td>
<td>(68.8)</td>
<td>(74.4)</td>
<td>(80.9)</td>
<td></td>
</tr>
</tbody>
</table>

Significantly different from resting value * = (p<0.05)
** = (p<0.01)

Data: mean(SD)
INFLUENCE OF INTENSE INTERVAL EXERCISE ON PLASMA CYTOKINE AND C-REACTIVE PROTEIN CONCENTRATIONS

A. BON GRAY\textsuperscript{1}, RICHARD D. TELFORD\textsuperscript{2}, and MAURICE J. WEIDEMANN\textsuperscript{1}

\textsuperscript{1} DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
SCHOOL OF LIFE SCIENCES
FACULTY OF SCIENCE
THE AUSTRALIAN NATIONAL UNIVERSITY,
GPO BOX 4 CANBERRA ACT 2601 AUSTRALIA.

\textsuperscript{2} DEPARTMENT OF PHYSIOLOGY AND APPLIED NUTRITION,
AUSTRALIAN INSTITUTE OF SPORT,
PO BOX 176 BELCONNEN ACT 2616 AUSTRALIA.

Running Title: Interval exercise and cytokines.
Abstract:


Eight trained male athletes of mean (SD) age = 31.5 (4.5) years; VO$_2$ max = 64.3 (3.8) mL.kg$^{-1}$.min$^{-1}$ undertook an intense interval exercise (treadmill running) protocol to exhaustion. Subjects completed an average of 15.6 one minute efforts. Plasma neopterin concentration was significantly increased at 6 and 24 hours post-exercise (p<0.05 and p<0.01 respectively) indicating activation of macrophages and T lymphocytes and implying an increase in IFN-γ synthesis. There was no significant change in plasma interleukin (IL)-1β concentration with exercise (p>0.05). Plasma TNF-α did not differ significantly from resting values at any of the post-exercise sampling points (p>0.05) and IL-6 was undetectable (<6 pg.mL$^{-1}$) in plasma either before or after exercise. Plasma creatine kinase activity was significantly elevated at 24 hours post-exercise (p<0.05) indicating muscle cell trauma but detectable plasma concentrations of C-reactive protein were not found at any sampling point. Intense interval exercise, in trained male subjects, appears to induce IFN-γ production, macrophage and T lymphocyte activation but has little effect on plasma concentrations of other
cytokines and C-reactive protein.

Key Words: anaerobic, interleukin-1, interleukin-6, interferon-γ, tumour necrosis factor, immune system activation, neopterin, creatine kinase.
INTRODUCTION:
Cytokines constitute a heterogeneous family of pleiotropic protein and glycoprotein factors that are intimately involved in regulation of the immune and other physiological systems. They include the interleukins, interferons, colony stimulating and tumour necrosis factors (3). Originally regarded as solely interleucocyte hormones they are now known to be produced by, and act on, a diverse range of human cells. Recent research has established cytokines as inter-system "communication" factors, providing links between immune, endocrine and nervous systems (4).

The cytokines interleukin (IL)-1 (produced by a range of cells including macrophages), tumour necrosis factor (TNF)-α (T lymphocytes, macrophages), IL-6 (macrophages, T lymphocytes and fibroblasts) and interferon (IFN)-γ (T cells) are known to be intimately involved in a diverse range of immune system activities (1) as well as induction of the acute phase response (APR) (26). The APR is a set of metabolic changes associated with inflammation, tissue damage, infection (18) and endurance exercise (21,33). A key manifestation of the APR is induction of hepatic acute phase protein (APP) synthesis involving increased production of acute phase proteins such as C-reactive protein (CRP). The APR appears to be protective in
nature although the specific role of many of the elements of the response remain to be determined (18).

Each of the cytokines IL-1, IL-6 and TNF-α appears to control particular components of the APR (21). Regulated release of cytokines appears to provide at least partial protection against inflammatory events such as overwhelming sepsis and bacterial and parasitic infections (9). In this study creatine kinase (CK), normally an intramuscular enzyme, will be used as an index of exercise-induced muscle damage (2).

Reports on the effects, of what to this time have been largely aerobic exercise protocols, on plasma cytokine concentrations are conflicting (see below). The area is complicated by a diversity of sampling times, use of exercise protocols of differing intensity and duration, and involvement of subjects with a range of physiological characteristics. In addition the use of different assay methodologies eg: in vitro bioassays assessing cytokine "activity" versus RIA/ELISA techniques assessing "immunoreactivity", has made interpretation of results difficult (23,27).

Despite reports of increases in IL-1 activity in plasma (7, 11, 33), and increased in vitro production following mitogenic stimulation (15,20), after extended
aerobic protocols, a number of recent reports have failed to find increases in plasma IL-1β immunoreactivity (25,27,29) following similar exercise. Similar conflicts exist in relation to the effects of aerobic exercise on plasma tumour necrosis factor (TNF)-α (8,10,27), IL-6 (15,27,29), and neopterin, produced by macrophages following stimulation by T-lymphocyte derived IFN-γ, (8,13,27,29).

Interval exercise constitutes a major component of the training regimes of many elite athletes and to this time the effects of this form of exercise on plasma cytokine concentrations, and possible induction of an APR, have not been determined.

The purpose of this study was to investigate the effect of intense interval exercise on plasma cytokine and CRP concentrations.
MATERIAL AND METHODS:

Subjects: Eight trained males, average age 31.5 (4.5) years were employed as subjects. All were, and had been for at least one year, engaged in a regular training program, involving combinations of swimming, running and cycling. All were free of symptoms of infection and none were taking medication. All refrained from food overnight and none had exercised within the prior 24 hours.

Exercise Tests: Subjects were familiarized with the testing procedures for both the maximal oxygen uptake and interval running tests, and all participants signed a consent form that detailed the scope of the study and its attendant risks. Procedures complied with human subject guidelines of the American College of Sports Medicine and were approved by the Ethics Committee of the Australian Institute of Sport.

a) Maximal Oxygen Uptake Test (Test #1): All subjects underwent a progressive treadmill running test employing increases in speed and gradient to determine their maximal oxygen uptake \((V_O_2 \text{ max})\). The initial treadmill speed was 11 km.hr\(^{-1}\) with an increase of 1 km.hr\(^{-1}\) each minute for the first seven minutes. At this stage the treadmill gradient was increased at a rate of 2% per minute. All subjects were exercised
until subjective estimation of exhaustion brought about voluntary termination of the test. Respiratory analysis was performed as described previously (16). Criteria for attainment of VO$_2$ max were: an R value in excess of 1.10, a "plateau" in oxygen consumption (an increment of less than 0.15 L.min$^{-1}$), or a heart rate equal to the predicted maximum.

b) **Interval Running Test (IRT) (Test #2):** This test was performed at least one week following Test #1. All subjects underwent a standard warm-up procedure involving 5 minutes running at both 7 and 11 km.hr$^{-1}$ and 3 minutes at 15 km.hr$^{-1}$. All warmup procedures were performed at 0% treadmill gradient. Subjects then performed alternating one minute periods of treadmill running and active (walk) recovery. Treadmill speed and gradient for each 1 minute exercise period were set at the levels under which VO$_2$ max. was achieved i.e.: same speed and gradient. Subjects were able to walk about the laboratory (at their own pace) during the 1 minute recovery periods. All subjects were informed of the elapsed time every 15 seconds and all received encouragement during the course of each repetition. Subjects were asked to perform as many exercise repetitions as possible, and continued until they could no longer maintain the required speed.
Blood Collection: Blood samples, obtained with subjects in the supine position, were obtained from venipuncture of an antecubital vein. Five samples were collected from each subject; before exercise (after the subject had been sitting quietly in the laboratory for 20 minutes), immediately after exercise, and 1, 6, 24 hours post-test. Samples were collected into EDTA Vacutainers (Becton Dickinson, Mountain View, Ca.) and were immediately separated by centrifugation at 4°C. Aliquots were stored at -20°C prior to analysis.

Full Blood Counts: A Coulter S550 cell counter (Coulter Electronics, Hialeah, Fl.) was employed to obtain a full blood count, including haematocrit.

Whole Blood Lactate: All blood samples were analysed for whole blood L-lactate with a YSI Model 23L L-lactate analyser (Yellow Springs Instrument Company, Yellow Springs, Ohio).

Assays: These were carried out in duplicate via use of commercially available assay kits as indicated. Only samples collected pre-, and 1, 6, 24 hrs post-test were assayed for cytokines, CRP and CK.

Interleukin-1β, interleukin-6, tumour necrosis factor-α: Immunoradiometric assays (IRMA) (Medgenix,
Australian Laboratory Services, Sydney, NSW) (27).

Neopterin: Radioimmunoassay (Henning, Australian Laboratory Services, Sydney, NSW) (27,29).

C-reactive protein: Rate nephelometry (Beckman Assay Systems, Beckman Instruments, Sydney, NSW).

Creatine Kinase Activity: Kodak Ektachem dry chemistry analyser (Eastman Kodak, Sydney, NSW).

Statistics: Data were analysed by a one-way ANOVA with repeated measures. Fisher’s PLSD was used for post-hoc comparisons. Significance was indicated at the p<0.05 level. Data are presented as mean (standard deviation).
RESULTS:

1. Physical and Physiological Characteristics of Subjects. Details of the subjects appear in Table 1. The intensity of interval exercise is indicated by the significantly elevated whole blood lactate concentration measured immediately post-IRT ($p<0.01$) which indicates a substantial contribution from anaerobic energy pathways during this protocol. Subjects completed an average of 15.6 one minute efforts.

2. Variation of Cytokines and Neopterin with Interval Exercise. Plasma neopterin was significantly elevated by 10% and 14% (relative to resting values) at 6 hours and 24 hours post-test respectively ($p<0.05$) (Table 2). These changes were not likely to be due to haemoconcentration, as haematocrits measured at 1, 6 and 24 hrs post-test, did not differ significantly from the value obtained at rest ($p>0.05$, data not shown). According to the method of van Beaumont (30), plasma volume was estimated to have changed as indicated: 1 hr ($-1.7\%$), 6 hr ($+3.3\%$) and 24 hr ($+0.5\%$).

There was no significant change in plasma IL-1B concentration with exercise ($p>0.05$) despite a 16% increase 6 hr post-test. Generally IL-6 could not be detected in the plasma of subjects either before or
after exercise. Only two subjects had resting plasma concentrations greater than 6 pg.mL\(^{-1}\), the minimum detection limit with the assay employed. TNF-\(\alpha\) was not significantly elevated from resting levels at any of the post-exercise sampling points despite a 17\% elevation in mean concentration at both 1 and 24 hours after exercise.

3. Variation of C-Reactive Protein and Creatine Kinase

The mean plasma concentration of CRP was below the detection limit of the assay employed (< 1 \(\mu\)g.mL\(^{-1}\)) at all sampling points, indicating that a demonstrable acute phase response was not associated with this form of exercise. However plasma CK activity was significantly elevated above resting levels at 24 hours post-exercise (p<0.01) suggesting post-exercise muscle trauma (Table 3).
DISCUSSION:
This study is one of the first to investigate the effects of intense interval exercise on plasma cytokine concentrations. We have shown that such exercise is associated with both a significant increase in plasma neopterin concentrations, and no significant change in plasma concentrations of either TNF-α or IL-1β.

Neopterin, derived from macrophages, has been routinely employed as a "marker" of IFN-γ production. Thus an elevation of plasma neopterin concentration is indicative of both a primary activation of T lymphocytes (known to produce IFN-γ) and a secondary activation of macrophages. Such activation is consistent with the ability of aerobic exercise to activate human monocytes (5) and macrophages (12). The specific effects of the exercise protocol employed in this study, on the activation-state of human macrophages remain to be determined.

Plasma neopterin concentration (immunoreactivity) has been reported to be similarly elevated (8, 24, 29), or unchanged (27) (as has IFN-γ; 32), following aerobic exercise. Other reports relating to the effects of aerobic exercise on in vitro production of IFN-γ following mitogenic stimulation (15), are largely irrelevant, as they assess only the potential of cells
to respond to, what are typically, non-physiological challenges.

The plasma concentration of IL-1β was not significantly altered by this interval exercise protocol. Thus our findings, even though based on an exercise protocol with a large anaerobic component, agree with an increasing number of recent reports that IL-1β immunoreactivity is unchanged by aerobic exercise (25,27,29). These reports are in conflict with others indicating increased IL-1 activity in plasma following extended aerobic exercise (7,20,33).

There are conflicting reports as to the effects of aerobic exercise on plasma TNF-α concentrations, with reports of increases (8,29) and no change (27) following exercise. In vitro production of TNF-α has been reported to be unaffected by exercise (15). As far as a comparison can be made our findings agree with those of Smith et al. (27) concerning no change in plasma TNF-α immunoreactivity with aerobic exercise.

We had difficulty in determining plasma IL-6 concentrations as did Smith et al. (27) and Sprenger et al. (29), all three studies employing the same assay methodology. The former reported being only able to detect IL-6 in the plasma of 4 of 8 control
(unexercised) subjects, while the latter reported a pre-exercise plasma concentration of $0 \text{ pg.mL}^{-1}$. Thus our findings are not without precedent.

The intense interval exercise protocol appears to have induced a differential activation of macrophages. Our results imply an exercise-induced activation of macrophages to produce IFN-$\gamma$ but apparently not IL-1$\beta$, IL-6 or TNF-$\alpha$. However as shown by Sprenger et al. (29) reference to only plasma cytokines may limit our understanding of the immunological processes operating during and after exercise. It is apparent from the study of Sprenger et al. (29) that urinary excretion of a range of cytokines increases after exercise, despite little change in plasma concentrations. Thus it is possible that macrophage and T cell activation occurs during and after exercise, but only in particular tissue microenvironments, so that plasma concentrations may only represent a "spill-over" from such sites.

The conflicting findings previously mentioned illustrate the need to differentiate between "immunoreactivity" and in vitro "activity" when considering assay methodology. The former method has the advantage of great specificity in identification of particular epitopes but the relationship between "immunoreactivity" and physiological effects are not,
in most cases, well established. The latter methods, predominantly involving in vitro bioassays are, as indicated by Northoff & Berg (23), often lacking in specificity, with more than one cytokine able to stimulate a biological response. This area of research is complicated by a plethora of exercise protocols, and differences in both the physiological characteristics of the subjects employed and the times at which blood was collected. In addition the limitations of particular assay techniques must be examined very carefully.

The mechanism by which exercise modulates plasma and urinary cytokine concentrations has not been determined. Each exercise protocol could be expected to exert a range of both "positive" and "negative" effects on cytokine production. The net result of these competing effects could be expected to depend on a number of, what are as yet, undetermined factors. Factors tending to promote cytokine production may include a post-exercise endotoxaemia (6), which may possibly play a role in elevation of neopterin (19) and other cytokines, as could post-exercise tissue damage (18). On the other hand elevation of immunosuppressive hormones such as cortisol and the catecholamines by this protocol (Gray et al., unpublished observations) may have directly inhibited cytokine production.
Support for this hypothesis comes from the findings of Cannon et al. (7), that combinations of hydrocortisone and epinephrine suppressed in vitro IL-1 production. This is supported by independent studies concerning the suppressive action of both catecholamines and corticosteroids in relation to IL-1 production (17,28), TNF-α (22) and IL-6 (14). The plasma concentration of both hormones is directly dependent on the intensity of exercise (31), suggesting a possibly mechanism for modulation of plasma cytokine concentrations in relation to this protocol.

Despite the significant elevation of plasma CK activity at 24 hours post-exercise, which is in agreement with previous findings (35), the induction of an APR could not be demonstrated. Liesen et al. (21) and Weight et al. (33) have shown that CRP concentrations were elevated maximally 24 hours after extended aerobic exercise. Thus while it could be postulated that the kinetics of an APR induced by intense interval exercise may differ from that previously reported (21,33), we failed to detect any elevation of CRP at 24 hours post-exercise. It appears that either the degree of post-exercise tissue damage was insufficient to induce an APR or other factors, possibly hormonal, were acting to suppress both production of cytokines and induction of the APR. However while the increase in plasma
neopterin concentrations tends to negate this latter argument it is possible that cytokine production may be affected differentially by hormones.

In conclusion, this study has demonstrated that intense, interval exercise is associated with an increase in plasma neopterin concentration, suggesting IFN-γ production and both macrophage and T lymphocyte activation. However this form of exercise appears to not produce significant increases in the plasma concentration of the cytokines IL-1, IL-6, TNF-α nor CRP.
ACKNOWLEDGEMENTS

The authors wish to thank David Pyne, Don Campbell and members of the Canberra Triathlon Club for helpful discussions and assistance with this investigation.

Correspondence to:—

A.B.Gray

Department of Physiology and Applied Nutrition,
Australian Institute of Sport,
PO Box 176, Belconnen ACT 2616 Australia.

ph: 011 616 252 1253
fax: 011 616 252 1603
REFERENCES:


**TABLE 1: Physical and Physiological Characteristics of Subjects**

<table>
<thead>
<tr>
<th>Maximal Oxygen Uptake (mL.kg(^{-1}).min(^{-1}))</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Repetitions Completed</th>
<th>La post-IRT (mmol.L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.3 (3.8)</td>
<td>31.5 (4.5)</td>
<td>74.0 (8.8)</td>
<td>15.6 (6.6)</td>
<td>7.6 ** (1.6)</td>
</tr>
</tbody>
</table>

La = lactic acid.

Significantly different from resting value: ** = p<0.01
TABLE 2: Variation of Cytokines and Neopterin with Exercise:

<table>
<thead>
<tr>
<th></th>
<th>Sampling time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rest</td>
</tr>
<tr>
<td>Interleukin-1β: (pg.mL⁻¹)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>(3.3)</td>
</tr>
<tr>
<td>Interleukin-6: (pg.mL⁻¹)</td>
<td>ND</td>
</tr>
<tr>
<td>Neopterin: (pg.mL⁻¹)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1507.9</td>
</tr>
<tr>
<td></td>
<td>(374.4)</td>
</tr>
<tr>
<td>Tumour Necrosis Factor-α: (pg.mL⁻¹)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>(2.5)</td>
</tr>
</tbody>
</table>

Significantly difference from rest: * = p<0.05

**= p<0.01

Not detectable = ND (< 6 pg.mL⁻¹).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rest</th>
<th>1 hour</th>
<th>6 hour</th>
<th>24 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (μg.mL⁻¹)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CK (U.L⁻¹)</td>
<td>124.0</td>
<td>155.8</td>
<td>179.4</td>
<td>246.3 **</td>
</tr>
<tr>
<td></td>
<td>(28.3)</td>
<td>(34.2)</td>
<td>(94.7)</td>
<td>(139.8)</td>
</tr>
</tbody>
</table>

Significantly different from resting value:  * = $p<0.05$
                                           ** = $p<0.01$
THE RESPONSE OF LEUCOCYTE SUBSETS AND PLASMA HORMONES TO INTERVAL EXERCISE.

A.B.GRAY 1, R.D.TELFORD 2, M.COLLINS 3, M.J.WEIDEMANN 1

1 DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY
SCHOOL OF LIFE SCIENCES
THE AUSTRALIAN NATIONAL UNIVERSITY,
GPO BOX 4 CANBERRA ACT 2601 AUSTRALIA.

2 DEPARTMENT OF PHYSIOLOGY AND APPLIED NUTRITION,
AUSTRALIAN INSTITUTE OF SPORT,
PO BOX 176 BELCONNEN ACT 2616 AUSTRALIA.

3 DEPARTMENT OF MATHEMATICS,
UNIVERSITY COLLEGE
AUSTRALIAN DEFENCE FORCE ACADEMY.
NORTHCOTT DRIVE, CANBERRA ACT 2600 AUSTRALIA

Running Title: Interval exercise and leucocyte subsets.
ABSTRACT:
Aerobic exercise has an established role in modulation of peripheral leucocyte concentrations. However the effects of intense interval exercise, as employed by athletes in a range of sports, has been given little attention. Eight trained male athletes of mean age (SD) = 31.5 (4.5) years; VO₂ max = 64.3 (3.8) mL.kg.⁻¹.min.⁻¹ undertook an intense interval exercise protocol (treadmill running) to exhaustion. Subjects completed an average of 15.6 one minute efforts. The protocol produced a biphasic leucocytosis: an initial (immediately post-test) leucocytosis resulting from mobilisation of lymphocytes (CD3⁺, CD4⁺, CD8⁺, CD3⁻CD16⁺/56⁺, CD3⁺HLA/DR⁺) (all p<0.01), with the later (6 hour) leucocytosis resulting from mobilisation of granulocytes and monocytes (both p<0.01). This protocol modified significantly the peripheral blood concentration of the hormones cortisol (both total and free), norepinephrine, DHPG and dopamine (all p<0.01). Modulation of peripheral leucocyte subsets induced by interval exercise correlated with both the number of exercise efforts performed and the concomitant changes in peripheral hormone concentrations. Sustained alterations in plasma catecholamine levels in the post-test period may have important metabolic and
immunological implications for athletes undertaking regular interval training.

