STUDIES
ON
THE REGULATION OF THE IMMUNE RESPONSE

The in vitro experiments described in Chapter VIII were
carried out in conjunction with Prof. J. Lathey. With this
exception, the experimental work described in this thesis was done
by myself.

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The in vitro experiments described in Chapter VIII were carried out in conjunction with Dr. K. J. Lafferty. With this exception, the experimental work described in this thesis was done by myself.

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ABSTRACT

The mechanisms that regulate an animal's response to antigen were studied in in vivo experimental situations. The efferent duct of the popliteal lymph node was cannulated and antigens were injected into the area drained by the lymph node. In this way, the immune response was confined to the node and the output of blast cells, plaque-forming cells and antibody could be measured in the lymph issuing from the node.

Specific IgM or IgG antibody complexed with chicken red blood cells was found to inhibit primary and secondary immune responses to the CRBC antigen whereas mixtures of IgM and IgG antibodies, complexed to chicken red blood cells enhanced the primary immune response but had no effect on the secondary immune response. When antigen-antibody complexes were injected 48 hours before the same or before a non-cross-reacting antigen, the response to both the complexed and the free antigen was enhanced above the response to the free antigen injected alone. Antigen-antibody complexes injected 260 hours before the same or a non-cross-reacting antigen inhibited the immune response to the free antigen. It was concluded that specific IgM or IgG antibody-antigen complexes did not inactivate immunocompetent cells but rather the antibody blocked the interaction between antigenic determinants and specific B lymphocytes whilst at the same time activating T lymphocytes.

The activated T lymphocytes appeared to produce factors which controlled the activities of specific B lymphocytes in a non-specific way. Thus 48 hours after the injection of antigen-antibody complexes, T lymphocytes produced factors which activated B lymphocytes that had antigen bound to their surface receptors, whilst 260 hours after the
injection of antigen-antibody complexes, T lymphocytes produced some factors(s) which prevented the differentiation and proliferation of those B lymphocytes that had antigen bound to their surface receptors.

A series of experiments was designed to examine the phenomena of antigenic competition and the allogeneic effect. The results of these experiments suggested that similar mechanisms control these phenomena. Factors which control the secretion of antibody by antibody-forming cells and factors which enhance DNA synthesis in PHA stimulated lymphocytes were found in lymph draining lymph nodes responding to a variety of antigens, these factors were non-specific in their action. Late in the response to antigen, factors which inhibited the secretion of antibody by antibody-forming cells were found in the lymph.

During primary and secondary responses to specific antigen, cells in the lymph produced immunoglobulins which showed no specificity for the antigen. Within this population of immunoglobulin-forming cells, cells were found which secreted antibody specific for antigens that had previously been injected into the animal. It was suggested that memory cells or cells that have been generated in response to some previous antigenic challenge, differentiate and proliferate under the influence of a response to a non-cross-reacting antigen. Furthermore, antibody-forming cells are induced to secrete their antibody under the influence of some factor(s) elaborated during the immune response to the unrelated antigen.

It was concluded that anamnestic responses can be elicited by a variety of non-specific antigenic stimuli.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td></td>
<td>(iii)</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>(iv)</td>
</tr>
</tbody>
</table>

* * *

CHAPTER I - INTRODUCTION

- The Evolution of Adaptive Immunity in Vertebrates 1
- Ontogeny of the Immune Response 3
- The Role of the Lymphatic System in the Mammalian Immune Response 5
- The Immune Response in Regional Lymph Nodes 9
- The Establishment of Systemic Immunity 13
- Antigenic Memory 14
- The Regulation of Metabolism and Differentiation in Cells 15
- Cellular Participants in the Immune Response 18
  1. Macrophages and other phagocytic cells 18
  2. The integrated function of lymphocytes in the immune response 22
- Antibody Mediated Suppression 27
- The Aims and Scope of the Thesis 30

CHAPTER II - MATERIALS AND METHODS 32

- Experimental Animals 32
- Chemicals, Solutions, Culture Media and Buffers 32
- General Methods 38
### Chapter 3 - The Normal Immune Response to Chicken Red Blood Cells

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>57</td>
</tr>
<tr>
<td>Results</td>
<td>58</td>
</tr>
<tr>
<td>The Immediate Effect of Chicken Red Blood Cells on the Cellular Content of Efferent Lymph Draining the Regional Lymph Node</td>
<td>58</td>
</tr>
<tr>
<td>The Primary Immune Response to $2 \times 10^9$ Chicken Red Blood Cells</td>
<td>59</td>
</tr>
<tr>
<td>1. Cellular response</td>
<td>59</td>
</tr>
<tr>
<td>2. Plaque-forming cell response</td>
<td>60</td>
</tr>
<tr>
<td>3. Antibody response</td>
<td>62</td>
</tr>
<tr>
<td>The Effect of Antigen Dose on the Primary Response</td>
<td>62</td>
</tr>
<tr>
<td>The Secondary Immune Response to $2 \times 10^9$ Chicken Red Blood Cells</td>
<td>63</td>
</tr>
<tr>
<td>Discussion</td>
<td>64</td>
</tr>
<tr>
<td>Significance of the Immediate Effects of Antigen</td>
<td>65</td>
</tr>
<tr>
<td>Cellular Events in the Primary Immune Response</td>
<td>66</td>
</tr>
<tr>
<td>Humoral Events in the Primary Response</td>
<td>67</td>
</tr>
<tr>
<td>The Plaque-Forming Cell Response</td>
<td>67</td>
</tr>
<tr>
<td>The Control of the Immune Response</td>
<td>69</td>
</tr>
<tr>
<td>1. Lymph node control, immediate effects of antigen</td>
<td>69</td>
</tr>
<tr>
<td>2. Suppression and expression of the proliferation of immunocompetent cells</td>
<td>69</td>
</tr>
</tbody>
</table>
CHAPTER IV - ANTIBODY MEDIATED SUPPRESSION OF THE IMMUNE RESPONSE

**INTRODUCTION** .. .. .. .. .. .. .. 70

**RESULTS** .. .. .. .. .. .. .. 71

1. Primary Immune Responses .. .. .. 71

<table>
<thead>
<tr>
<th>Response Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM antibody</td>
<td>71</td>
</tr>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgG antibody</td>
<td>72</td>
</tr>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM plus IgG antibody</td>
<td>72</td>
</tr>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells injected with homologous non-specific immunoglobulin</td>
<td>73</td>
</tr>
</tbody>
</table>

2. Secondary Immune Responses .. .. .. 73

<table>
<thead>
<tr>
<th>Response Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM antibody</td>
<td>73</td>
</tr>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgG antibody</td>
<td>74</td>
</tr>
<tr>
<td>The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM plus IgG antibody</td>
<td>75</td>
</tr>
</tbody>
</table>

**DISCUSSION** .. .. .. .. .. .. .. .. .. 75

<table>
<thead>
<tr>
<th>Discussion Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Suppressive Effect of Specific Antibody</td>
<td>76</td>
</tr>
<tr>
<td>The Enhancing Effect of Specific Antibody</td>
<td>77</td>
</tr>
</tbody>
</table>
CHAPTER V - ANTIGENIC COMPETITION

INTRODUCTION

RESULTS

1. The Primary Immune Response to Chicken and Rabbit Red Blood Cells

2. The Modification of the Immune Response to Chicken or Rabbit Red Blood Cells by Rabbit Red Blood Cells Injected Before or at the Same Time as the Chicken Red Blood Cells
   (a) Chicken and rabbit red blood cells injected together
   (b) Chicken red cells injected 24 hours after rabbit red cells
   (c) Chicken red cells injected 48 hours after rabbit red cells
   (d) Chicken red cells injected 260 hours after rabbit red cells

3. The Modification of the Immune Response to Chicken Red Blood Cells when Salmonella muenchen Heat Killed Organisms were Injected Before or at the Same Time as Chicken Red Blood Cells