**Keywords:** Anaerobic, lymphocyte, granulocyte, monocyte, hormone, flow cytometry, monoclonal antibody, cortisol, catecholamines.

--------------
INTRODUCTION:
Interval exercise (that is, alternating periods of activity and recovery) was popularised among track & field athletes by Gerschler in the 1930's. Interval exercise is now also used widely by athletes engaged in a range of sports including swimming and football. However little of the research into the effects of exercise on human physiological systems, including the immune system, has investigated interval exercise. Those few studies that employed interval exercise often used protocols that were irrelevant to the actual training regimes of athletes.

The ability of exercise to alter the circulatory dynamics and homeostasis of the human immune system is well established (16,23,25). Aerobic exercise produces a transient leucocytosis that is biphasic and dependent on the duration and intensity of the exercise. The response initially involves lymphocytes and then, later, granulocytes (see review by McCarthy and Dale, 25), monocytes (2), and alterations in the proportions of lymphocyte subsets [CD3+, T lymphocytes; CD4+, T helper-inducer lymphocytes; CD8+, T cytotoxic-suppressor lymphocytes; CD19+, B lymphocytes; CD16+/CD56+, natural killer (NK) cells; 16,23].

Exercise-induced immunomodulation is thought to be mediated by an interplay of hormones, cytokines, neural
and haematological factors (23). Many of the systems responsible for elaboration of these factors display both an activation threshold and a pattern of response that is related to exercise intensity (and hence the anaerobic energy contribution). For example both the sympatho-adrenal and hypothalamic-adrenocortial axes (which produce epinephrine and cortisol respectively) display both of these features (33) and are vitally involved in meeting the physiological demands of intense exercise.

Intense interval exercise, requiring a significant anaerobic energy contribution (30), may subject the immune system to a markedly different "internal milieu" from that encountered during aerobic exercise. These differences may be manifested in the leucocyte subsets mobilised within the peripheral circulation. It is hypothesised that intense interval exercise will lead to both qualitative and quantitative changes in the circulating concentrations of peripheral blood leucocyte subset concentrations from those existing at rest, and those produced by other more aerobic exercise regimes.

The purpose of this investigation was to study the effects of intense, interval exercise (running) on the peripheral blood concentrations of total leucocytes, leucocyte subsets and immunomodulatory hormones,
including the catecholamines (19,32) [epinephrine (Ep),
norepinephrine (NE), dopamine (DA, a precursor for Ep
and NE synthesis), 3,4-dihydroxy-phenylglycol (DHPG; a
neuronal metabolite of NE)] and cortisol (both total
and free) (34), that are likely to influence leucocyte
distribution and/or function.

MATERIALS AND METHODS:

Subjects: Eight trained male triathletes, mean age (SD)
= 31.5 (4.5) years, were employed as subjects. All
were, and had been for at least one year, engaged in a
regular training program, involving combinations of
running, cycling and swimming. Most were competing at
either district or state level. All were free of
symptoms of infection and none were taking medication.
All had refrained from food overnight and none had
exercised within the prior 24 hours.

Exercise Tests: Subjects were familiarised with the
testing procedures for both the determination of
maximal oxygen uptake (VO₂ max) and interval running
tests. All testing commenced between 08.00 and 09.00
hours. Procedures complied with human subject
guidelines of the American College of Sports Medicine
and were approved by the Ethics Committee of the
Australian Institute of Sport.
Maximal Oxygen Uptake Test (Test #1): All subjects underwent a progressive treadmill running test employing increases in speed and gradient to determine their VO\textsubscript{2} max. All exercised until a subjective estimation of exhaustion brought about voluntary termination of the test. Criteria for attainment of VO\textsubscript{2} max were: an R value in excess of 1.10, a "plateau" in oxygen consumption (an increment of less than 0.15 L.min\textsuperscript{-1}), or a heart rate equal to the predicted maximum. Procedures were as previously described (17).

Treadmill Interval Running Test (IRT) (Test #2): This test was performed at least one week following test #1. All subjects underwent a standard warm-up procedure involving 5 minutes running at both 7 km.hr\textsuperscript{-1} and 11 km.hr\textsuperscript{-1} and 3 minutes at 15 km.hr\textsuperscript{-1}. All warmup procedures were performed at 0\% treadmill gradient. Subjects then performed alternating one minute periods of treadmill running and active (walk) recovery. Subjects were allowed to walk freely about the laboratory between exercise repetitions. Treadmill speed and gradient for each repetition were individually selected using the same speed and gradient at which VO\textsubscript{2} max. was achieved. Subjects were informed of the elapsed time every 15 seconds and all received encouragement during the course of each exercise period. Subjects were asked to perform as many exercise efforts as possible, and continued until they could not
maintain the required speed.

**Blood Sampling and Analysis:** Blood samples, obtained with subjects in a supine position, were collected by venipuncture of an antecubital vein. Six samples were collected for each subject; before exercise (after the subject had been sitting quietly in the laboratory for 20 minutes), post warm-up, post-test, 1, 6, and 24 hours post-test. Samples were placed immediately into EDTA Vacutainers (Becton Dickinson, Mountain View, Ca.) prior to analysis, which was in all cases completed within 90 minutes for full blood counts, and within 3 hours for leucocyte enumeration.

Full blood counts (including haematocrit) were obtained using a Coulter S550 Cell Counter (Coulter Electronics, Hialeah, FL). Quality control was evaluated via daily analysis of a standard blood preparation; (4C, Coulter Electronics, Hialeah, FL.) and was in all cases within acceptable limits. Leucocyte differential were determined by flow cytometry.

**Leucocyte Enumeration:** Leucocytes were enumerated via use of the Simultest IMK Plus (Becton Dickinson, Mountain View, Ca.) which contained the following murine, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated, monoclonal antibodies to human leucocyte antigens (with associated primary
specificities):

anti-leucocyte (CD45⁺), anti-Leu-M3 (CD14⁺, monocytes), anti-Leu-4 (CD3⁺, T lymphocytes), anti-Leu-12 (CD19⁺, B lymphocytes), anti-Leu-3 (CD4⁺, T helper/inducer lymphocytes), anti-Leu-2 (CD8⁺, T cytotoxic/suppressor lymphocytes), anti-HLA/DR⁺ (B lymphocytes and activated T lymphocytes), anti-Leu-11 (CD16⁺) and anti-Leu-19 (CD56⁺; Natural Killer cells and cytotoxic T cells), FITC-labelled control IgG₁ and PE-labelled control IgG₂a monoclonal murine antibodies.

The monoclonal antibody preparation (20µl) and whole blood (100µl) were placed into polystyrene test tubes (12 x 75mm, Falcon # 2054 Bacto Laboratories, Sydney), and processed according to the manufacturers instructions. Samples were placed in paraformaldehyde (0.5ml/1%) before being stored in the dark, at 4°C, for analysis within 24 hours of staining.

**Flow Cytometry:** A FACS 440 cell sorter (Becton Dickinson, Mountain View, Ca), operated with appropriate forward and 90° light-scatter settings (to exclude red blood cells and debris) was used to identify lymphocyte, granulocyte and monocyte subpopulations. List mode data was collected on 20,000 cells per sample. Determination of leucocyte differentials (granulocytes, lymphocytes and monocytes)
and enumeration of specific, positively-stained leucocyte subsets was obtained through use of the WEFCS software package (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The set of six monoclonal antibody stainings obtained per subject were processed as previously described (21). This enabled an accurate determination to be made of the "purity" of the lymphocyte gate and correction of the values obtained previously.

**Blood Lactate Analyses:** All peripheral blood samples were analysed for whole blood L-lactate using a YSI L-lactate analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio).

**Hormone Analyses:** Both total cortisol (Ciba-Corning, Australian Diagnostics, Sydney) and cortisol binding globulin (Medgenix, Australian Laboratory Services, Sydney) were analysed by use of commercially available RIA kits.

Free cortisol was calculated using the following equations:-

* U = molar concentration of unbound cortisol.
* C = molar concentration of total cortisol
* T = molar concentration of cortisol binding globulin (CBG)
\[
Z = 0.0167 + 0.182(T-C)
\]
\[
U = (ZxZ + 0.0122C)^{1/2} - Z
\]

Catecholamines were assayed by gas chromatography using methods described previously (10).

**Statistics:** Data were analysed by a repeated measures one-way ANOVA. Fisher's PLSD was used for post-hoc comparisons. Correlation analyses (Pearson Product Moment), including assessment of partial correlations, were also carried out. Significance was indicated at the \( P<0.05 \) level.
RESULTS:

1. Physical and Physiological Characteristics of Subjects: The physical and physiological characteristics of the subjects are presented in Table 1. The intensity of interval exercise is indicated by the significant elevation immediately after completion of the IRT in whole blood lactate concentration (p<0.01). Lactate concentration, which was also elevated following the warm-up procedure (p<0.05), was not significantly different from resting values at other sampling points (data not shown).

2. Leucocyte Subpopulations:
Intense interval exercise resulted in a significant leucocytosis immediately post-test (p<0.01; 65% increase) with contributions from all main subpopulations as indicated: lymphocytes (129%), granulocytes (51%) and monocytes (45%) (Table 2). The significant increase in lymphocyte concentration at this time was composed of differential elevations in CD3+ (100%), CD4+ (37%), CD8+ (180%), CD19+ (36%), CD3+HLA/DR+ (activated T lymphocytes) (100%) and CD3−CD16+/56+ (NK cells) (295%) subpopulations (Table 3). The CD4+:CD8+ ratio decreased to 0.71 at this time. This lymphocytosis was transient. At 1 hour after completion of the test a relative lymphopenia had developed as a result of decreases in concentrations below resting values of the following subpopulations.
(percentage decrease); CD3⁺ (35%), CD4⁺ (25%), CD8⁺ (46%), CD19⁺ (18%), CD3⁻CD16⁺/56⁺ (52%). The CD4⁺:CD8⁺ ratio was significantly elevated at this time (p<0.01).

The initial leucocytosis was sustained at both 1 hour (71% elevation) and 6 hours (75% elevation) post-test (both p<0.01). In contrast to the lymphocyte changes granulocyte (120%) and monocyte (45%) concentrations were elevated significantly at 6 hours after cessation of exercise.

All leucocyte values, whether expressed on an absolute or a proportional basis had returned to resting values at 24 hours after the exercise test.

Haemoconcentration made only a minor contribution to the elevation of leucocyte subset concentrations observed throughout the period after exercise. Haematocrit was significantly different from rest only in those samples taken post-warmup and post-test (p<0.05). According to the method of van Beaumont (31) these changes corresponded to estimated decreases of 6.4% and 10.2% in plasma volume, respectively (data not shown).

3. Hormone Concentrations:
Total cortisol concentrations were elevated significantly immediately (43%) and 1 hour post-test
Free cortisol also increased significantly 1 hour post-test (82%) (p<0.01). There was a significant decrease in total cortisol, free cortisol and percent free cortisol (all p<0.01) at 6 hours post-test (late afternoon). No significant changes in cortisol binding globulin were detected at any post-test sampling point.

Ep did not differ significantly from resting values at any of the sampling points. Immediately post-test the plasma concentration increased by approximately 22% (p>0.05), but half of this change can be accounted for by haemoconcentration. NE was only significantly different from resting values at the immediate post-test sampling point where it was elevated by 108% (p<0.01). DA concentrations were significantly lower than resting values at all post-test sampling points, including at 24 hours (p<0.01) when it remained at only 31% of the pre-exercise value. DHPG was increased significantly only immediately post-exercise when it was elevated by 760% (p<0.01). The ratio of NE to DHPG, referred to as the "N" ratio, was significantly decreased below resting values immediately, 1 and 6 hours post-exercise.

4. Correlations:
The number of exercise efforts performed correlated significantly with both the total plasma cortisol
concentration immediately post-test (r=+0.73, p<0.05) and with the granulocyte concentration at 1 hour post-test (r=+0.72, p<0.05). The post-test total cortisol concentration was not significantly correlated with total leucocyte, lymphocyte or granulocyte concentrations whether assessed immediately, or 1 hr post-test (all p>0.05). Concentrations of neither lymphocytes nor granulocytes, when assessed immediately and 1 hr post-test), correlated significantly with post-exercise Ep and NE concentrations (all p>0.05). DHPG concentration immediately post-test was significantly correlated with lymphocyte and CD8⁺ concentrations (r=+0.73 and +0.72 respectively, both p<0.05) but not with either CD4⁺ or NK concentrations at this time (p>0.05). Immediately post-test Ep concentration correlated positively with the mobilisation (percentage change relative to rest) of granulocytes observed between rest and 1 hr post-test (r=+0.74, p<0.05) and total leucocytes between rest and 6 hr post-test (r=+0.80, p<0.05).

An analysis of partial correlations, to examine possible interactions between cortisol and the catecholamines in mobilisation of leucocyte subsets, did not reveal significant effects in this regard (data not shown).
DISCUSSION:

This investigation has established that intense, interval exercise, at the level of maximal oxygen uptake, produces significant alterations in the concentrations of both leucocyte subsets and immunomodulatory hormones in the peripheral circulation.

Despite this protocol requiring a significant anaerobic energy contribution, as indicated by the elevated post-test whole blood lactate concentration, the pattern of leucocyte mobilisation was very similar to that produced by other, more aerobic exercise protocols (16,23).

The mobilisation of leucocyte subsets occurred on a differential basis and gave rise to a biphasic leucocytosis. The leucocytosis is similar to that described by McCarthy & Dale (25), whose model of a biphasic leucocytosis rests on an initial catecholamine-dependent lymphocytosis, and later cortisol-dependent, granulocytosis. Our results support the former but not the latter mechanism. Ep has also been found to contribute to the redistribution of granulocytes from margined to circulating pools (1). Our data support this finding, as mobilisation of granulocytes at 1 hr post-test was significantly correlated with post-test Ep concentrations.
The initial leucocytosis was composed primarily of lymphocytes but also included increases in the peripheral concentration of both monocytes and granulocytes. The later leucocytosis (1-6 hrs) can be attributed to granulocytes (at 1 and 6 hours) and monocytes (6 hours). The delayed post-exercise monocytosis supports earlier findings of Bieger et al. (3) and suggests the existence of a marginated pool of monocytes or of enhanced monocytopoiesis.

The initial transient lymphocytosis may have been influenced by three factors. Firstly, increased cardiac output associated with exercise (12) producing elevated shear stress on, and mobilisation of, cells marginated on the vascular endothelium. A second factor may relate to the enhanced perfusion of "low flow" and storage areas such as the lungs (26) and spleen (27,32) respectively. Thirdly, an epinephrine dependent component as detailed by Crary et al. (5) and van Tits et al. (32), producing a similar lymphocyte mobilisation pattern to that described here, and possibly produced as a result of attenuated lymphocyte adherence to vascular endothelium (3).

The pattern of mobilisation of lymphocyte subsets, notably the elevated concentrations of CD3+, CD4+, CD8+ and CD3−CD16+/56+ lymphocytes and the significant decrease in the CD4+:CD8+ ratio immediately
post-exercise (attributable to a larger mobilisation of CD8+ than CD4+ lymphocytes), corresponds closely to changes detailed previously, in relation to exercise of a predominantly aerobic nature (23) and short term anaerobic exercise (14) and following infusion of β2-adrenergic agonists (32). The rapid increase in activated T lymphocytes (CD3−HLA/DR+) implies mobilisation from storage areas rather than activation of new cells.

The similarities to previous studies (16,23,25), in terms of leucocyte subset mobilisation, are not reflected in the hormonal, and specifically the catecholamine profile, measured immediately post-test. In spite of an exercise intensity well above the activation threshold for the adreno-sympathetic system (33), plasma Ep and NE concentrations, when assessed immediately post-test, were only minimally elevated. These concentrations were well below those previously reported following both largely aerobic (18) and anaerobic (22) exercise.

Our findings thus create something of a paradox. Previous investigations (17,24,32) have established that exercise-induced mobilisation of lymphocytes is largely a β2-adrenoceptor and spleen dependent process, produced predominantly by the actions of Ep rather than NE. Ep infusion produces a preferential mobilisation of
lymphocyte subsets expressing a relatively high density of $\beta_2$-adrenergic receptors (ie: CD8$^+$ and NK cells rather than CD4$^+$ lymphocytes), resulting in changes comparable to those produced by exercise. However, as we have shown, plasma Ep concentrations were not significantly elevated following this protocol, only those of NE. Thus to what agents can the mobilisation of lymphocytes produced by this intense interval exercise protocol be ascribed? Fortunately assessment of other compounds, that relate directly to the metabolism of catecholamines, may help to elucidate the processes involved.

A highly significant increase in the plasma concentration of DHPG, a neuronal metabolite of NE, immediately post-test, indicates a significant neuronal release of NE during this protocol. In addition the reduction in the N ratio (NE:DHPG) immediately post-test indicates that neuronal rather than extra-neuronal (ie: adrenal medullary) release of NE was predominant during this protocol.

The pattern of leucocyte subset mobilisation induced by the IRT, together with the known intensity of exercise, argues strongly for a significant elaboration of both Ep and NE during the IRT, thus contributing to leucocyte mobilisation. Cessation of exercise, which in this case was due to volitional exhaustion (ie: an
inability to continue at the required speed and gradient), may have been due to exhaustion of catecholamine reserves. The sustained post-test (24 hour) depression in circulating DA may indicate that the elaboration of Ep and NE during the IRT may have depleted stores of this important precursor for the synthesis of both Ep and NE. In this regard Lehmann et al. (20) have reported a reduction in urinary catecholamine excretion during periods of heavy training and competition. It is possible that repeated bouts of high intensity exercise will result in a reduced ability, at least in the short term, of the athlete to meet those metabolic demands mediated by catecholamine secretion, for example muscle and liver glycogenolysis (29).

A recent study by Fry et al. (13) has shown that a similar interval training protocol produced a transient suppression of lymphocyte proliferative ability in response to mitogenic stimulation. While neither lymphocyte activity, nor the ability to respond to antigenic stimulation, has been assessed in this study it is apparent that elevation of Ep concentrations during exercise has the potential to suppress a range of lymphocyte functions (4,11).

The lymphocytosis detected immediately post-exercise was short-lived. At 1 hour post-exercise a
transient relative lymphopenia (resolved by 6 hours post-test) and a significant granulocytosis had developed. Changes of this type observed in other more aerobic protocols have been ascribed to an exercise induced increase in circulating cortisol (25). Corticosteroids (eg: hydrocortisone) have an established role in producing both lymphopenia (8,9) and granulocytosis (6). Both processes are thought to involve the bone marrow, the former as a consequence of temporary sequestration within the bone marrow whilst the latter appears to result from release of cells from their site of production. However our results do not support a role for cortisol in post-exercise elevation of the peripheral concentration of either total leucocytes or granulocytes.

The exercise protocol used in the present study did induce significant, and sustained, elevations in concentrations of both free and total cortisol; the 65% elevation in free cortisol at 1 hour post-exercise indicates that the immune system was exposed to high relatively concentrations of free cortisol for a prolonged period post-exercise. Cortisol has an established role in producing a broad spectrum immuno-suppression (34). The susceptibility of athletes to infection during periods of intense training (7) and competition (28) may be related to a sustained post-exercise elevation of cortisol. The
immunosuppressive, as opposed to lymphopenic, actions of cortisol in relation to this protocol remain to be determined. However the negative correlations between total cortisol and circulating lymphocyte concentration both immediately-post, and 1 hr post-test did not reach significance and thus do not support the view that cortisol is a significant lymphopenic agent in relation to this exercise protocol.

The number of repetitions performed correlated positively with the granulocyte concentration at 1 hour post-exercise. This may be related to exposure to elevated concentrations of cortisol both during and after the exercise period. This explanation is supported, at least in part, by the significant correlation between the number of repetitions performed and the total cortisol concentration observed immediately post-test.

It must be emphasised that the hormonal and leucocyte changes described in this study result from a single interval exercise (training) session. Athletes commonly employ interval training on a regular, sometimes daily basis. The effects, on the immune system, of such repeated high intensity exercise remain to be determined.
These data indicate that exercise induced modulation of leucocyte subsets, possibly mediated via increased adreno-sympathetic activity, is correlated with changes in the peripheral concentrations of endocrine hormones and the number of exercise efforts completed.
ACKNOWLEDGEMENTS: The authors thank Mr.Antony Parker, Biochemistry Department, Australian Institute of Sport, and Mr.Geoff Osborne, Flow Cytometry Unit, Australian National University, for expert technical assistance.