4. The Modification of the Immune Response to Chicken Red Blood Cells when a Similar Dose of Chicken Red Blood Cells was Injected at the Same Time or After the Initial Antigenic Challenge

DISCUSSION

Enhanced Immune Responses

Suppressed Immune Responses
CHAPTER VI - THE EFFECT OF ANTIGEN-ANTIBODY COMPLEXES ON IMMUNE RESPONSES

INTRODUCTION

RESULTS

The Effect of Antigen-Antibody Complexes on the Immune Response to Chicken Red Blood Cells

1. Red cell-IgM complexes given 48 hours prior to chicken red blood cells

2. Red cell-IgG complexes given 48 hours prior to chicken red blood cells

3. Red cell-IgM complexes given 260 hours prior to chicken red blood cells

4. Red cell-IgG complexes given 260 hours prior to chicken red blood cells

DISCUSSION

CHAPTER VII - MEDIATORS OF HUMORAL IMMUNITY

INTRODUCTION

RESULTS

The Isolation of Factors which Influence Immune Responses

The Effect of Lymph upon the Primary Immune Response to Chicken Red Blood Cells

The Evaluation of Antibody-Containing Cells and Antibody-Secreting Cells during a Primary Immune Response to 2 x 10^9 Chicken Red Blood Cells

The Effect of Lymph on the Secretion of Antibody by Antibody-Forming Cells

The Effect of Lymph upon the Uptake of 3H Thymidine in Lymphocytes Stimulated Previously with Phytohaemagglutinin
** CHAPTER VII - continued **

The Effect of Lymph upon Normal Lymphocyte Transfer Reactions ... ... 108

The Effect of DEAE Fractions from Normal Sheep Blood Plasma on the Immune Response to Chicken Red Blood Cells ... ... ... ... ... 109
1. Primary responses ... ... ... ... ... 109
2. Secondary responses ... ... ... ... ... 110

The Effect of DEAE Fractions from Normal Sheep Blood Plasma on the Lymphocyte Transfer Reaction ... ... ... ... ... 110

The Effect of DEAE Fractions from Sheep Lymph and Blood Plasma on the Response of Lymphocytes to PHA ... ... ... ... ... 111

DISCUSSION ... ... ... ... ... ... ... ... ... 112

** CHAPTER VIII - ALLOGENEIC ENHANCEMENT AND INHIBITION OF HUMORAL IMMUNE RESPONSES ... ... ... ... ... 116

INTRODUCTION ... ... ... ... ... ... ... ... ... 116

RESULTS ... ... ... ... ... ... ... ... ... ... ... ... 118

In Vitro Studies of the Effect of Mitomycin C upon Lymphocytes ... ... ... ... ... ... ... ... 118

The Response of the Sheep Popliteal Lymph Node to Allogeneic Lymphocytes ... ... ... ... ... 120

The Immune Response to Rat Lymphocytes ... ... ... ... ... ... ... 121

The Effect of Allogeneic Lymphocytes on the Immune Response to 2 x 10^9 Chicken Red Blood Cells ... ... ... ... ... ... ... ... 122
1. Chicken red blood cells injected with normal allogeneic lymphocytes ... ... ... ... ... ... ... ... ... ... 122
2. Chicken red blood cells injected with allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C ... ... ... ... ... ... ... 123
3. Chicken red blood cells injected with allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C .. 123

4. Chicken red blood cells injected 24 hours after normal allogeneic lymphocytes .. .. .. .. .. .. 124

5. Chicken red blood cells injected 24 hours after allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C .. .. .. .. .. .. 124

6. Chicken red blood cells injected 24 hours after allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C .. .. .. .. .. .. 125

7. Chicken red blood cells injected 48 hours after normal allogeneic lymphocytes .. .. .. .. .. .. 125

8. Chicken red blood cells injected 48 hours after allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C .. .. .. .. .. .. 125

9. Chicken red blood cells injected 48 hours after allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C .. .. .. .. .. .. 126