Correspondence to:-

A.B.Gray
Department of Physiology and Applied Nutrition, Australian Institute of Sport, PO Box 176, Belconnen ACT 2616 Australia.

ph: 011 616 252 1253
fx: 011 616 252 1603
REFERENCES:


Table 1: Subject Characteristics:

<table>
<thead>
<tr>
<th>Maximal Oxygen Uptake (mL.kg(^{-1})min(^{-1}))</th>
<th>Age (years)</th>
<th>Mass (kg)</th>
<th>Intervals completed</th>
<th>La post-IRT (mmol.L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>64.3</td>
<td>31.5</td>
<td>74.0</td>
<td>15.6</td>
<td>7.6 **</td>
</tr>
<tr>
<td>(3.8)</td>
<td>(4.5)</td>
<td>(8.8)</td>
<td>(6.6)</td>
<td>(1.6)</td>
</tr>
</tbody>
</table>

La = lactic acid

Significantly different from resting value: ** = p<0.01
### Table 2: Leucocyte Concentrations:

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Post-test</th>
<th>1 hour post</th>
<th>6 hour post</th>
<th>24 hour post</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Leucocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.16</td>
<td>6.30</td>
<td>8.54**</td>
<td>8.83**</td>
<td>9.03**</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>(0.77)</td>
<td>(0.94)</td>
<td>(2.00)</td>
<td>(3.48)</td>
<td>(1.44)</td>
<td>(0.83)</td>
</tr>
<tr>
<td><strong>Lymphocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>1.61</td>
<td>3.05**</td>
<td>0.87</td>
<td>1.30</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>(0.42)</td>
<td>(0.49)</td>
<td>(1.25)</td>
<td>(0.26)</td>
<td>(0.44)</td>
<td>(0.39)</td>
</tr>
<tr>
<td><strong>Monocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>0.50</td>
<td>0.68*</td>
<td>0.62</td>
<td>0.77**</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>(0.15)</td>
<td>(0.19)</td>
<td>(0.27)</td>
<td>(0.37)</td>
<td>(0.33)</td>
<td>(0.16)</td>
</tr>
<tr>
<td><strong>Granulocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.36</td>
<td>4.09</td>
<td>5.09*</td>
<td>7.40**</td>
<td>6.96**</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>(0.48)</td>
<td>(0.67)</td>
<td>(0.95)</td>
<td>(3.24)</td>
<td>(1.18)</td>
<td>(0.53)</td>
</tr>
</tbody>
</table>

Data: mean (SD)

Concentrations: x 10^9 L^-1

Significantly different from resting value: * = p<0.05
** = p<0.01
Table 3: **Lymphocyte Subset Concentrations**

<table>
<thead>
<tr>
<th>CD3⁺</th>
<th>1.00 (0.32)</th>
<th>1.11 (0.38)</th>
<th>2.01** (0.99)</th>
<th>0.65 (0.22)</th>
<th>0.98 (0.36)</th>
<th>0.96 (0.30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺</td>
<td>0.54 (0.25)</td>
<td>0.58 (0.25)</td>
<td>0.74** (0.27)</td>
<td>0.40* (0.13)</td>
<td>0.60 (0.27)</td>
<td>0.54 (0.22)</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>0.45 (0.18)</td>
<td>0.57 (0.23)</td>
<td>1.29** (0.70)</td>
<td>0.24 (0.12)</td>
<td>0.35 (0.13)</td>
<td>0.42 (0.17)</td>
</tr>
<tr>
<td>CD19⁺</td>
<td>0.11 (0.05)</td>
<td>0.11 (0.05)</td>
<td>0.15* (0.05)</td>
<td>0.09 (0.03)</td>
<td>0.15* (0.07)</td>
<td>0.11 (0.05)</td>
</tr>
<tr>
<td>CD3⁻CD16⁺/56⁺:</td>
<td>0.21 (0.15)</td>
<td>0.36 (0.33)</td>
<td>0.83** (0.33)</td>
<td>0.10 (0.05)</td>
<td>0.14 (0.08)</td>
<td>0.18 (0.09)</td>
</tr>
<tr>
<td>CD3⁺HLA/DR⁺:</td>
<td>0.21 (0.18)</td>
<td>0.21 (0.21)</td>
<td>0.41** (0.35)</td>
<td>0.11 (0.08)</td>
<td>0.16 (0.08)</td>
<td>0.14 (0.11)</td>
</tr>
</tbody>
</table>

Sampling Time

<table>
<thead>
<tr>
<th>Rest</th>
<th>Post-warmup</th>
<th>Post-test 1 hour</th>
<th>6 hours post</th>
<th>24 hours post</th>
</tr>
</thead>
</table>

Data: mean (SD)

Concentration: x $10^9. L^{-1}$

Significantly different from resting value * = p<0.05

** = p<0.01
Table 4: Hormone Concentrations

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Post-test</th>
<th>1 hour post</th>
<th>6 hour post</th>
<th>24 hours post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cortisol (ng.mL(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>196.3</td>
<td>176.9</td>
<td>281.5**</td>
<td>263.9**</td>
<td>88.6**</td>
<td>184.3</td>
</tr>
<tr>
<td></td>
<td>(21.4)</td>
<td>(38.8)</td>
<td>(37.2)</td>
<td>(33.3)</td>
<td>(30.6)</td>
<td>(40.4)</td>
</tr>
<tr>
<td>Cortisol Binding Globulin (µg.mL(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>47.3</td>
<td>nd</td>
<td>nd</td>
<td>49.2</td>
<td>47.8</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>(3.1)</td>
<td></td>
<td></td>
<td>(4.2)</td>
<td>(3.7)</td>
<td>(5.6)</td>
</tr>
<tr>
<td>Free Cortisol (ng.mL(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.2</td>
<td>nd</td>
<td>nd</td>
<td>20.2**</td>
<td>3.9**</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>(2.2)</td>
<td></td>
<td></td>
<td>(6.4)</td>
<td>(1.9)</td>
<td>(4.5)</td>
</tr>
<tr>
<td>% Free Cortisol:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>nd</td>
<td>nd</td>
<td>7.5**</td>
<td>4.5**</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>(0.7)</td>
<td></td>
<td></td>
<td>(1.5)</td>
<td>(1.1)</td>
<td>(1.7)</td>
</tr>
<tr>
<td>Catecholamines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epinephrine (nmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td>0.36</td>
<td>0.50</td>
<td>0.34</td>
<td>0.30</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>(0.45)</td>
<td>(0.13)</td>
<td>(0.13)</td>
<td>(0.29)</td>
<td>(0.18)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>Norepinephrine (nmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.46</td>
<td>0.35</td>
<td>0.96**</td>
<td>0.34</td>
<td>0.43</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.15)</td>
<td>(0.42)</td>
<td>(0.12)</td>
<td>(0.17)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>DA (µmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.13</td>
<td>1.70</td>
<td>0.84*</td>
<td>1.05*</td>
<td>0.69**</td>
<td>0.65**</td>
</tr>
<tr>
<td></td>
<td>(1.98)</td>
<td>(1.40)</td>
<td>(0.73)</td>
<td>(0.71)</td>
<td>(0.48)</td>
<td>(0.41)</td>
</tr>
<tr>
<td>DHPG (nmol.L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.51</td>
<td>0.38</td>
<td>4.39**</td>
<td>0.73</td>
<td>0.84</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>(0.22)</td>
<td>(0.18)</td>
<td>(2.51)</td>
<td>(0.43)</td>
<td>(0.68)</td>
<td>(0.28)</td>
</tr>
<tr>
<td>N Ratio (NE:DHPG)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.12</td>
<td>1.04</td>
<td>0.30**</td>
<td>0.54*</td>
<td>0.63*</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>(0.75)</td>
<td>(0.64)</td>
<td>(0.18)</td>
<td>(0.20)</td>
<td>(0.29)</td>
<td>(0.58)</td>
</tr>
</tbody>
</table>

Significantly different from resting value: * = p<0.05
** = p<0.01

nd = not determined
GRANULOCYTE ACTIVATION INDUCED BY INTENSE INTERVAL RUNNING

AUTHORS: A.B.GRAY\textsuperscript{\dag}, R.D.TELFORD\textsuperscript{\S}, M.COLLINS\textsuperscript{\Y}, M.S.BAKER\textsuperscript{\Y}, M.J.WEIDEMANN\textsuperscript{\dag}

\textsuperscript{\dag} DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, SCHOOL OF LIFE SCIENCE, FACULTY OF SCIENCE, AUSTRALIAN NATIONAL UNIVERSITY, GPO BOX 4 CANBERRA ACT 2601 AUSTRALIA.

\textsuperscript{\S} DEPARTMENT OF PHYSIOLOGY AND APPLIED NUTRITION, AUSTRALIAN INSTITUTE OF SPORT, PO BOX 176 BELCONNEN ACT 2616 AUSTRALIA.

\textsuperscript{\Y} DEPARTMENT OF MATHEMATICS, UNIVERSITY COLLEGE, AUSTRALIAN DEFENCE FORCE ACADEMY, NORTHCOTT DRIVE, CANBERRA ACT 2600 AUSTRALIA.

\textsuperscript{\Y} DEPARTMENT OF BIOLOGY UNIVERSITY OF WOLLONGONG, WOLLONGONG NSW 2500 AUSTRALIA

Address correspondence to:--

A.B.Gray, Department of Physiology and Applied Nutrition, Australian Institute of Sport, PO Box 176 Belconnen ACT 2616 Australia.
Ph: 011 616 252 1253
Fx: 011 616 252 1603

Key Words: Flow cytometry, monoclonal antibody, lactoferrin, elastase, granules, degranulation, CD16, CD11b, CR3, Fc\textsubscript{R}.\textsuperscript{\Y}
ABSTRACT: Activation of granulocytes has been associated with normal immune function, inflammation and exercise induced muscle damage. The effect of intense interval running on granulocyte activation was examined via the use of flow cytometry, monoclonal antibodies and spectrophotometric techniques. Eight trained males (VO2 max (mean (SD) = 64.4 (3.6) ml.kg⁻¹.min⁻¹, age = 30.1 (4.8) years) undertook an intense interval exercise (treadmill running) protocol to exhaustion. Subjects completed an average of 16.5 one minute runs. Granulocyte expression of both CR3 (CD11b), receptor for complement component C3bi (6 and 24 hours post-test), and FcγRIII (CD16) (24 hours post-test), and the plasma concentration of elastase-inhibitor complex (1 hour post-test), increased significantly (all p<0.05). Subjects (8 of 8) exhibited a post-test decrease, at either 1 or 6 hours (p<0.01), and a 24 hour post-test significant increase (7 of 8;p<0.05) in granulocyte 90° light-scatter (LS). Plasma lactoferrin (Lf) concentration, although increasing by 16% at 6 hours post-test, was not significantly different from resting values at any sampling point. Changes in plasma Lf and median channel 90° LS were significantly correlated (r=-0.43, p=0.04), raising the possibility of monitoring exercise induced granulocyte activation (degranulation) using flow cytometry. Intense interval exercise appears to induce granulocyte activation, as manifested by release of
INTRODUCTION:

Exercise has an established role in modulating the peripheral concentration of leucocyte subsets (1). The effect of exercise on the incidence and severity of infections is the subject of considerable debate. Studies of subjects undergoing intense physical training and/or competition (2,3,4) report an increased incidence of infection. Other studies, involving more moderate exercise have reported a reduction in symptom days per incident of upper respiratory tract infection (5). Moreover a relationship between exercise and modulation of the immune system may, in part, explain the epidemiological finding that physical fitness (and presumably physical activity) is associated with a reduced rate of cardiovascular disease and cancer (6).

Evidence is emerging that exercise is capable of altering the level of activation of the human immune system (7). Such alterations may modulate the incidence of infection previously noted. The first line of defence against infection is provided by the polymorphonuclear leucocytes (granulocytes). These cells have an extremely short plasma half-life (approximately 6 to 10 hours) and normally constitute 60-80% of peripheral blood leucocytes (8).

Following stimulation of specific cell surface receptors by agents such as complement components (9),
for example CR3/CD11b for C3bi, and the Fc portion of immunoglobulins (10), granulocytes respond to infective challenge by phagocytosis and two main effector mechanisms. Firstly via release (into either a phagolysosome or extracellular fluid) of bactericidal reactive oxygen species (ROS; eg: superoxide anion, hydrogen peroxide), following the univalent reduction of oxygen by a membrane associated NADPH oxidase (11). Secondly via release, from either primary or secondary cytoplasmic granules, of proteolytic enzymes such as elastase, cathepsin G (primary granules) and lysozyme (primary and secondary) and the iron binding, bacteriostatic/bacteriocidal protein lactoferrin (secondary) (12). These effector mechanisms appear to be under differential regulation (13).

Granulocytes also appear to be involved in a broad spectrum of tissue damaging events, including inflammation (8), reperfusion injury (14) and exercise induced muscle damage (15) and adult respiratory distress syndrome (16), possibly as a consequence of activation of these same pathways mentioned above.

Previous reports of aerobic exercise induced elevations in the plasma concentration of elastase (17,18), derived from the primary granules of these granulocytes, appear to indicate an exercise-induced...
degranulation and activation of the polymorphonuclear components of the immune system.

To this time there has been no investigation of the effects of intense interval running on the activation state of peripheral blood granulocytes. This investigation involved sensitive monoclonal antibody, flow cytometric and spectrophotometric techniques to assess the activation status of granulocytes. This consisted, in-part, of measurement of the expression of cell-surface receptors for both complement (CR3;CD11b) and the Fc portion of IgG (FcRIII;CD16). Additionally the plasma concentration of the granule proteins elastase and lactoferrin as well as a related parameter, flow-cytometric 90° LS, were also assessed. A decrease in 90° LS has been related, via invitro experiments, to degranulation (19,20). The purpose of this investigation is to provide a profile of the effects of interval running on the activation status of granulocytes.
MATERIAL AND METHODS:

Subjects: Eight trained males, mean age (SD) 30.1 (4.8) years, were employed as exercise subjects. All were, and had been for at least one year, engaged in a regular training program. All were free of symptoms of infection and none were taking medication. All had refrained from food overnight and none had exercised within the prior 24 hours.

Exercise Tests: Subjects were familiarised with testing procedures for both determination of maximal oxygen uptake ($VO_2$ max) and interval running tests and all participants signed a consent form that detailed the scope of the study and its attendant risks. Procedures were approved by the Ethics Committee of the Australian Institute of Sport.

Maximal Oxygen Uptake Test (Test #1): All subjects underwent a progressive treadmill running test employing increases in speed and gradient to determine their maximal oxygen uptake ($VO_2$ max). The initial treadmill speed was 11 km.hr$^{-1}$ with an increase of 1 km.hr$^{-1}$ each minute for the first 7 minutes. At this stage the treadmill gradient was increased at a rate of 2% per minute. All subjects were exercised until subjective estimation of exhaustion brought about voluntary termination of the test. The respiratory analysis followed procedures described previously (21).
Criteria for attainment of VO$_2$ max were: an R value in excess of 1.10, a "plateau" in oxygen consumption (an increment of less than 0.15 L.min$^{-1}$), or a heart rate equal to the predicted maximum.

**Treadmill Interval Running Test (IRT; Test #2):** This test was performed at least one week following test #1. All subjects underwent a standard warm-up procedure involving 5 minutes running at both 7 km.hr$^{-1}$ and 11 km.hr$^{-1}$ and 3 minutes at 15 km.hr$^{-1}$. All warmup procedures were performed at 0% treadmill gradient. Subjects then performed alternating one minute periods of treadmill running and active (walk) recovery. Treadmill speed and gradient for each 1 minute exercise period were set at the levels under which maximal oxygen uptake was achieved ie: same speed and gradient. Subjects were able to walk around the laboratory (at their own pace) during the 1 minute recovery periods. All subjects were informed of the elapsed time every 15 seconds and all received encouragement during the course of each repetition. Subjects were asked to perform as many repetitions as possible, and continued until they could no longer maintain the required speed.

**Blood Sampling and Analysis:** Blood samples, obtained with the subjects in a supine position, were collected from venipuncture of an antecubital vein. Four samples were collected for each subject; before exercise (after
the subject had been sitting quietly in the laboratory for 20 minutes), and 1, 6 and 24 hours post-test. Samples were immediately placed into either EDTA vacutainers (Becton Dickinson, Mountain View, Ca.) prior to monoclonal antibody analysis and collection of plasma samples or into clotting tubes for collection of serum samples.

**Antibody Labelling**: All samples were processed at room temperature according to the suppliers recommendations. Whole blood (20µL) was incubated with saturating quantities of both fluorescein isothiocyanate (FITC) conjugated anti-FcγRIII (CD16) and phycoerythrin (PE) conjugated anti-complement receptor 3 (CR3;CD11b) monoclonal antibodies (Becton Dickinson, Mountain View, Ca.). Fluorochrome conjugated isotype controls using irrelevant antibodies were also employed.

**Flow Cytometric Analysis**: A FACS 440 cell sorter (Becton Dickinson, Mountain View, Ca.) operated with appropriate forward and 90° LS (to exclude red blood cells and debris) was used to identify the granulocyte subpopulation. List mode data on 10,000 cells per sample was collected. Data on each sample was obtained by processing of the list-mode data via use of the WEFCS software package (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). Each sample was analysed for granulocyte expression of both CD16
and CD11b as well as 90° LS characteristics.

Granule Protein Determinations:

Elastase: The plasma concentration of elastase, as complexed to inhibitor proteins, was assessed by use of a spectrophotometric method (Merck IMAC, BDH Chemicals, Melbourne; 22).

Lactoferrin: Serum samples, stored at -20°C prior to analysis, were employed for determination of lactoferrin concentrations (ELISA, AUSPHARM Institute of Mucosal Immunology, Newcastle; Taylor D, Cripps A., Clancy R., manuscript submitted).

Statistical Analysis: Data were analysed by a repeated measures one-way ANOVA. Fisher's PLSD was used for post-hoc comparisons. Student's paired "t" tests and Pearson product-moment correlation analyses were employed as appropriate. Significance was indicated at the p<0.05 level. Data are presented as mean (SD).
RESULTS:
Subjects, of age 30.1 (4.8) years, weight 74.1 (8.6) kg, and maximal oxygen uptake (VO₂ max) of 64.2 (3.6) ml.kg⁻¹.min⁻¹, completed a mean of 16.5 (9.7) 1 minute exercise repetitions.

Interpretation of the results of this investigation is complicated by the differing individual kinetics of response exhibited in relation to each parameter (Figures 1,2,3). Cognizance must be given to this point when evaluating the significance of the results obtained at each sampling point. Nevertheless there was a significant increase in the granulocyte expression of CR3 (CD11b) at 6 hour post-test, and both CR3 and CD16 at 24 hours post-test (all p<0.05) (Table 1).

Despite the significance of these results, investigation of individual kinetics revealed that 7 of the 8 subjects exhibited a decrease in CD16 expression at 1 hour post-test (however p>0.05; Figure 1). Expression of CD11b was increased in all subjects at either 1 or 6 hours post-test (Figure 2).

Median channel 90° LS for gated granulocytes decreased, from values obtained at rest, in all subjects at either 1 or 6 hours post-test (p<0.01). However due to differing individual kinetics of this parameter the
median channel, when assessed at 1 hour and 6 hours post-test, was not significantly different from that at rest. A majority of subjects, 7 of 8, showed an increase in this parameter at 24 hours post-test when median channel 90° LS had increased significantly (P<0.05; Figure 3).

The concentration of elastase-inhibitor complex was increased significantly only at 1 hr post-test (67% elevation; p<0.05) indicating release of primary granule constituents. There was a non-significant 16% increase in plasma lactoferrin concentration at 6 hours post-test (Table 2). A comparison of resting values with the single highest value obtained post-test (ie: at either 1, 6 or 24 hours) revealed a mean 43% increase (p=0.055) (data not shown).

However whilst plasma elastase and median channel 90° LS were not significantly correlated (r = -0.205, p>0.30), serum lactoferrin and median channel 90° LS, expressed as a percentage of resting values, were significantly correlated (r = -0.43, p=0.04, n=24).
DISCUSSION:
This investigation has established that intense interval exercise is associated with granulocyte degranulation and changes in $90^\circ$ LS and expression of receptors for both complement fragment C3bi (CD11b/CR3) and the Fc portion of immunoglobulin G (CD16). These changes indicate that granulocyte activation was induced by intense interval running.

The presence of increased plasma concentrations of elastase-inhibitor complex following exercise clearly indicate neutrophil activation. However the measured changes in receptor expression and granularity probably reflect a combination of the effects of neutrophil activation and release of "new" populations of neutrophils from the bone marrow or other storage sites (23).

Terstappen et al. (1990;24) have carried out a phenotypic examination of neutrophil maturation using antibodies against CD11b and CD16 as well as assessing light-scatter characteristics. While the study of Terstappen et al. (1990;24) may prove useful in elucidating the phenotypic nature and source of neutrophils mobilised by exercise, the question of whether neutrophils in the peripheral circulation, prior to exercise, exhibit the same phenotypic characteristics as mature bone-marrow neutrophils was
not addressed in that study. Normal human physiology requires a basal level of immune system activation, producing small but measurable plasma concentrations of lactoferrin, elastase and other neutrophil-derived proteins. Hence it appears likely that neutrophils in the peripheral circulation may exhibit slightly different phenotypic characteristics from their mature, bone-marrow resident counterparts. Thus at this stage it is not possible to determine the relative contributions of neutrophil activation and mobilisation from the bone marrow to the phenotypic changes induced by this exercise protocol.

The post-exercise elevation in the concentration of the elastase-inhibitor complex clearly indicates degranulation of primary granules. Intense interval exercise thus joins extended aerobic exercise (17,18) as a form of exercise capable of inducing degranulation of primary granules. Elastase release was used as a "marker" for the degranulation process. Clearly, other primary granule proteins such as cathepsin G, lysozyme, defensins and myeloperoxidase (25) will also be released in association with elastase. Both elastase and cathepsin G are serine proteases, whilst lysozyme has powerful anti-bacterial action. This implies a potential for enzymatic degradation of tissue, once protective anti-proteases (eg: α2-macroglobulin and α-1
proteinase inhibitor) are either fully saturated or inactivated.

Repeated bouts of intense interval exercise, as performed by athletes involved in swimming and track & field, may increase the potential for granulocyte-induced tissue damage by overwhelming normal defences against proteolytic damage. Indeed Smith (1991;15) has implicated granulocyte derived lysosomal proteins in the production of delayed onset muscle soreness.

The dynamics of plasma lactoferrin concentration varied from subject to subject. Whilst the 16% increase in lactoferrin at 6 hrs post-test was not statistically significant there was a 43% increase observed when the individual maximum post-test values were compared with those obtained prior to exercise. The fact that all subjects displayed an initial (at either 1 or 6 hours post-test) decrease in granulocyte median channel 90° LS (Figure 3;p<0.01) and the significant correlation between plasma lactoferrin and this parameter, provides evidence of a degranulation of secondary granules. Decrease in granulocyte 90° LS has previously been related, by invitro experiments, to degranulation of primary as opposed to secondary granules (19). To our knowledge this is the first time that 90° LS has been correlated with invivo concentrations of lysosomal
proteins. These results clearly create the possibility of rapid monitoring of exercise-induced granulocyte degranulation by flow cytometry.

Secondary granules contain lactoferrin in the iron free, apo-lactoferrin form. Thus, release of lactoferrin may be associated with sequestration of any free iron, a situation that clearly does not favour iron catalysis of hydroxyl radical production (26), reducing the potential for tissue damage (15). Aside from its iron chelating properties, which may have significance in relation to immunomodulation (27,28), lactoferrin has also been found to have direct anti-bacterial action (25) which may have significance in the post-exercise period.

The secondary granule perigranular membrane is the storage site for pre-formed CR3 (29). Increased cell-surface expression of CR3 is thought to result, at least in part, from translocation of these to the cell membrane (29). Hence up-regulation of CR3 may provide an index of secondary granule release. Thus the increase in CD11b expression detected at 6 and 24 hours post-test (Table 1) appears to indicate a post-test degranulation, and has been reported to be induced by inflammation (30). Increased expression of the receptor for the complement fragment C3bi may allow for enhanced removal of complement cleavage fragments, the plasma
concentration of which have been reported to increase following aerobic exercise (31).

CR3 is a heterodimer consisting of a 165kD \( \alpha \) chain (CD11b) and a 95kD \( \beta \) chain (CD18), common to CR4 and lymphocyte function associated antigen (LFA-1). Together these membrane proteins constitute a family of leucocyte adhesion molecules (32). The role of these structures in leucocyte adhesiveness has been the subject of debate (33,34). The physiological importance of exercise-induced modulation of these structures remains to be determined.

Following this interval training protocol, granulocytes, having undergone at least partial degranulation appear to be present in the peripheral circulation. Such systemic, as opposed to tissue localised, degranulation may increase the potential for generalised rather than site specific granulocyte-mediated tissue damage. The net effect of post-exercise degranulation on overall immunocompetence of the athlete is open to conjecture. Loss of myeloperoxidase from primary granules may compromise the ability of granulocytes to generate hypochlorous acid, a key bacteriocidal agent (35). Alternatively exercise-induced degranulation may be beneficial in removing latent infective agents.
Median channel 90° LS was significantly greater than pre-exercise levels when assessed 24 hours post-test. This together with the normalisation of peripheral granulocyte concentration at this time (Gray A.B., Telford R.D., Collins M., Weidemann M.J., manuscript submitted), may indicate recruitment of a new population of granulocytes possibly from the bone marrow or other storage site, with a greater degree of granularity, and selective removal of less granular (possibly degranulated) cells from the circulation.

Increased Fc\textsubscript{\(\gamma\)}R (eg:CD16) expression may provide an avenue for both degranulation and phagocytic removal of IgG coated particles and immune complexes. Immune complexes have been found to be capable of activating the human NADPH oxidase (36). The mechanism of increased CD16 and CD11b expression remains to be determined. Upregulation of both receptors involved a relatively long time course, reaching significance at 6 and 24 hours, in relation to CD11b and CD16, respectively.

The reported release of Fc\textsubscript{\(\gamma\)}RIII (CD16) from the granulocyte membrane following stimulation by either phorbol myristate acetate (PMA) or f-met-leu-phe (37) may indicate that the decrease in CD16 expression noted in most subjects (7 of 8) at 1 hour post-test (Figure 1), is a consequence of exercise-induced activation.
The increase in CD16 expression at 24 hours post-test may indicate the presence of a new population of the cells in the peripheral circulation.

Interferon-γ (IFN-γ) has been reported to augment both lysosomal enzyme release (degranulation) and oxygen metabolism (NADPH oxidase activity) in human neutrophils (38,39). The time course of these changes was in the range of 90 to 240 minutes (38). Interestingly there are reports of an increase in the plasma concentration of the IFN-γ "marker" neopterin at both 6 and 24 hours post-exercise (Gray A.B., Telford R.D., Weidemann M.J., manuscript submitted). However while the above indicates a possible role for IFN-γ in the exercise-induced changes, this hypothesis still requires a primary stimulus, such as provided by opsonized zymosan in the experiments of Kowanko & Ferrante (1987; 39), to induce granulocyte activation. Nevertheless whilst the nature of the primary stimulus has yet to be determined it is possible that IgG based immune complexes may be triggering degranulation. Alternatively a post-exercise endotoxaemia (40) or complement activation (31) may be implicated here.

The role of endocrine hormones in modulation of neutrophil activation has been of increasing interest. Jansson (1991;41) reports an estrogenic hormone induced release of the primary granule protein, myeloperoxidase
from resting neutrophils. The exercise protocol employed in this study has previously been found to lead to a significant and sustained increase in peripheral β-estradiol concentration (Gray A.B., Telford R.D., Weidemann M.J., manuscript submitted). Thus the degranulation observed in this study may have an estradiol dependent component.

In summary this study has demonstrated that granulocyte activation, as indicated by release of granule proteins, changes in receptor expression and 90° LS, is induced by a single interval running session. The net effect of such activation on immunocompetence in the post-exercise period remains to be elucidated.
ACKNOWLEDGEMENTS:

The authors thank:

Dr Allan Cripps and Ms Dianna Taylor of the AUSPHARM Institute of Mucosal Immunology, Newcastle for lactoferrin analyses.

Mr Jeff Osborne and Ms Sabine Gruenger, Flow Cytometry Unit, John Curtin School of Medical Research, Australian National University, and Mr Don Campbell, Biochemistry Department, Australian Institute of Sport, for expert assistance.

--------
REFERENCES:


----------
<table>
<thead>
<tr>
<th>Sampling point</th>
<th>Pre-exercise</th>
<th>1 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16</td>
<td>91.6 (10.9)</td>
<td>90.3 (10.4)</td>
<td>92.0 (10.3)</td>
<td>95.0* (11.1)</td>
</tr>
<tr>
<td>CR3 (CD11b)</td>
<td>97.0 (7.3)</td>
<td>96.1 (9.0)</td>
<td>101.3* (8.4)</td>
<td>100.9* (12.0)</td>
</tr>
<tr>
<td>90° light scatter</td>
<td>123.9 (24.5)</td>
<td>120.5 (23.3)</td>
<td>121.8 (23.7)</td>
<td>130.0* (29.0)</td>
</tr>
</tbody>
</table>

Data represents median channel of log (CD16 and CR3) and linear (granulocyte 90° light scatter) fluorescence. Data: mean (SD)

* = p<0.05
Table 2: Plasma concentration of granule proteins

<table>
<thead>
<tr>
<th></th>
<th>Pre-exercise</th>
<th>1 hr</th>
<th>6 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase- (ng.ml(^{-1})) inhibitor complex</td>
<td>33.8 (27.7)</td>
<td>55.6* (32.3)</td>
<td>35.5 (21.1)</td>
<td>nd</td>
</tr>
<tr>
<td>Lactoferrin (µg.ml(^{-1}))</td>
<td>0.66 (0.27)</td>
<td>0.62 (0.38)</td>
<td>0.77 (0.35)</td>
<td>0.61 (0.17)</td>
</tr>
</tbody>
</table>

Data: mean(SD)
* = p<0.05
nd = not determined.
FcR III (CD16)

Sampling Time

Median Channel

- subject 9
- subject 10
- subject 11
- subject 12
- subject 13
- subject 14
- subject 15
- subject 16

Sampling Times:
- rest
- 1 hr
- 6 hr
- 24 hr
90 DEGREE LIGHT SCATTER

Medlan Channel

Sampling Time

subject 9
subject 10
subject 11
subject 12
subject 13
subject 14
subject 15
subject 16

rest 1 hr 6 hr 24 hr
LEGENDS TO FIGURES

FIGURE 1: CD16 (FcγRIII) ASSOCIATED FLUORESCENCE; (Individual responses). Variation of median channel log fluorescence with sampling time.

FIGURE 2: CD11b (CR3) ASSOCIATED FLUORESCENCE; (Individual responses). Variation of median channel log fluorescence with sampling time.

FIGURE 3: 90° FLOW-CYTOMETRIC LIGHT-SCATTER; (Individual responses). Variation of median channel linear fluorescence with sampling time.

----------
ACTIVATION OF NEUTROPHILS INDUCED BY INTENSE INTERVAL RUNNING: FURTHER CHARACTERISATION.

A.BON GRAY1, DAVID B.PYNE2, JOHN A.SMITH1, MAURICE J.WEIDEMANN1, RICHARD.D.TELFORD2.

1Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, Canberra ACT 0200 Australia.

2Department of Physiology and Applied Nutrition, Australian Institute of Sport, PO Box 176 Belconnen ACT 2616 Australia.

Running Title: Interval running and neutrophil activation.

Correspondence to:

A.B.Gray.
Faculty of Nursing and Health Sciences, Griffith University, PMB 50 Gold Coast Mail Centre, Gold Coast, QLD 4217, Australia.

Ph: 011 6175 948 921
Fx: 011 6175 948 908
ABSTRACT: Moderate aerobic exercise has recently been recognised as being capable of modulating the activity of neutrophils. However the effects of intense interval running on neutrophil activity have only been investigated minimally. Nine trained males [mean (SD) age = 35.3 (8.3) yrs, VO₂ max. = 62.1 (6.7) mls.kg.⁻¹.min.⁻¹] performed an interval running session consisting of 10 x 400 metres. Relative to pre-exercise values, the ability of neutrophils isolated after exercise to generate reactive oxygen species (ROS), was either significantly decreased [ferricytochrome C reduction/PMA stimulation;p < 0.05 and chemiluminescence (CL)/opsonized zymosan (OZ);p < 0.05], or unchanged (ferricytochrome C reduction/OZ and DCFH-DA/PMA; both p > 0.05) depending on the measurement procedure. Neutrophil expression of CD11b (increased; p < 0.05) and 90° light-scatter (decreased; p < 0.05), but not neutrophil expression of CD16 (p > 0.05), were altered significantly by this protocol. Evaluation of a similar interval running session (8 x 400 m) using another group of trained male subjects [mean age = 22.8 (4.8) years], revealed a significant post-exercise decrease in neutrophil expression of LECAM-1 (p < 0.05) and significant increases in the concentrations of lymphocytes and monocytes (both p < 0.01) in the peripheral circulation. Incubation of neutrophils with recombinant human growth hormone did not modulate significantly the expression of either CD11b or CD16,
although it did "prime" the cells for increased ROS production. Intense interval exercise appears to induce a multi-faceted activation of circulating neutrophils consisting of changes in 90° light-scatter indicative of degranulation, altered expression of CD11b and LECAM-1 as well as a post-exercise "refractory" period in the ability of neutrophils to generate ROS.


Key Words: degranulation, receptor, CD16, CD11b, LECAM-1, 90° light-scatter, priming, NADPH-oxidase.
INTRODUCTION:
Neutrophils possess two main effector mechanisms mediating the destruction of potentially pathogenic agents. Phagocytosed particles are exposed to the combined actions of a range of reactive oxygen species (ROS), the first of which, superoxide ($O_2^-$) is produced by a membrane-bound NADPH-oxidase (1,2). The second effector mechanism involves the release (degranulation) of an array of bactericidal and bacteriostatic proteins from a system of cytoplasmic granules (3). These two effector mechanisms interact to produce a range of chlorinated amines with powerful bactericidal activity (4).

Neutrophil effector functions, including NADPH-oxidase activity, are regulated in part by cell surface receptors for ligands such as complement (CR3, CD11b/CD18; 5), immunoglobulins (FcRIII/CD16; 6,7), hormones (eg: growth hormone; 8) and cytokines (9,10). The manifestations of neutrophil activation are numerous. They include production of ROS following translocation of components of the NADPH-oxidase to the plasma membrane (11), increased adherence to endothelial tissue [mediated via LECAM-1 (leucocyte-endothelial cell adhesion molecule) and CR3; 12,13], degranulation (3), chemotaxis (14), and characteristic changes in receptor expression, including increased expression of CD11b (15) and
decreased expression of CD16 (16). Neutrophils may be "primed" for enhanced production of ROS, following subsequent stimulation, by a number of agents including growth hormone (GH; 8,17), tumour necrosis factor (TNF; 9) and granulocyte-macrophage colony stimulating factor (GM-CSF; 10). Both TNF (18) and GM-CSF (10) are also able to increase neutrophil expression of CR3, a key molecule mediating neutrophil adherence to the endothelium (15). It was therefore hypothesised that the increases (approximately 20-fold) in the plasma concentration of GH, induced by a similar interval exercise protocol to that employed in the present study (19), mediate the increase in expression of CR3 on neutrophils noted after interval exercise. It was also hypothesised that the increased adhesiveness of neutrophils reported after incubation with GH in vitro (8), is mediated by increased expression of CR3.

Earlier investigations have generally found that aerobic exercise alters the activation state of neutrophils. This includes degranulation and release of granule proteins into plasma (20, 21), changes in phagocytosis (22,23) and chemotaxis (23), as well as changes in the NADPH oxidase system ("priming", 24; "suppression", 20,22). It has been established recently that intense interval running carried out in the laboratory is also able to induce activation of neutrophils (25). The present investigation was
designed to establish firstly, whether similar changes are induced by a typical interval running session conducted in the "field", secondly to further characterise the effects of this form of exercise on neutrophils, and thirdly to examine whether GH in vivo has the capacity to induce changes in the expression of receptors on neutrophils.
MATERIALS AND METHODS:

Materials: Ferricytochrome-C (horse heart), luminol (5-amino-2,3,dihydro-1,4-phthalazine dione), phorbol myristate acetate (PMA), superoxide dismutase (SOD) and zymosan-A were all purchased from Sigma (St.Louis, Mo). Ficoll-paque was obtained from Pharmacia (Uppsala, Sweden) and Mono-poly resolving medium from ICN Biomedicals (Sydney, Australia). Dichlorofluorescein diacetate (DCFH-DA) was purchased from Serva (Heidelberg, Germany) and the stock solution prepared according to methods described previously (26). Dihydrorhodamine was purchased from Molecular Probes (Eugene, Or). Hanks balanced salt solution containing 5mM D-glucose (HBSS) and phosphate buffered saline (PBS; Sigma, St.Louis, Mo) were prepared by standard methods. Opsonized zymosan (OZ) and luminol were prepared as described previously (24). PMA was dissolved in dimethyl sulfoxide to a concentration of 1mM. Subsequent dilutions were performed with HBSS.

Subjects: Two groups of subjects were used. Group 1 consisted of nine trained males. Most were competitive triathletes undertaking regular, usually daily, exercise involving combinations of running, cycling and swimming. These activities involved between 7 and 20 hours of training per week. Group 2 consisted of eight trained males. These subjects were runners who performed an average of 50 km.wk\(^{-1}\). All were free of
symptoms of infection and none were taking medication. All refrained from food overnight and none had exercised within the previous 24 hours.

**Exercise Tests:** Subjects were familiarised with the testing procedures for both the determination of maximal oxygen uptake (VO$_2$ max.; for Group 1 subjects) and the interval training session (both Groups). Testing commenced between 0800 and 0900 hours for Group 1 subjects and between 1700 and 1800 hours for Group 2 subjects. All subjects signed a consent form that detailed the scope of the study and its attendant risks. Procedures were approved by the Ethics Committee of the Australian Institute of Sport.

**Maximal Oxygen Uptake Test:** Subjects from Group 1 underwent a progressive treadmill running test employing increases in speed and gradient to determine their VO$_2$ max. The initial treadmill speed was 11 km.hr$^{-1}$ with an increase of 1 km.hr$^{-1}$ each minute for the first seven minutes. At this stage the treadmill gradient was increased at a rate of 2% per minute. All subjects were exercised until a subjective estimation of exhaustion brought about voluntary termination of the test. Criteria for attainment of VO$_2$ max were: a respiratory exchange ration (R) value in excess of 1.10, a "plateau" in oxygen consumption (an increment of less than 0.15 L.min$^{-1}$), or a heart rate equal to
the predicted maximum. Procedures for respiratory analysis have been described previously (27).

**Interval Training Session #1 (Group 1):** All subjects completed an interval training session consisting of 10 x 400 metres running on a 3 minute "cycle" (ie: 3 minutes was allowed for each run and recovery), with a 200 metres jog recovery between each run. The training session was preceded by a warmup jog/run of approximately 1200 metres. The time for each 400 metre run was recorded and the 3 minute cycle strictly enforced by a supervisor.

**Interval Training Session 2 (Group 2):** Another group of trained male subjects undertook a similar session of interval running (8 x 400 metres, 200 metre jog recovery, on a 3 minute "cycle"). Subjects performed a warm-up and warm-down, both consisting of 1200 metres of easy running and jogging.

**Blood Sampling:** Blood samples were collected by venepuncture of an antecubital vein before and after each training session. Samples from Group 2 subjects were used only for full blood counts, assays of complement components in plasma and neutrophil cell-surface expression of LECAM-1. Samples were collected into tubes containing EDTA or lithium heparin (Becton-Dickinson, Mountain View, Ca) as appropriate.
The pre-exercise sample was collected before the warm-up procedure and after the subject had been sitting quietly for 15 minutes. In relation to Group 1 subjects post-exercise samples were collected immediately after the final 400 metre run. Post-exercise blood samples from the Group 2 subjects were collected after the warm-down (approximately 10 minutes post-exercise). This was to allow dissipation of accumulated lactic acid which tended to interfere with the isolation of neutrophils.

Preparation of Neutrophils: Heparinized blood (25 mL) was layered over a cushion of Mono-poly resolving medium (15mL) and Ficoll-paque (5mL) in a sterile tube and centrifuged (30 minutes, 600 x g, 4°C). Erythrocytes were removed by hypotonic lysis, the cells washed in PBS, and resuspended in HBSS (with 5mM glucose) before being stored on ice prior to use. The purity of the preparation, as assessed by flow cytometric light-scatter was greater than 95%.