10. Chicken red blood cells injected 120 hours after allogeneic lymphocytes .. .. .. .. .. .. 126

11. Chicken red blood cells injected 260 hours after allogeneic lymphocytes .. .. .. .. .. .. 127

The Immune Response to $2 \times 10^9$ Chicken Red Blood Cells injected 48 hours after $3 \times 10^9$ Rat Lymphocytes .. .. .. .. .. .. 127

DISCUSSION .. .. .. .. .. .. .. 129
| CHAPTER IX | THE SYNTHESIS OF NON-SPECIFIC IMMUNOGLOBULINS DURING THE IMMUNE RESPONSE TO SPECIFIC ANTIGENS | 135 |
| INTRODUCTION | 135 |
| RESULTS | 136 |
| The Secondary Immune Response to Horse Radish Peroxidase | 136 |
| The Primary Immune Response to $3 \times 10^8$ Viable Allogeneic Lymphocytes | 137 |
| The Primary Immune Response to $3 \times 10^8$ Viable Allogeneic Lymphocytes Injected 5 Weeks after the Injection of $2 \times 10^9$ Chicken Red Blood Cells | 138 |
| The Production of Cells Secreting Antibody against Rabbit Red Blood Cells following the Injection of $2 \times 10^9$ Chicken Red Blood Cells | 140 |
| DISCUSSION | 141 |
| CHAPTER X | SUMMARY AND CONCLUSIONS | 144 |
| BIBLIOGRAPHY | 154 |
CHAPTER 1

INTRODUCTION

The Evolution of Adaptive Immunity in Vertebrates

The study of the evolution of adaptive immunity in vertebrates has provided an insight into the association that exists between a functional immune capacity and the morphological development of the lymphoid apparatus.

All animals must have, by virtue of their continued evolution and presence in terrestrial environments, the ability to effectively cope with foreign or infectious substances within their general environment (Bibliography). Vertebrates, including invertebrates, have as yet revealed little evidence for the presence of adaptive immunity within the phyla. Although some evidence has been obtained that bactericidal substances are produced by Amphibian skin (Evans, Palos, and Anson, 1968), amniotes (Evans, Palos, and Anson, 1968), and insects (Stott, 1958; Bragg and Dodds, 1957; Nira, 1970), these bactericidal substances, however, are not similar to the mechanisms proposed in vertebrates.

Invertebrate animals are capable of dealing with such foreign and pathogenic substances and their ability may be related to the ability to degrade such substances by enzymes (Bibliography). The objectives of this study include the determination of the ability of various invertebrate phyla to offer a partial immunity for the elimination of foreign and native substances that may affect the animal (Bibliography).

Probably one of the first steps in the development of an immune system was the development of a capability to distinguish between native and foreign substances (Bibliography). Immunological mechanisms have not been demonstrated in invertebrate animals but it is
The Evolution of Adaptive Immunity in Vertebrates

The study of the evolution of adaptive immunity in vertebrates has provided an insight into the association that exists between a functional immune capacity and the morphological maturation of the lymphoid apparatus.

All animals must have, by virtue of their continued evolution and present existence, mechanisms whereby they can effectively cope with foreign or infectious substances within their natural environment (Hildemann, 1966). Extensive studies on invertebrates have as yet revealed little evidence for the presence of adaptive immunity within the phylum although some evidence has been obtained that bactericidal substances are produced by Sipunculids (Evans, Cushing and Evans, 1973), crustacea (Evans, Painter, Evans, Weinheimer and Acton, 1968), and insects (Briggs, 1958; Briggs, 1964; Chadwick, 1967; Hink, 1970). These bactericidal substances, however, are not similar to the antibodies produced by vertebrates.

Invertebrate animals are capable of dealing with many foreign and pathogenic substances but this ability may be related to the degradation of these substances by enzymes (Hildemann and Reddy, 1973). The ubiquitous mechanism of non-specific phagocytosis is also of capital importance for the elimination of foreign and noxious materials that may invade the animal (Hildemann and Reddy, 1973).