Chemiluminescence (CL): Luminol-enhanced chemiluminescence detects principally hydrogen peroxide and hypochlorous acid when neutrophils are stimulated with PMA (28), indicating a dependence on myeloperoxidase activity for ROS production. CL was measured in a 1251 Luminometer (LKB, Wallac, Turku, Finland) at 37°C with continuous stirring of the
reaction mixture as described previously (24). Briefly, neutrophils (10^5) suspended in HBSS were added to polystyrene luminometer tubes (Clinicon 2174-086 Turku, Finland) containing luminol (225 µM). OZ (100 OZ particles per cell) was used as a stimulant and the final reaction volume was 1.0 mL. The chemiluminescence signal [expressed in millivolts (mV)] was recorded continuously up to peak velocity on an LKB 2210 potentiometer chart recorder (LKB-Produkter AB, Bromma, Sweden).

**Superoxide Assay:** Extracellular superoxide production was determined via the SOD-inhibitable reduction of ferricytochrome C. Isolated neutrophils (10^6) in a final volume of 1.0 mL were incubated at 37°C (30 minutes with continuous stirring) with ferricytochrome C (50 mM) with and without SOD (0.2 mg). The tubes were centrifuged for 30 seconds and the optical densities of the supernatants measured spectrophotometrically at 550 nm, using the tubes containing SOD as blanks. Two series of experiments were performed; one using PMA (100 nM), the other using OZ (100 OZ particles per cell) as agonists. An extinction co-efficient of 18.5 mM^{-1} cm^{-1} for cytochrome C was employed for all calculations.

**Dichlorofluorescin Diacetate (DCFH-DA):** This method has been employed previously to detect intracellular H_2O_2.
production (26). Neutrophils (5 x 10⁵) were incubated in 1.5 mL micro-centrifuge tubes using horizontal agitation with DCFH-DA (10µM) at 37°C for 20 minutes in a volume of 1.0 mL. PMA (100 nM) was then added and the incubation continued for a further 20 minutes. Neutrophils incubated with DCFH-DA but unstimulated were used as a negative control. After the second incubation cells were placed immediately on ice prior to flow cytometric analysis.

Flow cytometry: Samples were analysed using a Coulter Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Laser alignment was maintained by daily use of "Immunocheck" polystyrene microspheres (Coulter Electronics, Hialeah, FL). Identification of the neutrophil population was accomplished by appropriate gating on the forward-90° light-scatter profile.

Receptor analysis: EDTA anticoagulated whole blood was incubated with saturating concentrations of anti-CD16 FITC, anti-CD11b PE (both Becton-Dickinson, Mountain View, Ca.) and anti-LECAM-1 FITC (Immunotech, Marseille, France; provided by Mr Peter Chisholm of Haem Pty Ltd, Melbourne) conjugated monoclonal antibodies. Fluorochrome conjugated isotype control monoclonal antibodies were also employed.
Complement Assay: Both pre- and post-exercise EDTA plasma samples from Group 2 subjects were assayed within 24 hours of collection (after storage at 4°C) for the complement fragments C3a and C3a des arg by a commercially available kit (Amersham, Sydney).

Growth Hormone and Receptor Expression on Neutrophils: Duplicate samples of neutrophils (5 x 10⁵ in a final volume of 1.0mL) obtained from subjects prior to exercise were incubated (1 hr, 37°C) with recombinant human GH (rhGH; provided by Dr Ken Ho, Garvan Institute of Medical Research, Sydney) at a concentration of 500 ng.mL⁻¹. The incubation was stopped by the addition of 2.0mL ice-cold PBS and the sample centrifuged immediately (300g, 5 minutes, 4°C). The supernatant was aspirated leaving the cell pellet plus approximately 100 µL of liquid with which saturating concentrations of monoclonal antibodies (anti-CD11b, anti-CD16) were incubated (45 minutes, in the dark, on ice). The incubation was stopped by addition of ice-cold PBS (1 mL) before centrifugation (as above) and resuspension in PBS before being placed on ice. Samples were analysed immediately by flow cytometry.

Growth Hormone and Priming of ROS Production by Neutrophils: Dihydrorhodamine (DHR) coupled with flow cytometry has been reported recently to be a very sensitive means of assessing the neutrophil respiratory...
burst (29). Duplicate samples of neutrophils (5 x 10^5) were incubated (1 hr, 37°C, continuous agitation) with rhGH (500 ng mL^-1). Cell were then incubated with DHR (1 µM; 5 minutes, 37°C) followed by addition of PMA (100 nM) and further incubation (20 minutes, 37°C, continuous agitation). Samples were then immediately placed on ice, prior to flow cytometric analysis.

**Activation Studies - Receptor Expression:** In order to establish the changes in receptor expression associated with activation of neutrophils, isolated cells (5 x 10^5 in a final reaction volume of 1.0 mL), obtained from subjects prior to exercise, were incubated with PMA (100 nM) for 15 minutes at 37°C with horizontal agitation. The expression of both cell-surface CD16 and CD11b were determined as described for the GH studies (above).

**Full Blood Count:** Full blood counts (including total and differential leucocyte counts) were determined by use of a Coulter JT haematology analyser (Coulter Electronics, Hialeah, Fl).

**Whole Blood Lactate:** Earlobe micro blood samples (25 µL) were analysed for whole blood L-lactate with a YSI 23L L-lactate analyser (Yellow Springs Instrument Company, Yellow Springs, Ohio).
**Statistics:** Results from the cytochrome c and DCFH-DA studies were analysed by use of the Sign Test. This test has the advantage that it makes no assumptions concerning the way in which the data are distributed (30). Due to large inter-individual variation the data obtained from the chemiluminescence experiments were "normalised" by conversion to natural logarithms and analysed by Student's paired "t" test. Data obtained from the GH and receptor expression studies (relating to the effects of GH, PMA and exercise) were analysed by use by Student's "t" tests (as above). Significance was indicated at the $p<0.05$ level. Data are presented as mean (SD).
RESULTS:

1. **Group 1:** These subjects had the following physical characteristics: mean (SD) age = 35.3 (8.3) years, weight = 73.3 (4.6) kg, VO₂ max = 62.1 (6.7) ml.kg⁻¹.min⁻¹. They completed the 10 x 400 metre runs in an average time of 72.4 (2.3) seconds. Whole blood lactate concentrations obtained immediately after exercise were typically in the range 14.0 to 16.0 mmol.L⁻¹ (data not shown).

1.1 **ROS Production:** The ability of isolated neutrophils to reduce ferricytochrome c in response to stimulation by PMA was decreased significantly after exercise (p<0.05), while that relating to stimulation with OZ was not significantly different from rest (p>0.05). This was despite 7 of 9 subjects showing a post-exercise decrease. The ability of isolated neutrophils to produce ROS, expressed as the change from baseline values, assessed by chemiluminescence using OZ as a stimulus, was significantly decreased from resting values when assessed post-exercise (p<0.05). Baseline (unstimulated) chemiluminescence decreased after exercise in every case (p<0.01). Mean channel log fluorescence obtained after exercise, using the dye DCFH-DA and PMA to stimulate neutrophils, was not significantly different from resting values (Table 2).
1.2 **Receptor Expression** Relative to values obtained at rest prior to exercise neutrophil cell-surface expression of receptors changed as follows after exercise: CR3 (CD11b) increased significantly \((p<0.05)\), whilst FcγRIII (CD16) was not significantly changed \((p>0.05)\), again despite 7 of 9 subjects showing a decrease following exercise (Table 1).

1.3 **Light-Scatter:** Neutrophil mean channel 90° light-scatter (linear fluorescence) decreased significantly following interval exercise \((p<0.01)\) (Table 2).

2. **Group 2:** These subjects had the following physical characteristics: mean age 22.8 (4.8) years, weight = 64.4 (7.5) kg, and height = 173.8 (9.4) cm, completed the 8 x 400 metres as a group. Individual times for each 400 metres were not recorded as the subjects ran in a group rather than individually. The estimated average time was approximately 73 seconds for each 400 metre run.

2.1 **Leucocyte Subsets:** After exercise there were significant increases in the circulating concentration of the following leucocyte subsets: total leucocyte \((p<0.05)\), lymphocytes \((p<0.01)\), monocytes \((p<0.01)\), whilst the concentration of granulocytes was not elevated significantly \((p>0.05)\) (Table 3).
2.2 Complement components: After exercise the plasma concentration of complement activation products C3a and C3a des arg was not significantly different from those obtained pre-exercise (p>0.05). The plasma concentration at rest was 1352.8 (133.8) ng.mL$^{-1}$ and after exercise it was 1456.0 (136.2) ng.mL$^{-1}$.

2.3 Neutrophil expression of LECAM-1: Neutrophil expression of LECAM-1, as indicated by mean channel log fluorescence, decreased significantly after exercise (p<0.01) (Table 2).

3. GH Studies: GH produced no significant difference in neutrophil cell-surface expression of either CD16 or CD11b (Table 4). However incubation with rhGH at a concentration of 500 ng.mL$^{-1}$ did "prime" neutrophils for significantly increased production of ROS (p<0.01), as assessed by use of DHR, when they were subsequently stimulated with PMA (Table 4).

4. PMA Studies: Incubation of isolated neutrophils with PMA in five separate experiments induced a significant decrease in expression of CD16, and a significant increase in CD11b expression (both p<0.01; Table 5).
DISCUSSION:

This study has demonstrated that intense interval exercise, involving protocols that are used regularly by athletes in many sports, is associated with a multi-faceted activation of neutrophils during exercise. In addition, the protocol caused a significant mobilisation of total leucocytes, consisting principally of lymphocytes and monocytes, a finding consistent with those of McCarthy & Dale (31), concerning predominantly aerobic exercise.

Our data indicate that intense interval exercise reduces the ability of a substantial proportion of circulating neutrophils to generate ROS in vitro in response to exogenous stimulation and are consistent with reports of the effects of moderate aerobic exercise on neutrophil function (20, 22). This reduced ability to generate ROS is manifested as a decrease in both the maximum rate and the total production of ROS following stimulation as assessed by the CL and ferricytochrome c reduction assays, respectively. Our findings are consistent with previous reports of studies that used chemiluminescent techniques which have found a close correspondence between these two measures (32).

A decrease in the ability of neutrophils isolated after exercise to reduce ferricytochrome c (in response to
PMA) indicates a primary deficiency in the NADPH-oxidase system of neutrophils as this assay technique is dependent on the generation of superoxide. The nature and location of this deficiency has not been determined. However O2 and PMA activate differentially the NADPH-oxidase system of neutrophils, the former by binding to cell-surface receptors for both IgG and complement, and the latter by direct action on protein kinase C (PKC) (1). Our results indicate that the deficiency lies distal to PKC in the signal transduction pathway. This implicates events such as translocation of either PKC or oxidase components to the plasma membrane or possibly phosphorylation of endogenous proteins as possible locations of this exercise-induced deficiency (1). Defects in NADPH-oxidase activity could be expected to decrease the production of other ROS such as hydrogen peroxide and hypochlorous acid given that superoxide is a precursor for their formation (2). However moderate aerobic exercise has also been found to produce an increase in the plasma concentration myeloperoxidase (MPO; 33), indicating degranulation of the primary granules of neutrophils. While this investigation provides no direct evidence for release of MPO into the plasma following interval exercise, a similar exercise protocol did induce a significant increase in the plasma concentration of elastase (25), another protein derived from the primary granules of neutrophils. The
significant reduction in neutrophil 90° light-scatter noted in this investigation is indicative of degranulation of primary granules (34) and consistent with other reports of increases in the plasma concentration of primary granule proteins following moderate aerobic exercise (20). Thus, the ability to generate a chemiluminescent signal, which is dependent on the presence of MPO for production of hypochlorous acid, might be expected to be reduced on the basis of a decreased availability of MPO to act as a catalyst and hydrogen peroxide to act as a substrate.

The decreased ability of the NADPH-oxidase system of neutrophils isolated after exercise to generate ROS in vivo can be hypothesised to be mediated in at least two ways. It could be due to a direct suppression of this system, possibly via the action of immuno-suppressive hormones such as cortisol (35,36), the concentration of which is elevated significantly after strenuous aerobic (37) and in particular after intense interval exercise (Gray et al., unpublished observations). Alternatively, it may represent a "refractory" period, following activation of the cells during exercise. This contention is consistent with changes in expression of complement receptors and LECAM-1 and a reduction in the 90° light-scatter of neutrophils induced by intense interval exercise. This hypothesis is also supported by the findings of Prasad
et al. (38), who have established in an animal study that neutrophils subject to \textit{in vivo} activation are "refractory" to further stimulation \textit{in vitro}. It is possible that elevated plasma concentrations of cortisol induced by exercise may accentuate the duration or depth of the post-exercise refractory period, thereby leaving athletes susceptible to infection at this time.

After intense interval exercise neutrophils displayed changes in cell-surface expression of both CR3 (CD11b/CD18) and LECAM-1. The significant increase in expression of CR3 is indicative of both activation (39, and as shown here with PMA) and degranulation of secondary granules, where these receptors are stored preformed in association with these granules (40). \(\beta_2\)-Integrins such as CR3 are critical to the process of extravasation – whereby neutrophils migrate across the endothelium – mediated by an interaction of the CD11b/CD18 complex with structures on the surface of the endothelium such as ICAM-1 (intercellular adhesion molecule) (41).

The increase in expression of CR3 on neutrophils induced by interval exercise is much less than that induced by PMA (Tables 4 & 5), a powerful promoter of secondary granule degranulation (34). While this indicates that interval exercise induces only a partial
release of CR3 from secondary granules consistent with a partial activation of neutrophils, our flow cytometric data indicate that this activation occurs across the whole neutrophil population. i.e. we have no evidence of a selective activation of particular subsets of neutrophils by interval exercise.

The significant decrease in the expression of cell-surface LECAM-1 noted in this study is a further indication of changes that are characteristic of neutrophil activation in vivo (15). In vivo, selectins (e.g. P-selectin, E-selectin, and L-selectin/LECAM-1) mediate initial contact between the endothelium and neutrophils (42). These "rolling" interactions appear to be a prerequisite for later, stronger interactions involving the $\beta_2$-integrins (42), although this may not be true in pathological conditions (41). In either event these interactions culminate in adhesion to and probably migration through, the endothelium. The implications of decreased LECAM-1 expression have not been explored in detail, although Kishimoto et al. (13) have reported that neutrophils with a reduced expression of LECAM-1 were unable to migrate normally to sites of inflammation. Jutila et al. (43) have demonstrated that treatment of neutrophils with antibodies against LECAM-1 decreased their accumulation in tissues and caused a corresponding increase in the concentration of these cells in the circulation. This
finding raises the possibility that the reduced expression of LECAM-1 on neutrophils induced by exercise may act to limit the migration of these cells to sites of inflammation, thereby contributing to the leucocytosis associated with exercise (31). The increased expression of CR3, the trend toward a reduction in the expression of CD16 and the reduction in 90° light-scatter induced by this "field" protocol are very similar to those described previously following intense interval exercise on a treadmill conducted in the laboratory (25).

In an effort to establish possible agents responsible for triggering activation of neutrophils in vivo during intense interval exercise, we investigated the effects of this protocol on the complement cascade, elements of which have been shown to activate neutrophils (5) and to be increased in plasma following aerobic exercise (21). We found no evidence of complement activation with exercise via assessment of the plasma concentrations of the complement cleavage products C3a and C3a des arg. Hence we conclude that the apparent activation of neutrophils induced by intense interval exercise is not mediated by the complement cascade.

Despite conducting experiments that used conditions and concentrations under which GH had been shown previously (and in this study) to prime neutrophils (500 ng.mL⁻¹;
we could find no change in the expression of either cell-surface CR3 or CD16 on neutrophils. It appears that changes in neutrophil adhesiveness due to the effects of GH on CR3 must be mediated by changes in receptor conformation as has been suggested for homotypic neutrophil aggregation (44) rather than simple up-regulation. Similarly the increase in expression of CR3 on neutrophils that is produced by intense interval exercise does not appear to be mediated by GH. Thus both the mechanisms associated with, and implications of, CR3 up-regulation following exercise are unclear. Reduction in neutrophil expression of CD16 is indicative of activation (16) and despite a non-significant decrease after exercise (in 7 of 9 subjects) the trend towards reduced expression is consistent with our other observations indicating activation of neutrophils in vivo.

In summary it appears that intense interval running induced a multi-faceted activation of neutrophils, characterised by changes in receptor expression and 90° light-scatter and a reduced ability to generate ROS in the post-exercise period.
REFERENCES:


**TABLE 1: Assessment of ROS Production by Neutrophils.**

<table>
<thead>
<tr>
<th>Method/Stimulus</th>
<th>Rest</th>
<th>Post-ex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Superoxide Production: (nM/30 mins)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c (PMA)</td>
<td>14.9 (4.3)</td>
<td>11.2* (6.9)</td>
</tr>
<tr>
<td>Cytochrome c (OZ)</td>
<td>6.1 (3.9)</td>
<td>4.5 (5.4)</td>
</tr>
<tr>
<td><strong>Chemiluminescence: (mV) (change from baseline)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OZ</td>
<td>357.9 (351.7)</td>
<td>218.3* (320.3)</td>
</tr>
<tr>
<td>Baseline (unstimulated)</td>
<td>124.2 (122.7)</td>
<td>54.7** (74.3)</td>
</tr>
<tr>
<td><strong>DCFH-DA: (mean channel log fluorescence)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>1.95 (0.65)</td>
<td>1.84 (0.20)</td>
</tr>
</tbody>
</table>

Data: mean (SD)

Significantly different from rest:  * = p<0.05  
** = p<0.01
**TABLE 2: Neutrophil Receptor Expression and Light-scatter.**

<table>
<thead>
<tr>
<th></th>
<th>CD16</th>
<th>CD11b</th>
<th>LECAM-1</th>
<th>90°LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>11.9</td>
<td>10.4</td>
<td>4.8</td>
<td>153.1</td>
</tr>
<tr>
<td></td>
<td>(11.6)</td>
<td>(8.3)</td>
<td>(1.0)</td>
<td>(12.7)</td>
</tr>
<tr>
<td>Post-ex</td>
<td>11.5</td>
<td>12.2*</td>
<td>4.1**</td>
<td>149.0**</td>
</tr>
<tr>
<td></td>
<td>(11.1)</td>
<td>(8.8)</td>
<td>(0.9)</td>
<td>(12.6)</td>
</tr>
</tbody>
</table>

Values represent mean channel log (receptor expression) and linear (90° light-scatter, LS) fluorescence.

Significantly different from rest: * = p<0.05  
** = p<0.01
TABLE 3: Leucocyte Subset Concentrations:

<table>
<thead>
<tr>
<th></th>
<th>Total Leucocytes</th>
<th>Lym.</th>
<th>Mono.</th>
<th>Granulo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
<td>8.4 (0.8)</td>
<td>3.2 (0.6)</td>
<td>0.4 (0.1)</td>
<td>4.8 (1.2)</td>
</tr>
<tr>
<td>Post-ex</td>
<td>10.8* (2.0)</td>
<td>4.5** (0.8)</td>
<td>0.6** (0.2)</td>
<td>5.7 (1.6)</td>
</tr>
</tbody>
</table>

Data: $x \times 10^9 \cdot L^{-1}$

Significantly different from rest: * = $p<0.05$
** = $p<0.01$
Table 4: Effect of GH on neutrophil receptor expression and stimulated ROS production.

<table>
<thead>
<tr>
<th></th>
<th>- GH</th>
<th>+ GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHR (PMA)</td>
<td>17.7 (2.2)</td>
<td>21.6** (4.6)</td>
</tr>
<tr>
<td>CD11b</td>
<td>4.94 (1.30)</td>
<td>4.69 (1.21)</td>
</tr>
<tr>
<td>CD16</td>
<td>3.66 (1.59)</td>
<td>3.55 (1.49)</td>
</tr>
</tbody>
</table>

Data: Mean (SD) channel log fluorescence.

Significantly different from samples without GH:

\[** = p<0.01\]
Table 5: Effect of PMA on Neutrophil Receptor Expression.

<table>
<thead>
<tr>
<th></th>
<th>-PMA</th>
<th>+PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>13.40</td>
<td>47.25**</td>
</tr>
<tr>
<td></td>
<td>(3.21)</td>
<td>(6.58)</td>
</tr>
<tr>
<td>CD16</td>
<td>5.53</td>
<td>3.61**</td>
</tr>
<tr>
<td></td>
<td>(1.01)</td>
<td>(0.42)</td>
</tr>
</tbody>
</table>

Data: mean (SD) channel log fluorescence.