Probably one of the first steps in the development of an immune system was the development of a capacity to distinguish between native and foreign substances (Hildemann and Reddy, 1973); immunoglobulin molecules have not been demonstrated in invertebrate animals but it is
conceivable that the immunoglobulins that are synthesised by certain lymphocytes in the higher vertebrate animals may have had a common evolutionary origin with those molecules synthesised by the immune reactive cells of the higher invertebrate animals (Burnet, 1968).

In recent years extensive comparative studies on adaptive immune responses of primitive and advanced vertebrate animals have been undertaken (Finstad, Papermaster and Good, 1964; Good and Papermaster, 1964; Good, Finstad, Pollara and Gabrielsen, 1966). The results of these studies have suggested that there has been both progressive and parallel evolution within the vertebrates and that the majority of vertebrate species are capable of adaptive immune responses.

Immunoglobulin and antibody synthesis and secretion appeared early in the evolution of vertebrates but plasma cells seem to be a later phylogenetic development (Good, Finstad, Pollara and Gabrielsen, 1966). The pressures imposed upon biological systems for the development of lymphoid and plasma cell systems could have been primarily intrinsic, that is, a systematic control of aberrant cell development in the animal, rather than a defence against invasion of micro-organisms (Thomas, 1959; Burnet, 1962; Finstad, Papermaster and Good, 1964). Good, Finstad, Pollara and Gabrielsen (1966) have considered the evolution of the immune system through the vertebrate phylum and demonstrated that there is an association between an animal's ability to undergo adaptive immune responses and the development of the lymphoid system. The increase in the structural organisation of lymphoid tissues is paralleled by an increase in immunologic vigour and potential. This concept is in all probability of major importance in attempting to define regulatory mechanisms incorporated within the immune apparatus. The immune system appears to have evolved in conjunction with a phagocytic
system already well established in the invertebrates and lower vertebrates. As the complexity of the immune system increased so regulatory mechanisms must have evolved so as to maintain the efficiency of the system. It may be that during evolution the functions of several independent regulatory mechanisms have been incorporated into a single mechanism. Inherent within this concept is the risk of extensive immune malfunction, should the regulatory controls fail. Such malfunctions may be represented by the various organ and cell hyperplasias.

Ontogeny of the Immune Response

Investigations into the ontogeny of the immune response (Silverstein and Prendergast, 1969), gave support to the view that a high degree of immunocompetence is not necessarily accompanied by a well established and highly organized lymphatic apparatus. Silverstein and Prendergast showed that in the foetal sheep there is a definite sequential appearance of immunological competence to a variety of antigens which is related to foetal age and which continues after birth, at which time the lymphoid apparatus is fully operational although not so well developed as that of the adult sheep.

Thymectomy at the end of the first third of the gestation period seems to have little effect on the growth and development of the lamb (Cole and Morris, 1971a). Thymectomy at 60-70 days post-conception does, however, have a detrimental effect upon the development of lymphoid tissues such as Peyers patches and lymph nodes, which remain small up to 56 weeks after birth; the spleen develops at the normal rate (Cole, 1969). Neonatal thymectomy severely depletes the cortical regions of lymph nodes and there is some reduction in the number of lymphocytes in the medulla. The low levels of lymphocytes in the blood and lymph remain low throughout foetal and post-natal life, at least up until
around one year of age (Cole, 1960; Cole and Morris, 1971a). It appears that the thymus plays a dominant role in controlling the development of the lymphoid apparatus and the levels of circulating lymphocytes in the lamb. It is important, however, to recognise that growth still continues in the thymectomised lamb albeit at a much reduced rate (Cole and Morris, 1971a).

The data available suggests that although the thymus has an important influence upon the rate of development of the lymphoid tissues, it is not essential in its development or in the appearance or maturation of immune competence (Cole and Morris, 1971a; 1971b). The function of the immunological system is dependent ultimately upon the presence of a lymphoid apparatus which depends in turn upon the function of the thymus. The relationship between the ability of an animal to give an immune response and the state of development of the lymphoid tissues is not a simple one (Silverstein and Prendergast, 1969).

Primary and secondary immune responses of lambs thymectomised 60-80 days post-conception were found to be essentially the same as the responses of normal lambs (Silverstein and Prendergast, 1969; Cole and Morris, 1971b); as are those of thymectomised lambs immunised in utero (Silverstein and Kraner, 1965).

Interpretation of the experimental data obtained in the sheep indicates that although the thymus holds no unique position in respect to the development of immunological competence, it does nonetheless have an important role in the generation of lymphocytes in this species. The general acceptance that the thymus generates a specific class of cell capable of regulating antibody synthesis does not appear consistent with experimental findings in the sheep (Cole and Morris, 1973). Thymic dependency may, however, be a phenomenon which is species dependent and
not all antigens which are thymus dependent in one strain of one species are thymus dependent in another strain of the same species. Thymectomy in mice gives rise to severely impaired primary humoral antibody responses to certain antigens which have subsequently been defined as thymus dependent antigens (Miller, 1961; Parrot, 1962; Miller and Osoba, 1967).

Evidence has been described in which the immune capacity of an animal has been claimed to be restricted by the morphological development of the lymphoid apparatus with respect to the animal's position on the vertebrate scale of evolution. Such a concept appears untenable in the light of evidence from ontogenetic studies in which the state of lymphoid development appears to bear little relationship to the immune capacity of the animal.

Information is lacking on the degree of heterogeneity shown in immune responses at the various levels of vertebrate evolution. It may be that in the evolution of vertebrates the development of increasing heterogeneity of immune responsiveness may have been of more importance than the development of a complex lymphoid apparatus.

The evolution of a complex lymphoid apparatus may permit a more efficient and more precise control of immune responsiveness by providing a framework in which the various cellular and humoral components of the immune system can interact.

The Role of the Lymphatic System in the Mammalian Immune Response

Anatomically the lymphatic system is comprised of two components: a vascular component and a cellular component. The vascular component includes the lymphatic absorbing terminals, lymphatic capillaries and the main lymph trunks, whilst the cellular component is
comprised of fixed and mobile cells of the reticuloendothelium. Fixed
cells are found in the spleen, lymph nodes, Peyers patches, tonsils,
appendix, kidney and other organs with associated lymphatic tissues
whereas the mobile elements, lymphocytes, monocytes and macrophages
circulate between the blood, tissues and lymph.

Peripheral lymph (lymph that has not passed through any
organised lymphoid tissue) is formed in the capillary beds of the
tissues and organs of the body and contains far fewer cells than central
lymph (lymph that has passed through at least one organised lymphoid
tissue). The cells of peripheral lymph are mainly lymphocytes with a
few basophilic blast cells and 15-20% macrophages, a cell type rarely
seen in central lymph (Haynes and Field, 1931; Yoffey and Drinker, 1939;
Ottaviani and Satta, 1959; Hall and Morris, 1963). Cellular traffic
from the blood stream through the tissues to the peripheral lymph occurs
continually but at a low level under normal conditions; it can, however,
be quite extensive in conditions of regional or general inflammation.
The fate of the macrophage in peripheral lymph is not known at the
present although they do not leave the lymph nodes in the efferent
(central) lymph. The cell content of central lymph is comprised of 90-
95% small and medium lymphocytes under normal conditions (Morris, 1972).

It is now well established that a crucial attribute of the
lymphocyte is its ability to migrate and it was first demonstrated in
the rat that blood lymphocytes are continually migrating into the
lymphatics (Gowans, 1957; Gowans, 1959). It would appear that the
lymphocytes in central lymph have three origins, peripheral lymph
entering the node through the pericapsular sinus contributes a minor
proportion of this population, some 5 - 10%, although in some cases,
as in the efferent hepatic lymph in sheep, the peripheral lymph cells
may contribute as much as 40% of the total efferent lymph cell population (Smith, McIntosh and Morris, 1970). Mitotic division in the node itself contributes less than 5% of the central lymph lymphocyte population in the sheep (Hall and Morris, 1965b). In the antigen stimulated node, however, cell proliferation occurs at a high rate and many of the newly formed cells appear in the efferent lymph, mainly as basophilic blast cells (Hall and Morris, 1965b). Emigrant lymphocytes from the blood make up about 90% of lymphocytes found in central lymph (Hall and Morris, 1965b). Blood lymphocytes have been shown to enter the lymphatics by traversing the endothelium of post-capillary venules in the cortical and cortico-medullary regions of lymph nodes (Gowans and Knight, 1964); they do this by migrating between the high endothelial cells of the post-capillary venules (Sugimura, Furuhata, Kudo, Takahata and Mifune, 1964; Schoeffl, 1972).