Significantly different from samples without PMA:

** = p<0.01
EXERCISE INDUCES TRANSLOCATION OF P47-PHOX IN NEUTROPHILS.

A.Bon Gray¹, David B.Pyne², John A.Smith¹, Gary D.Buffington², June R.Hornby², Maurice J.Weidemann¹, Richard D.Telford³.

¹ Division of Biochemistry and Molecular Biology, School of Life Sciences, and
² Department of Clinical Sciences, John Curtin School of Medical Research, Australian National University, Canberra ACT 0200, Australia.
³ Department of Physiology and Applied Nutrition, Australian Institute of Sport, PO Box 176, Belconnen ACT 2616, Australia.

RAPID COMMUNICATION

Running Title: Translocation of p47-phox and exercise.

Correspondence to A.B.Gray,
Dept.Nursing & Health Sciences, Griffith University, PMB 50 Gold Coast Mail Centre, Gold Coast QLD 4217 Australia. ph: 6175 948 921; fx: 6175 948 908
exercise is capable of inducing a post-exercise refractory state in neutrophils in which their ability to produce reactive oxygen species (ROS) in response to exogenous stimulation is reduced. It was hypothesised that this refractory period is secondary to an exercise-induced assembly and activation of the membrane-bound NADPH-oxidase system of neutrophils. This investigation sought to establish whether exercise was able to induce assembly of the NADPH-oxidase as indicated by translocation of a key component of this system (p47-phox), from the cytosol to the plasma membrane. Three trained male subjects (mean age = 29.9 yrs; range 23.0–36.4 yrs) undertook intense interval running (10 x 400 metres) and moderate aerobic cycling (1 hr, 60% VO₂ max) protocols. Both forms of exercise were found to induce translocation of p47-phox from cytosol to membrane fractions indicating assembly and possibly activation of the NADPH oxidase system of neutrophils during exercise.

Index Terms: NADPH-oxidase, reactive oxygen species, interval, aerobic exercise.
**INTRODUCTION:** Exercise is being recognised increasingly as a behaviour capable of activating neutrophils. Recent reports indicate that both predominantly aerobic exercise and intense interval exercise induce neutrophil degranulation (2,5,8), increased expression of complement receptor 3 (CR3; CD11b/CD18; 2) and a post-exercise "refractory" period in which the ability of neutrophils stimulated *in vitro* to produce reactive oxygen species (ROS) is reduced (3,5).

Production of ROS by neutrophils occurs via a multi-component membrane-bound NADPH-oxidase, which prior to activation, has separated components located in the plasma membrane and cytosol (7). Translocation of a 47kD protein constituent of the phagocyte-oxidase (p47-phox) from the cytosol to the plasma membrane is mandatory for (1), and clearly indicative of (12), oxidase activation.

The decreased ability of neutrophils isolated after exercise to produce ROS may represent a "refractory" period following activation *in vivo* (9) or alternatively, the refractory period may represent a post-exercise immunosuppression, possibly mediated by hormones such as cortisol (15), that is not preceded by the activation of neutrophils. Previous investigations of the effects of exercise on stimulated ROS production by isolated neutrophils have not been able to
discriminate between these possibilities. In order to address this question, the possible translocation of p47-phox from cytosol to plasma membrane, as an indicator of NADPH oxidase assembly and activation, was assessed.

MATERIALS & METHODS:

**Materials:** Phorbol myristate acetate (PMA), phosphate buffered saline (PBS), phenylmethylsulphonyl fluoride (PMSF), D-glucose, 1,4-piperazine diethane sulfonyl acid, ethylene bis[oxethylenenitrilo]tetraacetic acid (EGTA) and goat anti-rabbit horseradish peroxidase conjugated antibody were purchased from Sigma (St Louis, Mo.), Ficoll-paque from Pharmacia (Uppsala, Sweden) and Mono-poly resolving medium from ICN Biomedical (Sydney, Australia). PMA was dissolved in dimethyl sulphoxide to a stock concentration of 1mM. Further dilutions were performed with PBS. The rabbit anti-human p47-phox (B3) antibody was generously supplied by Prof W.M. Nauseef, University of Iowa, USA.

**Subjects:** Trained males (n=3) of mean age = 29.9 yrs (range 23.0 - 36.4 yrs) were used as subjects. All were engaged in a program of regular running that had continued for at least three years. Subjects were categorised as moderately to well trained. All were free of symptoms of infection and none were taking medication. All had refrained from food overnight and
none had exercised within the previous 24 hours. All subjects signed a consent form that detailed the scope of the study and its attendant risks. Procedures were approved by the Ethics Committee of the Australian Institute of Sport.

Exercise Tests: All tests were conducted in the morning (0800 - 1000 hrs) and two protocols were employed:

1. Interval Training: Following a warm-up jog of approximately 1200 metres, subjects completed 10 x 400 metre runs, each followed by a 200 metre jog recovery, on a 3 minute "cycle" (ie: 3 minutes for run and recovery), followed by a 1200 metre jog warm-down. Subjects were supervised throughout the session which was conducted on a 400 metre running track. Heart rates were monitored with a Sportstester PE 4000 heart rate monitor (Polar Electro, Finland).

2. 1 hour Cycling: No warm-up was allowed and subjects completed 1 hour cycling on a geared cycle ergometer at a heart rate of approximately 140 beats per minute. This represented a power output requiring approximately 60% of each subject's maximal oxygen uptake (VO$_2$ max). Power output was monitored with an Ex-50 work monitor unit (Exertech, Melbourne) and heart rates were measured with a Sportstester PE 4000 heart rate meter (Polar Electro, Finland).
**Blood Collection:** Blood samples, obtained with subjects in a supine position, were collected using lithium heparin as an anticoagulant (Vacutainer, Becton Dickinson, Mountain View, Ca), by venipuncture of an antecubital vein. Blood samples (25 mL) for the interval training protocol were collected prior to the warm-up and after the warm-down, to avoid problems with neutrophil isolation associated with elevated blood lactate levels. In relation to the cycling test blood was collected immediately before and immediately after the test.

**Neutrophil Isolation:** Heparinized blood (25 mL) was layered over a cushion of Mono-poly Resolving Medium (15 mL) and Ficoll-paque (5 mL) in a sterile tube and centrifuged for 30 minutes (600 x g, 4°C). Erythrocytes were removed by hypotonic lysis (one minute in ice-cold distilled water). Isotonicity was restored with double-strength PBS. The purity of the preparation, as assessed by flow cytometric light-scatter, was found to be greater than 95%. Cell counts were performed with a Coulter JT haematology Analyser (Coulter electronics, Hialeah, Fl). Immediately following isolation neutrophils were treated with a 1:100 dilution of PMSF (100mM) for 20 minutes on ice. Cells were centrifuged (5 min, 500 x g, 4°C) and the cell pellet was resuspended in PBS (with 5mM glucose) and kept on ice.
**PMA Studies:** As a positive control isolated neutrophils suspended in PBS plus glucose (reaction volume 1mL) were incubated (37°C, 20 minutes) with PMA (100 nM) using continuous agitation. PMA has an established ability to induce translocation of p47-phox from the cytosol to the plasma membrane (1). Incubation was stopped by addition of ice-cold PBS (3 mL). Cells were separated by centrifugation (5min, 500 x g, 4°C) and resuspended in PBS plus glucose, before storage on ice.

**Neutrophil Preparations:** Neutrophil membrane and cytosolic fractions were prepared by methods described previously (1), except that the two high speed centrifugation steps (110,000 x g) were extended from 6 to 20 minutes. Briefly, isolated cells were centrifuged (5 min, 4°C, 500 x g) and the pellet resuspended in 0.5mL relaxation buffer (KCl 100mM, NaCl 3mM, MgCl₂ 3.5 mM, EGTA 1.25mM, 1,4-piperazine diethane sulfonic acid 10mM, pH=7.3). Cells were disrupted by use of a microprobe sonicator (2 x 15 second cycles, 4°C). The sample was recentrifuged, as above, to remove unbroken cells and nuclei. The supernatant was subjected to ultra-centrifugation (110,000 x g, 20 min, 4°C) using a TL-100 ultracentrifuge with a TLA 100.2 rotor (Beckman Instruments, Palo Alto, Ca.). This supernatant represented the cytosolic fraction. The pellet was resuspended in 0.5mL relaxation buffer, vortexed, and
recentrifuged (110,000 x g) as above. The resulting pellet, constituting the membrane fraction, was resuspended in 0.5mL relaxation buffer.

**Electrophoresis and Immunoblotting:** Membrane and cytosolic fractions were processed as described previously (13), being subjected to SDS-PAGE (9% gel, reducing conditions) and immunoblotting using the primary B3 antibody (at a dilution of 1:1000) and second-step goat anti-rabbit horseradish peroxidase conjugate before detection using chemiluminescence (ECL system; Amersham, Sydney).

**Whole Blood Lactate:** Earlobe micro blood samples (25µL) were analysed for whole blood L-lactate with a YSI 23L L-lactate analyser (Yellow Springs Instrument Company, Yellow Springs, Ohio).
RESULTS:

1. Exercise Studies: During the interval exercise test subjects derived a substantial proportion of their energy requirements from anaerobic metabolism, as indicated by the whole blood lactate concentrations determined immediately after exercise, which were in the range 14.0 to 16.0 mmol.L⁻¹ (data not shown). Maximal heart rates obtained during the interval running test were in the range 175-185 bpm. The one hour cycling test required predominantly aerobic metabolism with subjects exercising at a heart rate of approximately 140 bpm.

The responses of subjects to the two exercise protocols differed. p47-Phox was evident in membrane preparations obtained from two of the three subjects used for each exercise test. p47-Phox was also detectable in some cases, at relatively low levels, in membrane preparations obtained before exercise. Figure 1 shows a representative result. The amount of p47-phox detected in membrane preparations was greater following the 1 hr test than after the interval running test. p47-Phox was also detected in the cytosolic preparations before and after both forms of exercise, indicating that only a portion of the total cytosolic "pool" of p47-phox was translocated to the membrane. It was not possible to quantitate the proportion of p47-phox translocated, or
the percentage of cells in which translocation occurred, in response to the different exercise tests.

2. **PMA Studies**: p47-Phox was detectable in the cytosolic fractions of stimulated and unstimulated neutrophils. P47-Phox was clearly evident in membrane preparations obtained from neutrophils stimulated with PMA. It was also detectable, in some cases, at low levels in membrane preparations obtained from unstimulated neutrophils. It appears that PMA stimulation induced translocation of at least some of the cytosolic p47-phox to the membrane (Figure 1).
DISCUSSION:
The results presented here suggest that extended aerobic cycling and intense interval running are both associated with translocation of p47-phox from the cytosol to the plasma membrane of neutrophils isolated after exercise. The quantity of p47-phox detected in the membrane fraction after exercise appeared to be greater following 1 hr of moderate cycling than after the intense interval exercise, which involved only 10-15 minutes of high speed running and approximately 30 minutes of jogging. This may indicate that exercise duration is a factor modifying the translocation of p47-phox to the plasma membrane. Alternatively the significantly increased plasma concentrations of cortisol induced by this exercise protocol (Gray et al., unpublished observations) may act to inhibit NADPH-oxidase activity (15). We have not determined however, whether our results reflect a greater degree of translocation on a per cell basis following the cycling test, or represent the involvement of a greater number of cells following this test. The presence of p47-phox in some of the membrane preparations obtained prior to exercise probably reflects a combination of a basal level of neutrophil activation in vivo and activation induced by the procedures used to isolate neutrophils (14).
Our findings are clearly indicative of assembly and activation of the NADPH-oxidase during exercise, and imply an increase in production of ROS by neutrophils during exercise. Clark et al. (1) and Tyagi et al. (12) have shown that there is a strong correspondence between translocation of p47-phox and generation of the superoxide anion following activation of the oxidase. Failure of p47-phox to translocate (as in certain forms of chronic granulomatous disease, CGD) has been shown to be associated with failure of the cells to generate superoxide (11). Whilst it is possible that exercise may constitute a mechanism that dissociates translocation of p47-phox to the membrane from NADPH-oxidase activation, our findings are consistent with other manifestations of neutrophil activation that are induced by exercise (2,3,5).

Prasad et al. (9) have shown that neutrophils are refractory to restimulation for up to 90 minutes (as assessed by ROS production), following an initial stimulation in vivo. An activation of the NADPH-oxidase system during exercise may explain the reduced ability of neutrophils isolated after exercise to produce ROS when stimulated in vitro. A reduction in the ability of neutrophils to produce ROS, and hence to be able to respond fully to infective challenge, may increase the susceptibility of athletes to infection in the post-exercise period (4). However this contention must
be considered in the light of the fact that we have not quantified the proportion of neutrophils, in the peripheral circulation after exercise, in which p47-phox was translocated. In addition plasma concentrations of neutrophils typically undergo a sustained elevation following exercise (6), which may help to counter any reduction in the ability of a proportion of these cells to produce ROS when stimulated.

The different forms of exercise used in this study (cycling and running, extended aerobic and intense interval running requiring substantial anaerobic metabolism) appear to induce assembly and activation of the NADPH-oxidase system. While it appears that exercise has a general ability to induce activation of neutrophils, further investigations are required to establish whether activation of these cells is amenable to modulation by altering the form, quantity or intensity of exercise.

Production of ROS and release of proteolytic enzymes by neutrophils has an established ability to induce tissue damage (16) and indeed neutrophil activation during exercise has been suggested as contributing to the development of delayed onset muscle soreness (10). Our findings provide additional support for this contention.
In summary our findings indicate that both aerobic and intense interval exercise induce assembly of the NADPH-oxidase system of neutrophils and provide further evidence that exercise is associated with increased production of ROS by neutrophils during exercise.
REFERENCES:


Legends:

Figure 1: Effect of exercise and PMA on the localisation of p47-phox within neutrophils.

a & b: Cytosolic preparations obtained before and after exercise, respectively. Both show the presence of p47-phox.

c & d: Membrane preparations obtained before and after exercise, respectively. Lane "c" shows the presence of some p47-phox in the membrane before exercise. Lane "d" shows an increased amount of p47-phox in membrane after exercise, clearly indicating translocation with exercise.

e: Membrane preparation showing the presence of p47-phox, obtained after incubation of neutrophils with PMA.

Molecular weight standards (kD) are shown at left.
20. DISCUSSION
This study has demonstrated that intense interval exercise by trained male subjects leads to a series of biochemical and morphological changes consistent with the multi-faceted activation (as opposed to priming) of neutrophils. Such activation may have important implications for the status of the immune, haematological and other systems of athletes in the post-exercise period. We have demonstrated, in addition, that a single session of intense interval running leads to significant perturbation of other physiological systems known to be capable of altering immune function. This was manifested as a significant elevation in the plasma concentrations of several hormones, minor fluctuations in plasma cytokine concentrations and iron status parameters as well as a characteristic pattern of mobilisation of leucocyte subsets (FIGURE 2).

This study employed two slightly different intense interval exercise protocols each requiring substantial anaerobic metabolism, as indicated by the significantly elevated whole blood lactate concentrations measured after exercise (7.6 mmol.L⁻¹ and 14.0 - 16.0 mmol.L⁻¹, respectively). The first protocol was laboratory based using a treadmill, the second was a "field" test that utilised a 400 metre running track. The protocols produced similar changes in both peripheral concentrations of leucocyte subsets and phenotypic
FIGURE 2: Relationship of physiological systems affected by intense interval exercise.
characteristics of neutrophils. These similarities indicate the close comparability of the laboratory-based interval training protocol to those which are employed in the field.

Intense interval exercise produced only minor changes in several parameters that relate to iron status, with elevations being observed in the plasma concentrations of red blood cells, haemoglobin and serum transferrin that can be attributed largely to haemoconcentration. Despite an increase in serum transferrin concentration, the physiologically more relevant parameter, transferrin saturation, was not altered significantly by this protocol. There was also no significant change in serum ferritin concentration. These findings are of interest for at least two reasons: firstly, a single interval training session failed to induce changes in the iron status parameters measured that can be considered physiologically important. However measurement of additional parameters such as haptoglobin may help to further elucidate the effects of interval exercise on iron status (Taylor et al., 1987). Our findings indicate that the established association between endurance exercise and a reduced iron status (our findings; Clement & Sawchuk, 1984) apparently relates more to either aerobic training regimes or to the cumulative effects of a number of training sessions; secondly, the availability of iron,
to act as a catalyst in the formation of hydroxyl radicals by the Haber-Weiss reaction (Halliwell & Gutteridge, 1986) does not seem to be modified by this exercise protocol. Thus the increased formation of lipid peroxidation products, which has been associated with exercise (Lovlin et al., 1987; Jenkins, 1988), does not seem to be due to an increased availability of iron in the circulation. However our findings do not exclude the possibility of either free iron or iron within iron-binding proteins inducing lipid peroxidation in particular tissue microenvironments.

The findings of the present study, together with those of other investigators, suggest that release of granule proteins into the circulation, together with activation of the neutrophil NADPH-oxidase system may provide an explanation for the low-iron status (hypoferremia) associated with infection, inflammation (Lipschitz, 1990) and chronic exercise (Clement & Sawchuk, 1984; Kaaden, 1988). The combined effects of neutrophil-mediated oxidative (NADPH-oxidase linked) and non-oxidative (enzymatic damage) have been found to increase immunoglobulin binding to "altered" cell-surface structures on erythrocytes (Weiss et al., 1992), offering a possible explanation for the accelerated destruction of these cells during inflammation and the associated anaemia (Lipschitz, 1990). It is possible that the low iron status
exhibited by some endurance athletes may be mediated by repeated exercise-induced activation of neutrophils. However, the relative contributions of neutrophil-mediated and other putative mechanisms [(eg: foot-strike haemolysis, loss through sweat etc (Kaaden, 1988)] to the relatively low iron status of athletes requires further investigation.

It has been reported that low pH conditions, such as those found in tissue micro-environments associated with the activation of the NADPH-oxidase and concurrent \( H^+ \) transfer to the extra-cytoplasmic side of the plasma membrane (Henderson et al., 1988) or those which occur during intense anaerobic exercise (with associated reduction in plasma pH) favour mobilisation of iron from transferrin and lactoferrin (Aruoma & Halliwell, 1987). Thus training regimes which rely principally on anaerobic, rather than aerobic, metabolism may potentiate a reduction in iron-status. Even though low iron levels may act to inhibit bacterial growth there is no evidence that iron deficiency provides protection against infection (Chandra, 1991). In fact low iron-status compromises the ability of the immune system to meet an infective challenge (Chandra, 1991), specifically in relation to the iron requirements of proliferating lymphocytes (Brock, 1981; Pattanapanyasat et al., 1992), activated NK cells (Chandra, 1991) and neutrophils (van Asbeck et al., 1984).
Our finding of an exercise-induced release of granule proteins, from both primary (elastase) and secondary granules (implied by up-regulation of CR3 on neutrophils), indicates that neutrophils have the potential to exert multi-component bactericidal (Ellison & Giehl, 1991; Selsted, 1988) and bacteriostatic (Brock & De Sousa, 1986) activity during and following exercise. Activation of neutrophils by exercise may constitute a "surveillance" or protective function, and may provide immediate protection from potential (but latent) pathogens present at the time exercise is undertaken.

Adherence of neutrophils to the endothelium and extravasation to sites of tissue damage are controlled by at least two structures on the surface of the neutrophil (CR3 and LECAM-1) and others on the endothelium (Pardi et al., 1992). We have shown that intense interval exercise leads to decreased expression of LECAM-1 and increased expression of CR3 on the surface of neutrophils. These changes, interpreted in conjunction with the effects of PMA on CR3 and FcR, are consistent with a partial activation of neutrophils during exercise (Kishimoto et al., 1989). There are a number of reports indicating that blocking of LECAM-1 (by the use of specific antibodies) inhibits extravasation of neutrophils, thereby increasing their concentration in the peripheral circulation (Jutila et
al., 1989; Kishimoto et al., 1989). Our findings indicate that the down-regulation of LECAM-1 induced by exercise may inhibit extravasation of neutrophils, possibly contributing to the sustained elevation of neutrophils noted in this and other studies (McCarthy & Dale, 1988).

Previous investigations that focused on aerobic exercise have reported that it is generally (but not always; Smith et al., 1992) associated with increases in plasma cytokine "activity" or immunoreactivity (Cannon & Kluger, 1983; Dufaux & Order, 1989; Sprenger et al., 1992). Typically these findings have been interpreted as reflecting an activation of cellular immunity following aerobic exercise. Neither the initiating mechanisms nor the consequences of such activation have been elucidated. However the results reported in this study do not support a role for intense interval exercise in elevating plasma cytokine concentrations generally, except in relation to those processes that lead to an elevation of neopterin. Neopterin, which is produced by macrophages following stimulation by lymphocyte-derived IFN-γ, was elevated at 6 and 24 hours after exercise. The relatively late increase in neopterin may reflect a significant activation of IFN-γ-producing T lymphocytes that is secondary to tissue damage induced by exercise. The occurrence of such damage, which can be inferred from
the increased CK activity detected in plasma post-exercise, is likely to lead to the infiltration of phagocytic cells such as granulocytes and macrophages to sites where muscle fibres have been damaged, and a localised inflammatory response (Smith, 1991). A response of this kind has been implicated in the development of delayed onset muscle soreness (DOMS; Smith, 1991). The origin and components of this putative response have yet to be fully characterised, and the relationship between the symptoms observed and neutrophil activation during exercise, if any, has yet to be determined. Recruitment of phagocytic cells to sites of exercise-induced tissue damage may play some role in the normalisation of peripheral granulocyte concentrations at 24 hrs post-test, as described in this investigation.

It appears that while down-regulation of LECAM-1 may inhibit extravasation of neutrophils (Jutila et al., 1989), this process may be counteracted by the ability of localised tissue damage to act as a chemoattractant, hence promoting extravasation (Smith, 1991). The net effect of these two opposing processes has yet to be determined.

Our study of the effects of the interval training session on endocrine system revealed significant elevations in plasma concentrations of most of the
hormones examined, including total cortisol, free cortisol, norepinephrine, DHPG, growth hormone, prolactin, estradiol, testosterone and SHBG. However, in the case of testosterone and SHBG, the increases can be attributed to the effects of haemoconcentration. In addition, a reduction in the metabolic clearance rates for both testosterone (Sutton et al., 1976) and estradiol (Keizer et al., 1980) would tend to favour an elevation in the plasma concentration of these hormones in the period immediately post-exercise. It is unlikely that sustained elevation of estradiol can be explained simply on the basis of reduced clearance, however. It appears to be unrelated to the activation of neutrophils, which was only observed immediately post-exercise, although the possibility of an interaction between these phenomena cannot be ruled out. The increases induced by interval exercise in the plasma concentrations of most of the other hormones assayed (GH, PRL, cortisol) were consistent with earlier studies that had revealed a distinct exercise-intensity related activation threshold and an intensity-dependent increase in plasma concentration (Viru, 1985).

While most of the hormones investigated have an ability to modulate some aspects of immune system activity, our investigations in relation to this ability, were centred on growth hormone. Despite earlier reports that
growth hormone treatment \textit{in vitro} induced increases in neutrophil adhesiveness (Weidermann \textit{et al}., 1991), we were unable to demonstrate any changes in the expression of \( Fc_{y}R \) or CR3 on neutrophils, that could be ascribed to GH added \textit{in vitro}. Thus, increases in neutrophil adhesion do not appear to be related simply to up-regulation of CR3, although this is one of the main molecules implicated in mediating cell adhesion. This explanation agrees with the conclusions of Buyon \textit{et al}. (1988), who have implicated a conformational change in the receptor rather than receptor up-regulation as a key event in increased adhesion. We verified the earlier findings of Spadoni \textit{et al}. (1991) and Weidermann \textit{et al}. (1991) concerning the ability of GH to "prime" neutrophils for enhanced ROS production. Thus while GH does not appear to be involved in direct activation of neutrophils, increases in plasma GH concentrations induced by exercise (see below) may potentiate the production of ROS during exercise.

One of the most surprising findings of the present study was the relatively modest elevation observed in NE, and the lack of significant elevation of Ep, in plasma samples collected immediately after exercise. One of the primary metabolites of neuronal NE, DHPG, was elevated significantly at this time, indicating that significant activation of the sympathetic nervous system had occurred during the interval exercise. In
addition, the plasma concentration of a precursor for formation of both Ep and NE, DA, was decreased significantly at all post-exercise sampling points. Taken together these findings indicate that cessation of exercise, which in the case of the laboratory-based protocol was due to volitional exhaustion (ie: an inability to continue at the required speed and gradient), may have been due to depletion of catecholamine reserves. This may have, in turn, decreased the ability of the body to mobilise energy reserves, in processes that are dependent on catecholamines or have affected neurotransmission within the sympathetic nervous system (Ganong, 1983). This finding is consistent with a report that the urinary excretion of catecholamines is reduced during periods of intense training and competition (Lehmann et al., 1992). The fatigue experienced during and following single or multiple sessions of interval training, may be mediated by either the endocrine or nervous system. Interval exercise produced a different "profile" of plasma catecholamines than that which results typically from aerobic exercise (Viru, 1985). The unexpected plasma catecholamine "profile" obtained following intense interval exercise serves to illustrate the differing physiological responses induced by various forms of exercise (eg: extended aerobic versus interval training).
This study has shown that, with regard to intense interval exercise, the mobilisation of leucocyte subsets is correlated significantly with both the number of exercise efforts performed and plasma catecholamine (eg: DHPG) (but not cortisol) concentrations. A biphasic leucocytosis, that is an initial increase in lymphocytes, and a later (6 hr) and sustained increase in granulocytes, induced by this exercise protocol, is in agreement with the conclusions reached by McCarthy & Dale (1988). The elevated concentrations of lymphocytes and granulocytes in the peripheral circulation after exercise have been ascribed to the effects of catecholamines and cortisol, respectively (McCarthy & Dale, 1988). Our findings, however, support the former but not the latter mechanism.

The mobilisation of lymphocyte subsets measured in this study related most closely to changes in plasma catecholamine concentrations, and is probably similar to the $\beta_2$-adrenergic mediated, spleen-dependent process, described by Van Tits et al., (1990). The increases in CD4$^+$, CD8$^+$, and NK cells produced by this exercise protocol are in agreement with other investigators (Mackinnon & Tomasi, 1988). It is to some extent surprising that the mobilisation of granulocytes correlated more strongly with changes in plasma catecholamines, than with plasma cortisol
concentrations, as this finding is in conflict with that of Moorthy & Zimmerman (1978), although it might have been predicted on the basis of Ep infusion studies carried out earlier (Athens et al., 1961).

We have found that granulocytes displayed changes in their phenotypic characteristics (eg: in cell-surface expression of receptors and light-scatter) after exercise. While these changes appear to be due to neutrophil activation, the possible contribution of phenotypically different neutrophils released from the bone marrow or other storage sites, to the circulating "pool" must not be overlooked. It is likely that both processes are involved. This question may be resolved by using flow cytometry with three colour analysis, which would enable the simultaneous assessment of CD11b, CD15 and CD16 expressed by neutrophils, the expression of which varies with the maturational stage of these cells (Terstappen et al., 1990).

The nature of the agents inducing neutrophil activation during exercise have not been determined. Our findings imply that cytokines, as indicated by the relatively small changes in their plasma concentrations, are unlikely to be involved in the activation of neutrophils during intense interval exercise, although they may potentiate the response (Kowanko & Ferrante, 1987). Neutrophils were apparently activated during the
course of exercise, yet cytokine concentrations increased significantly only in relation to neopterin, and only at 6 and 24 hours after exercise. However, a role for cytokines in exercise-induced activation of neutrophils (particularly as might occur in specific tissue microenvironments) cannot be excluded, given the findings of Sprenger et al. (1992), indicating increased urinary excretion of cytokines after exercise.

It also appears unlikely that activation of the complement cascade mediates neutrophil activation during this protocol, given that the plasma concentration of C3a and C3a des arg were not significantly changed by interval exercise. Alternatively an exercise-induced endotoxemia (Bosenberg et al., 1988) may act to induce such activation.

Our finding that circulating neutrophils isolated immediately after exercise displayed a reduced ability to produce ROS when exposed to exogenous stimulation is indicative of the cells NADPH-oxidase system having undergone activation in vivo, which is consistent with the work of Prasad et al. (1991). Our findings indicate that the "refractory" period is mediated by events distal to PKC in the signal transduction pathway culminating in ROS production. Translocation of the
NADPH-oxidase component p47-phox to the membrane is mandatory for (Clark et al., 1990), and clearly indicative of (Tyagi et al., 1992), oxidase activation. This is the first investigation in which changes in key components of the NADPH-oxidase complex in response to exercise have been assessed. Our findings are consistent with activation of the NADPH-oxidase, and production of ROS by neutrophils, during exercise. Such activation, coupled with the above mentioned degranulation, not only leaves neutrophils as a newly identified source of ROS during exercise (Sjodin et al., 1990), but also provides support for the contention that neutrophils contribute to exercise-induced tissue damage. In particular the sustained elevation in neutrophil concentration observed post-exercise may increase the potential for tissue damage.

An interaction of proteolytic enzymes and ROS derived from neutrophils has been identified previously as playing a key role in tissue damage (Weiss et al., 1992). It is thus possible, and in agreement with the conclusions of Smith (1991), that the co-ordinated action of these effector mechanisms is responsible for some of the tissue damage induced by exercise. In this regard, Cannon et al. (1990) have postulated that the higher plasma CK activity after exercise in subjects receiving dietary supplementation with α-tocopherol may
be due to an increase in ROS production. Such increases are thought to result from the protection, by α-tocopherol, of neutrophil membranes against oxidative "attack" by self-generated ROS, which may limit the self-destruction and potentiate an increase in total ROS production and release. This controversial interpretation implicates neutrophils as primary agents in exercise-induced muscle damage, but provides no direct evidence that NADPH oxidase activation occurs during exercise. The present work provides evidence for just such an activation of the NADPH-oxidase and hence production of ROS during exercise. However, the role of neutrophils, and the relative contribution of oxidative and non-oxidative effector mechanisms, in promoting tissue damage requires further investigation.

It appears that both aerobic (Kokot et al., 1988) and interval exercise protocols cause activation of neutrophils. How can these observations be reconciled with the apparently differential effects of exercise intensity on human immune function? i.e.: gentle aerobic exercise being associated with a reduced incidence, and intense exercise with an increased incidence, of infections. As discussed previously exercise of low intensity, provided it is not continued for a protracted length of time, is associated with little, if any, elevation in the plasma concentrations of the immunosuppressive hormones, cortisol or catecholamines,
whilst it significantly increases the plasma concentration of GH (Viru, 1985), for which immunopotentiating properties have been described (Weidemann et al., 1991). In contrast, our results indicate that intense exercise induces significant, and in the case of cortisol, sustained, elevation in the plasma concentration of this immunosuppressive hormone, that may over-ride the potentiating effects of GH. It can be hypothesised that the post-exercise "refractory" period, induced in neutrophils subsequent to activation (Prasad et al., 1991), may be prolonged or accentuated by immunosuppressive hormones. A refractory period that becomes longer or more pronounced as the exercise intensity increases may increase the susceptibility of subjects to infection at these times (Douglas & Hanson, 1978; Heath et al., 1991; Nieman et al., 1990a; Peters & Bateman, 1983).

Thus activation of the immune system induced by moderate exercise may serve to remove latent, potential pathogens from the body. Any refractory period that develops under these conditions may be of limited magnitude and duration, and possibly counterbalanced by the "priming" effects of immunopotentiating hormones such as GH. It is possible that the benefits of moderate aerobic exercise in reducing the incidence and/or severity of upper respiratory infections (Nieman et al., 1990b; Nehlsen-Cannarella et al., 1991) relate
in some way to a mild exercise-induced activation of the immune system. High intensity exercise, with an accentuated refractory period due to the associated hormonal milieu, may leave subjects susceptible to infection for an extended time after exercise (Nieman et al., 1990a).

This hypothesis is in contrast to those put forward previously (Smith & Weidemann, 1990) in that it proposes an immunosuppression (refractory period) as a direct consequence of exercised-induced activation of neutrophils, rather than an induction of a simple immunosuppression (without neutrophil activation) as a consequence of exercise. This difference is important. Exercise-induced activation of neutrophils may have wide implications for the field of exercise physiology, particularly in relation to regulation of iron status, the susceptibility of physically active subjects to infection and the origin of tissue (including muscle) damage associated with exercise.

In summary, intense interval exercise resulted in modulation of a number of physiological systems and induced a broad-spectrum activation (rather than mere priming) of neutrophils, subsequent to which the cells were refractory to further stimulation. Activation of the oxidative and non-oxidative effector mechanisms of
neutrophils during exercise may have important implications for both tissue damage induced during, and immunocompetence immediately after, interval exercise.
REFERENCES:


APPENDIX
Anaerobic exercise causes transient changes in leukocyte subsets and IL-2R expression

A. B. GRAY, Y. C. SMART, R. D. TELFORD, M. J. WEIDEMANN and T. K. ROBERTS

Department of Biological Sciences, University of Newcastle, NSW, 2308 AUSTRALIA; Discipline of Surgical Science, Faculty of Medicine, University of Newcastle, NSW, 2300 AUSTRALIA; Department of Physiology and Applied Nutrition, Australian Institute of Sport, Belconnen, ACT, 2616 AUSTRALIA; and Division of Biochemistry and Molecular Biology, School of Life Sciences, Faculty of Science, The Australian National University, Canberra, ACT, 2601 AUSTRALIA

ABSTRACT

GRAY, A. B., Y. C. SMART, R. D. TELFORD, M. J. WEIDEMANN, and T. K. ROBERTS. Anaerobic exercise causes transient changes in circulating leukocyte subsets and IL-2R expression. *Med. Sci. Sports Exerc.*, Vol. 24 No. 12 pp 1332-1336, 1992. There is evidence that the stress of intense athletic competition and training depresses cellular immunity and predisposes athletes to increased infection. This paper reports changes in circulating leukocyte subsets of trained (group I: VO₂max = 67.2 ± 5.4 ml·kg⁻¹·min⁻¹; age = 22.0 ± 6.2 yr) and untrained (group II: VO₂max = 55.0 ± 4.9 ml·kg⁻¹·min⁻¹; age = 21.4 ± 2.0 yr) males following 1 min of bicycle ergometry at maximum effort. Significant post-exercise increases in concentrations of total leukocytes, monocytes, lymphocytes, CD3⁺, CD4⁺, CD8⁺ lymphocytes were observed in both groups (all P < 0.01). The CD4⁺/CD8⁺ ratio decreased significantly (P < 0.01) but the granulocyte concentration was not altered (P > 0.05). Despite groups I and II not differing in either peak power or total work performed during the exercise test (P > 0.05), group II had a significantly greater concentration and percentage of CD8⁺ lymphocytes immediately after exercise (P < 0.01). All of the early changes were transient, with normalization occurring within 1 h. Only trained subjects showed a significant decrease in the percentage of CD25⁺ lymphocytes following PHA stimulation of whole blood obtained 6 h post-exercise. Alterations in leukocyte subpopulations found in response to predominantly anaerobic exercise appear to be associated with a significant, but possibly transient, alteration in the mitogen responsiveness of lymphocytes that is restricted to aerobically trained subjects.
ANAEROBIC EXERCISE AND IMMUNE FUNCTION

to “prime” peripheral blood neutrophils [which enhances their rate of formation of reactive oxygen species upon subsequent stimulation (28)], to enhance natural killer cell activity (17), and to moderate the responses of lymphocytes to mitogenic stimulation (17,31).

Most investigations of the effects of exercise on the immune system have largely ignored intense anaerobic exercise. The protocols employed have been predominantly aerobic in nature and often irrelevant to the actual training regimes of all athletes (21).

The highly stressful nature of anaerobic exercise [and the concomitant elevation of the plasma concentrations of immunomodulatory “stress” hormones such as cortisol and epinephrine (33)] is likely to lead to significant changes in leukocyte subsets and a reduction in the ability of lymphocytes to respond to mitogenic challenge (6,11,31). In a practical sense, this type of exercise relates most closely to the training and competition of athletes in a wide variety of sports, as these individuals derive a significant proportion of their metabolic energy from anaerobic pathways during both training and competition. It has been reported that some 72–87% of metabolic energy is derived from anaerobic sources during 30 s of bicycle ergometry at maximum effort (2) and that exercise of this intensity sustained for 60 s will completely exhaust anaerobic energy reserves (32).

The present study investigates the effects of 1 min of bicycle ergometry at maximal effort on circulating concentrations of leukocyte subpopulations. Trained and untrained male subjects were employed to address the possible effects of training and competition on the response of these parameters to intense anaerobic exercise. In particular, special reference has been made to the effects of this regime on CD3+ lymphocyte subsets and lymphocyte activation following mitogenic stimulation. A key element of the lymphocyte proliferative sequence, the expression of interleukin-2 receptors (IL-2R/CD25) (34), has been assessed. This is one of the first attempts to investigate a discrete element of this sequence rather than the assessment of total proliferation by the uptake of radioactive nucleotides (31).

MATERIAL AND METHODS

Study participants. All subjects were male and consisted of two groups. Group I: competitive cyclists (mean age ± SD) = 22.0 ± 6.2 yr) all of whom had been in regular training for at least 1 yr (4–5 h·d−1 representing between 500 and 800 km·wk−1). All were competing at a minimum of district level, with one recently returned from the World Junior Championships. Group II: untrained subjects (21.4 ± 2.0 years) with no history of regular physical training. All subjects were asked to refrain from exercise throughout the 24 h prior to the test. All were free of symptoms of infection, and none were taking medications or drugs of any kind.

Exercise tests

Subjects were familiarized with the testing procedures for both VO2max and 60-s cycling tests, and all participants signed a consent form that detailed the scope of the study and its attendant risks. Procedures complied with human subject guidelines of the American College of Sports Medicine and were approved by the Ethics Committee of the University of Newcastle.

Determination of maximal oxygen uptake (VO2max). Subjects underwent progressive bicycle ergometry, according to methods described previously (15), during which the workload was incremented, from an initial 100 W, by 25 W each minute until they could continue no further. Criteria for attainment of VO2max were: R value in excess of 1.10, a “plateau” in oxygen consumption (an increment of less than 0.15 l·min−1), or a heart rate equal to the predicted maximum.

Anaerobic cycling test. The anaerobic exercise tests utilized a purpose-built bicycle ergometer (22), which allowed the on-line measurement of both peak power output and cumulative work for each subject during 1 min of cycling. All values reported were normalized to standard temperature and pressure (i.e., atmospheric pressure = 760 mm Hg; temperature = 25°C). Testing of subjects was carried out within 2 wk of the VO2max test. Subjects arrived at the testing laboratory at approximately 0900 hours, at least 2 h after a light breakfast. All blood samples were obtained by venepuncture of an antecubital vein with subjects in the supine position. The first blood sample (at rest) was only taken after the subject had been sitting quietly in the laboratory for at least 30 min. Subjects then began a 10-min warm-up at 3 W·kg−1 (group I) and 2 W·kg−1 (group II), these levels approximating a power output corresponding to 60% VO2max. A second blood sample was drawn and the subject was positioned for the 60-s anaerobic cycling test. Subjects, using toe clips, were asked to remain seated throughout the test, and were instructed to pedal with maximum effort from commencement of the test. Subjects were informed of the elapsed time at 10-s intervals until completion of the test, at which time data acquisition was automatically terminated. They cycled slowly at the end of the test for 2–3 min to facilitate venous return. The third blood sample was collected 3–5 min after termination of the test. Samples 4 and 5 were collected 1 h and 6 h after completion of the test, respectively. Subjects were allowed to leave the laboratory between samples 4 and 5, but were asked to refrain from further exercise.

Blood sampling and analysis. Venous blood for leukocyte subset and lactate analysis was collected into an EDTA-containing test-tube (Disposable Products,
Sydney) and mixed continuously with a Coulter mixer (Coulter Electronics, Hialeah, FL) prior to analysis on an Coulter S550 Counter (Coulter Electronics). All peripheral blood samples were analyzed for whole blood L-lactate using a YSI Model 23L L-lactate analyzer (Yellow Springs Instrument Company, Yellow Springs, OH). The method of analysis involved the addition of whole blood (25 µl) to a buffer solution (50 µl) containing YSI 2326 lactate buffer, sodium fluoride, and Triton-X. All samples were analysed within 5 min of collection.

Flow cytometric analysis of leukocyte subpopulations. A fluorescence-activated cell sorter (FACS 440; Becton-Dickinson, Mountain View, CA) equipped with an argon laser emitting at 488 nm at 200 mW (Spectra Physics) was used for all flow cytometric determinations. Fluorescent polystyrene beads (Polysciences, Warrington, PA) were used to align and calibrate the machine. During the analysis of CD3+ lymphocyte subsets, list mode data was collected on 20,000 cells. Histograms of forward and 90° scattered light and log green fluorescence were collected using the ACQ4 program of the CONSORT 40 computer and analyzed using the DISP4 program (Becton-Dickinson). For the differential determination of the major leukocyte subpopulations, list mode data on 50,000 cells were collected, with appropriate gating of the forward and 90° scatter profile to exclude debris and to allow differentiation of the lymphocyte, granulocyte and monocyte populations.