The number of lymphocytes in the recirculating pool has been defined for a number of species by thoracic duct cannulation. Estimates of $1.5 - 2 \times 10^9$ cells for rats (Caffrey, Rieke and Everett, 1962), $1 - 2 \times 10^8$ for mice (Miller, Mitchell and Weiss, 1967), have been quoted for small mammals and it has been estimated that in large mammals such as the sheep, the number of lymphocytes in the recirculating pool exceeds $5 \times 10^{10}$ (Morris, 1972). The daily output of cells in lymph from a particular region of the body is remarkably constant under normal conditions with the concentration of cells in the lymph being proportional to the rate of lymph flow. It is possible that short term increases in lymphocyte output from various regions can be evoked by various mechanical effects such as massage and exercise (Kous, 1908; Haynes and Field, 1931; Morris, 1972). In general, the number of lymphocytes in lymph is related to the number of lymphocytes in the blood.
The lymphatic system probably evolved to complement the blood circulatory system and functions to clear the tissues of large and small protein molecules and cells and other materials that may escape into the extracellular fluid through the walls of small blood vessels. Lymphatic vessels are present in most tissues of the body where they form a fine network of thin-walled vessels draining into thicker-walled collecting ducts that eventually return the lymph to the venous system. These lymphatic vessels provide a means whereby those components that escape from the blood into the extracellular fluids can be returned to the blood (Yoffey and Courtice, 1970).

Antigenic and foreign materials which gain access to the body very rapidly enter the lymph stream (Angevine, 1936; Freund and Angevine, 1938; Trevella and Morris, 1971). This rapid entry is due to the highly permeable nature of the lymphatic terminals and the existence of a pressure gradient between the tissue spaces and the lymphatic lumen. Foreign material having gained access to the lymphatic is very rapidly propelled along the ducts to the regional lymph node, a movement brought about by the intrinsic rhythmic contractions of the lymphatic ducts (Hall, Morris and Woolley, 1965), a process which may be aided by aspiration effects of muscle or respiratory movements in some cases (Yoffey and Courtice, 1956; Rusznyak, Földi and Szabo, 1960; Mayerson, 1963). These rhythmic contractions propel lymph from the periphery to the venous system; reflux of lymph is prevented by the presence of valves in the lymphatics. The system operates to control the removal of tissue fluid at a rate proportional to its rate of formation in the tissue spaces (Hall, Morris and Woolley, 1965; Morris, 1968). Soluble foreign material and recirculating lymphocytes are thus rapidly transported to the blood compartment. Small amounts of foreign particulate material are quantitatively retained in lymph nodes (Trevella and Morris, 1971) where it may be taken up by
phagocytic cells lining the medullary sinuses and dendritic macrophages in the cortex; here the material, if antigenic, may initiate an immune response.

When large amounts of particulate material are introduced into the peripheral lymphatics there is an increasing chance that some of this will escape being filtered in the regional and subsequent lymph nodes. This can occur when infective foci release large numbers of micro-organisms; those that escape phagocytosis in the lymph nodes may reach the blood giving rise to bacteraemia or viraemia (Barnes and Trueta, 1941).

Peripheral and central lymph from all regions of the body passes into several major collecting ducts; lymph from the peritoneal cavity, pleural cavity and thoracic viscera enters the right lymph duct, lymph from the tissues of the head, neck, mouth, pharynx and nose enters the cervical ducts, lymph from the intestines, liver, hind limbs and other visceral areas enters the thoracic duct. These major lymphatic ducts return the lymph to the venous system.