Preparation of blood cells for T lymphocyte (CD3) subset analysis by flow cytometry. The monoclonal antibodies, OKT3 (specific for mature T lymphocytes, anti-CD3); OKT4 (T helper/inducer lymphocytes, anti-CD4); OKT8 (T cytotoxic/suppressor lymphocytes, anti-CD8) were pretitred before incubation with whole blood. Optimal amounts of monoclonal antibody were reacted with EDTA anti-coagulated whole blood (100 µl) according to methods described previously (5). The indirect immunofluorescence method employed involved the addition of autologous plasma (20 µl) to overcome nonspecific effects and a second-step poly-clonal antibody (goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Tago, Medos Company, Sydney) (50 µl of 1:100 dilution). Samples were suspended in 1% paraformaldehyde (0.5 ml) and stored at 4°C in the dark for analysis within 2 d. A sample from a normal, unexercised subject was included in each experiment as a control measure.

Expression of interleukin-2 receptors (CD25). The method used to investigate the expression of receptors for interleukin-2 (IL-2R, CD25) was a modification of that described by Park and Good (26). Duplicate samples of sterile heparinized whole blood (50 µl) were mixed with RPMI 1640 culture medium (CSL, Sydney) (50 µl containing 50 µg·ml⁻¹ phytohaemagglutinin (PHA) (Sigma Chemical Company, St. Louis, MO) and gentamycin at 50 µg·ml⁻¹ (Sigma)), in a sterile tissue culture tube (75 × 12 mm, Disposable Products) that had been equilibrating in the incubator for approximately 12 h. The tube was vortexed gently, then incubated for 24 h at 37°C with 5% CO₂. Controls containing no PHA were prepared and treated in the same way. At the end of the incubation period optimal amounts of FITC conjugated anti-CD25 monoclonal antibody (Coulter) were used to enumerate CD25+ lymphocytes by flow cytometry as described above. Cell viability as assessed by trypan blue exclusion was in excess of 95% in all cases.

Statistical analysis. A two-way analysis of variance (ANOVA) with repeated measures was employed. A Tukey T post-hoc test was used to determine the identity of any significant F-value obtained in the ANOVA. Additionally Student's t-tests were employed to assess the significance of the differences between means for the parameters listed in Table 1. Data are presented as mean (±SD).

RESULTS

A comparison of the anthropometric and performance characteristics of groups I and II is presented in Table 1, including the evaluation of the significance of the differences observed between the mean values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Group I</th>
<th>Group II</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>yr</td>
<td>22.0 (6.2)</td>
<td>21.4 (2.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Height</td>
<td>cm</td>
<td>178.5 (5.7)</td>
<td>180.9 (9.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight</td>
<td>kg</td>
<td>71.3 (8.0)</td>
<td>73.8 (7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>VO₂max</td>
<td>ml·kg⁻¹·min⁻¹</td>
<td>57.2 (5.4)</td>
<td>55.0 (4.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1-Min test:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak power</td>
<td>W</td>
<td>891.1 (116.2)</td>
<td>966.6 (74.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Peak power per kilogram</td>
<td>W·kg⁻¹</td>
<td>12.4 (1.5)</td>
<td>13.2 (1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>% Power decrease (peak to end of test)</td>
<td>60.0 (7.6)</td>
<td>73.6 (7.4)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Total work</td>
<td>kj</td>
<td>33.3 (3.6)</td>
<td>31.9 (3.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Total lactate (post exercise)</td>
<td>m·mol·l⁻¹</td>
<td>12.2 (1.3)</td>
<td>15.0 (2.5)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NS = P > 0.05.
Trained (group I) subjects had a significantly greater maximum oxygen uptake (VO2max) (P < 0.001) than untrained (group II) subjects, reflecting their higher aerobic fitness. Group II subjects experienced a significantly greater decrease (relative to their peak power) in power output at the conclusion of the one minute test compared with group I subjects (P < 0.01). However, the performance of group I and II subjects during the test was not significantly different with respect to either peak power output or total work completed, whether expressed on an absolute basis or per kilogram body weight.

There was a significant increase in blood lactate concentration in both groups immediately after exercise (P < 0.01). The blood lactate concentration reached a higher value in the untrained than the trained subjects (P < 0.05).

When assessed at rest, there were no statistically significant differences between group I and II subjects with respect to any leukocyte subset, whether expressed in absolute or percentage terms (Tables 2 and 3). All leukocyte parameters were within normal reference ranges when assessed at rest (5.13).

High-intensity anaerobic exercise undertaken for 1 min produced significant alterations in both the concentrations and proportions of most of the leukocyte subsets examined (Tables 2 and 3). The significant increase in leukocyte concentration seen immediately post-exercise (P < 0.01 for both groups) can be attributed to a transient, but marked, increase in lymphocyte

### TABLE 2. Leukocyte subset concentration.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Group</th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Immediately Post-test</th>
<th>1 h Post</th>
<th>6 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>I</td>
<td>6.0(1.0)</td>
<td>7.8(2.0)</td>
<td>10.8(2.7)**</td>
<td>5.7(1.9)</td>
<td>8.4(2.9)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>6.4(1.0)</td>
<td>7.3(1.6)</td>
<td>11.6(2.3)**</td>
<td>9.4(3.4)</td>
<td>10.3(2.9)**</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>I</td>
<td>1.9(0.4)</td>
<td>2.9(0.7)</td>
<td>5.0(1.4)**</td>
<td>1.9(0.5)</td>
<td>2.2(0.4)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>2.0(0.4)</td>
<td>2.4(0.7)</td>
<td>5.1(2.1)**</td>
<td>1.9(0.7)</td>
<td>2.4(0.6)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>I</td>
<td>3.6(1.5)</td>
<td>4.3(1.7)</td>
<td>4.9(2.1)</td>
<td>4.3(2.0)</td>
<td>5.5(2.9)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>4.0(0.9)</td>
<td>4.4(1.2)</td>
<td>5.7(1.4)</td>
<td>5.9(3.1)</td>
<td>7.9(2.8)**</td>
</tr>
<tr>
<td>Monocytes</td>
<td>I</td>
<td>0.4(0.2)</td>
<td>0.5(0.2)</td>
<td>0.7(0.3)**</td>
<td>0.4(0.2)</td>
<td>0.4(0.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.3(0.1)</td>
<td>0.3(0.1)</td>
<td>0.5(0.3)**</td>
<td>0.3(0.2)</td>
<td>0.4(0.2)</td>
</tr>
<tr>
<td>CD3+</td>
<td>I</td>
<td>1.2(0.4)</td>
<td>1.5(0.5)</td>
<td>2.2(0.9)**</td>
<td>1.3(0.5)</td>
<td>1.4(0.3)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1.2(0.3)</td>
<td>1.5(0.5)</td>
<td>2.8(0.7)**</td>
<td>1.2(0.4)</td>
<td>1.5(0.3)</td>
</tr>
<tr>
<td>CD4+</td>
<td>I</td>
<td>0.8(0.2)</td>
<td>1.0(0.3)</td>
<td>1.3(0.5)**</td>
<td>0.9(0.3)</td>
<td>0.9(0.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.7(0.2)</td>
<td>0.8(0.2)</td>
<td>1.2(0.3)**</td>
<td>0.6(0.2)</td>
<td>0.9(0.3)</td>
</tr>
<tr>
<td>CD8+</td>
<td>I</td>
<td>0.4(0.1)</td>
<td>0.7(0.3)</td>
<td>0.9(0.3)**</td>
<td>0.4(0.2)</td>
<td>0.5(0.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.5(0.1)</td>
<td>0.7(0.3)</td>
<td>1.7(0.6)**##</td>
<td>0.5(0.2)</td>
<td>0.5(0.2)</td>
</tr>
</tbody>
</table>

Concentrations = x1000 μl/l; data = mean (±SD); group I (trained); group II (untrained).
** = significantly different from rest (P<0.01).
## = significantly different from group I (P<0.01).

### TABLE 3. Leukocyte subset proportions.

<table>
<thead>
<tr>
<th>Subset as a percentage of total leukocytes</th>
<th>Group</th>
<th>Rest</th>
<th>Post-warmup</th>
<th>Immediately Post-test</th>
<th>1 h Post</th>
<th>6 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>I</td>
<td>33.6(8.4)</td>
<td>38.5(6.9)</td>
<td>47.8(12.6)**</td>
<td>30.7(11.1)</td>
<td>29.7(9.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>31.5(6.6)</td>
<td>33.0(8.0)</td>
<td>43.8(6.8)**</td>
<td>25.5(11.3)</td>
<td>23.1(7.5)</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>I</td>
<td>57.8(10.5)</td>
<td>53.1(9.8)</td>
<td>43.5(12.5)**</td>
<td>61.2(11.7)</td>
<td>52.4(11.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>62.0(7.3)</td>
<td>62.1(7.2)</td>
<td>49.3(7.3)**</td>
<td>58.4(11.7)</td>
<td>71.4(8.4)</td>
</tr>
<tr>
<td>Subsets as a percentage of total lymphocytes</td>
<td>CD3+</td>
<td>62.9(7.1)</td>
<td>54.2(6.5)</td>
<td>44.4(4.8)**</td>
<td>65.5(4.9)</td>
<td>64.6(4.7)</td>
</tr>
<tr>
<td>CD4+</td>
<td>I</td>
<td>64.8(14.0)</td>
<td>58.6(4.3)</td>
<td>48.4(7.1)**</td>
<td>58.0(8.9)</td>
<td>63.6(5.4)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>39.4(3.8)</td>
<td>34.5(5.5)</td>
<td>25.8(6.2)**</td>
<td>43.2(6.1)</td>
<td>41.4(5.6)</td>
</tr>
<tr>
<td>CD8+</td>
<td>I</td>
<td>34.5(4.4)</td>
<td>32.0(4.2)</td>
<td>24.1(2.8)**</td>
<td>34.4(8.0)</td>
<td>36.9(4.2)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>22.9(5.8)</td>
<td>23.8(6.7)</td>
<td>21.1(6.5)</td>
<td>22.0(5.1)</td>
<td>21.9(6.4)</td>
</tr>
<tr>
<td>CO25+</td>
<td>I</td>
<td>26.5(5.4)</td>
<td>28.6(6.3)</td>
<td>32.5(7.3)**#</td>
<td>24.4(5.6)</td>
<td>23.2(3.6)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>30.4(2.2)</td>
<td>27.8(9.3)</td>
<td>27.2(8.6)</td>
<td>25.3(11.1)</td>
<td>20.7(5.1)*</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>I</td>
<td>1.7(0.4)</td>
<td>1.5(0.5)</td>
<td>1.2(0.4)**</td>
<td>1.9(0.6)</td>
<td>1.8(0.5)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1.3(0.3)</td>
<td>1.2(0.2)</td>
<td>0.8(0.2)**</td>
<td>1.4(0.2)</td>
<td>1.6(0.2)</td>
</tr>
</tbody>
</table>

Group I (trained); group II (untrained); data = mean (±SD).
Significantly different from resting value = *(P<0.05), **(P<0.01).
Significantly different from Group I = ##(P<0.01).
CD25 in response to mitogenic stimulation was lower immediately after exercise than did untrained subjects; (ii) the changes in both the absolute number and percentage of lymphocytes followed at 6 h post-exercise were significant for both groups. There were, however, three significant effects of training: (i) aerobically trained subjects exhibited a significantly lower concentration of lymphocytes at 6 h after exercise. The trained and untrained subjects did not differ significantly from each other at any sampling time from the value obtained at rest. When each subset was expressed as a percentage of the total lymphocytes (Table 3), both trained and untrained groups experienced a decrease in the proportion of lymphocytes represented by CD3+ cells (P < 0.01). Groups I and II differed significantly only immediately after exercise, and then only in relation to CD8+ cells; at this time the untrained (group II) subjects had a higher absolute concentration of CD8+ cells than did group I subjects, which also represented a significantly higher percentage of the total lymphocytes (Tables 2 and 3; P < 0.01 for both conditions). At no other time did groups I and II differ significantly in relation to the CD4/CD8 ratio, even though, at all sampling times, group II had a lower ratio than group I (however, P > 0.05). The only significant change in this ratio, which was common to both groups, occurred immediately post-exercise, when the value decreased significantly (P < 0.01).

With reference to the expression of IL-2R/CD25 following PHA stimulation of whole blood, groups I and II did not differ significantly from each other (P > 0.05), nor did this parameter differ significantly in group II subjects at any sampling time from the value obtained at rest (P > 0.05). Group I subjects, however, had a significantly lower percentage of CD25+ lymphocytes (P < 0.05) following mitogenic stimulation of whole blood obtained at 6 hours post-exercise compared with those obtained at rest.

DISCUSSION

This study demonstrates that high intensity anaerobic exercise of only 1 min's duration leads to significant changes in both the absolute number and percentage of most leukocyte subpopulations, irrespective of the training status of the subjects tested. There were, however, three significant effects of training: (i) aerobically trained subjects exhibited a significantly lower concentration and percentage of CD8+ lymphocytes immediately after exercise than did untrained subjects; (ii) the reduction in the percentage of lymphocytes expressing CD25 in response to mitogenic stimulation was restricted to the trained subjects measured at 6 h post-exercise; and (iii) untrained, as compared with trained, subjects exhibited significantly elevated concentrations of total leucocytes and granulocytes at 6 h after exercise.

The biphasic nature of the leukocytosis observed here (i.e., an initial increase composed predominantly of lymphocytes followed at 6 h by an increase in granulocytes) may be related to the differing sensitivities of leukocyte subpopulations to changes in circulating concentrations of specific hormones. These observations are in agreement with both previous investigations of largely aerobic exercise (17), and a model of exercise-induced leukocytosis proposed by McCarthy and Dale (21) that involves an initial catecholamine-dependent lymphocytosis and a later cortisol-dependent granulocytosis. The differential leukocytosis may be the result of an interplay between hemodynamic and hormonal factors. Hemodynamic factors associated with exercise include increased cardiac output (14), which, together with possible differential localization of leukocyte subsets in putative storage and low-flow areas (23), may be responsible for the acute leukocytosis evident immediately after exercise. Increases in peripheral blood hormone levels (e.g., cortisol and catecholamines) in response to high-intensity exercise (33) may also make a significant contribution to both the biphasic nature of the leukocytosis (3,4,7) and the reduction in the percentage of CD25+ lymphocytes seen in the trained subjects (12,19,30) following mitogenic stimulation of whole blood.

Both groups displayed similar post-exercise increases in concentrations of CD3+ and CD4+ lymphocytes and similar reductions in the CD4/CD8 ratio. Such changes correspond closely to those produced by aerobic exercise (17). The significant reduction in the percentage of CD3+ lymphocytes, which occurred immediately post-exercise in both groups, implies that other non-CD3+ lymphocytes such as B-lymphocytes and natural killer cells are mobilized into the circulation, again in a manner consistent with that already established for aerobic exercise (17).

The only significant difference in leukocyte subpopulations attributable to training occurred immediately post-exercise, and consisted of a significantly greater concentration and percentage (P < 0.01) of CD8+ lymphocytes in untrained (group II) than trained subjects. The trained and untrained subjects did not differ in relation to the total work performed, or peak power, during the 1-min test. Thus, this differential mobilization of CD8+ lymphocytes cannot be attributed to differences in physical performance during the exercise test. However, trained subjects were better able to maintain their power output (Table 1), which given their significantly lower post-exercise blood lactate levels, suggests an enhanced ability of these subjects to produce metabolic energy by aerobic pathways. It is likely that clearance of lactate, during and immediately after exercise of this duration and intensity, is only a minor factor in affecting the blood lactate concentration. The possibility that elevated lactate or a fall in
blood pH may contribute, either directly or indirectly, to the elevated release of CD8+ lymphocytes in untrained subjects requires further investigation. The frequency and intensity of training and competition undertaken by the trained subjects may have modified the response of the immune system to exercise of this intensity. However, whether these modifications occur simply as a result of a reduced reliance on anaerobic metabolism in these (aerobically) trained subjects, or are a consequence of other hormonal and metabolic processes, remains to be determined. The functional implications of such a training response are difficult to predict because of the transient nature of the perturbation and the heterogeneous nature of the CD8+ population (which consists of cytotoxic and suppressor subsets). Altered mobilization could lead, in principle, to a reduction in suppressive or cytotoxic capacity or, indeed, simply to maintenance of the status quo. The nature of the mobilized cells must be defined more closely.

The leukocytosis (primarily of granulocytes) observed in untrained, as compared with trained, subjects at 6 h after exercise may indicate that the untrained subjects apparently greater reliance on anaerobic metabolism, or those factors referred to previously, modified the pattern of mobilization of these cells.

The significant reduction in the proportion of lymphocytes expressing IL-2R/CD25 in response to mitogenic stimulation in trained subjects at 6 h post-exercise indicates that this group may be significantly more vulnerable to the impact of anaerobic exercise on the capacity of the immune system to respond to infective challenge. This decrease reflects a real reduction in the absolute concentration of CD25+ lymphocytes in the mitogen-stimulated cultures, as there were no differences in the circulating concentrations of any lymphocyte subset between rest and 6 h post-exercise.

Expression of IL-2R is a critical step in lymphocyte proliferation. Antigenic stimulation (e.g., infective challenge) leads to clonal (or polyclonal with mitogens) expansion that is selective for proliferation of lymphocytes specific for the antigen (or mitogen) employed. Mitogens allow an in vitro appraisal of the ability of leukocytes to respond to polyclonal challenge. In vitro mitogenic assays provide a "model" system in which to investigate cell function.

Proliferation is fundamental to both subsequent elaboration of immunomodulatory cytokines (18) and the immune response as it allows the expansion of leukocyte subpopulations that are antigenically suited to dealing with the infective challenge (34). Inhibition of this sequence is likely to increase the incidence and severity of infectious episodes. The lymphocyte proliferative sequence has a number of discrete steps including IL-1 production by macrophages (18), receptor stimulation (1), lymphocyte production of the cytokine IL-2, expression of receptors for IL-2 and transferrin, and DNA replication (24). Depper et al. (8) have found a clear correlation between proliferation, the amount of IL-2 elaborated, and the percentage of CD25+ cells.

While CD25 is associated with the β-chain of the high affinity IL-2R, a heterodimer consisting of the constitutively expressed α-chain and the β-chain (29), increased expression of the β-chain is the key factor in the ability of lymphocytes to express a biologically relevant receptor for IL-2. Many previous investigations, employing radioactive nucleotide precursors (31), have evaluated only DNA replication leaving important intermediate steps unexamined. Investigation of the diurnal variation between the hours of 0900 and 1500 h failed to account for either the elevation of granulocyte concentration or the change in the proportion of CD25+ lymphocytes seen in the mitogen-stimulated leukocytes collected at 6 h post-exercise (data not shown).

Reviews of the effects of exercise on lymphocyte response to mitogenic stimulation have concluded that exercise is immunosuppressive (17,31). Our findings are consistent with these conclusions. In particular our findings also agree with those of Lewicki et al. (20) in which, following a progressive cycling protocol to exhaustion, both PHA-induced IL-2R (CD25) expression and in vitro IL-2 production were significantly reduced.

A reduction in the percentage of CD25+ lymphocytes indicates an exercise-induced inhibition of a specific element of the lymphocyte proliferative sequence. It appears that training has altered the response of lymphocytes to mitogenic challenge. More generally it is possible that the intense anaerobic exercise undertaken regularly by trained subjects may contribute to a transient lowering of immunocompetence that would leave athletes more susceptible to infection in the immediate post-exercise period. This may be related to the increased incidence of infection noted following periods of intense training (10) and competition (27).

Athletes' training regimes often involve repeated anaerobic efforts, often once or twice daily. Given the effects on the immune system of a single minute of such exercise, effort must now be directed to examining the effects of actual training regimes on the immune system. A sustained decrease in the percentage of CD25+ lymphocytes following mitogenic stimulation might have important implications for the susceptibility of athletes in regular training to infection.

In conclusion, intense anaerobic exercise is associated with significant changes in the peripheral concentrations and proportions of most leukocyte subpopulations irrespective of the training status of the subjects tested. Trained subjects exhibited a significantly decreased percentage of CD25+ lymphocytes following mitogen stimulation of peripheral blood obtained 6 h post-exercise. Post-exercise mobilization of CD8+ lympho-
cytes differed between the two groups. Given that these groups did not differ significantly in relation to either the amount of work performed, or peak power, during the exercise test, the differences in CD8+ mobilization may be due to the large volume of training undertaken by group I subjects or to group II subjects' apparently greater reliance on anaerobic metabolism during the exercise test.

REFERENCES