The Immune Response in Regional Lymph Nodes

Following the introduction of antigenic material into the tissues underlying the skin there may be an outpouring of polymorphonuclear cells and macrophages into the tissues; this is particularly pronounced with bacterial and viral antigens. These cells ingest many of the micro-organisms that they encounter. Polymorphonuclear cells, macrophages and antigen enter the peripheral lymph and are transported to the regional lymph node. These reactions occur within minutes of the introduction of antigenic material. In some instances a continuing reaction may occur in the tissues where the antigen becomes localised, giving rise to the formation of a chronic granuloma. Lymphocyte
proliferation and differentiation occurs at such sites and blast cells and plasma cells are produced (Smith, McIntosh and Morris, 1970). These cells may appear subsequently in the efferent lymph and are concerned with antibody formation. Most of the mature plasma cells formed in the granuloma or indeed in the lymph node itself, appear to remain fixed in the area where the antigen is localised and they are seen very rarely in central lymph (Morris, Moreno and Bessis, 1968).

Particulate antigen is carried by the peripheral lymph and by cells to the subcapsular sinus of the regional lymph node. Any free material is taken up by macrophages and phagocytic cells in the medullary sinuses; there is little evidence for the presence of particulate antigen passing through the lymph node into the efferent lymph unless large amounts are introduced.

Polymorphonuclear cells and macrophages may appear subsequently in the central lymph in which they may be present for 48 hours or longer. The polymorphonuclear cells in the central lymph originate from those cells which enter the node via the peripheral lymph (Smith and Wood, 1949a; 1949b). Other polymorphs are seen in the intermediary and medullary sinuses and some of these are derived directly from the blood within the lymph node. These cells pass through capillaries and post capillary venules through which normally only lymphocytes pass (Marchesi and Gowans, 1964). The arrival of antigen at the regional lymph node gives rise to an immediate and drastic reduction in cellular output from the lymph node which normally occurs without any reduction in the rate of lymph flow (Hall and Morris, 1965a). The extent of this effect seems to be related to some characteristic of the antigen; influenza virus virtually eliminates cells from the efferent popliteal lymph of sheep for up to 24 hours (Smith and Morris, 1970). This phenomenon may act to facilitate
contact between antigen fixed within the node and immunocompetent cells migrating into the lymph node (Ada, Byrt, Mandel and Warner, 1971).

Between 12-48 hours after antigenic stimulation, the cell output in the efferent lymph increases to a level which may reach some ten times pre-stimulation levels. The cell content of the lymph at this time is comprised almost entirely of small and medium lymphocytes representing the circulating lymphocyte population recruited into the node from the blood. The recruitment of large numbers of lymphocytes from the blood would provide the maximum opportunity for those cells that are competent for the antigen to be brought into contact with it, so initiating the processes of cellular differentiation and proliferation, probable prerequisites for specific antibody synthesis. At about 60 hours after antigen enters the lymph node there is increased DNA synthesis in efferent lymph lymphocytes (Hay, Murphy, Morris and Bessis, 1972; Morris, 1972), although this cannot be correlated with any morphological changes at this time. In the secondary immune response increased DNA synthesis in efferent lymph lymphocytes may be observed as early as 24-36 hours after antigenic challenge (Morris, 1972).

During the period between 70-170 hours after primary antigenic challenge large numbers of basophilic blast cells appear in the efferent lymph and many of these are in mitosis. These cells may increase to levels representing around 30% of the total efferent lymph cell population (Smith and Morris, 1970). In secondary immune responses the blast cells usually appear at an earlier time, rise to higher levels and decline within 100 hours of antigenic challenge (Hall and Morris, 1962; Smith and Morris, 1970). The free floating lymphocytes produced during the immune response play an important part in the establishment of immunity. Some of these cells actively synthesise and secrete antibody and they may be retained in other lymph nodes along the lymphatic pathway or they may pass
into the blood compartment (Hulliger and Sorkin, 1965; Kearney and Halliday, 1965; Hall, Morris, Moreno and Bessis, 1967). Within the regional lymph node itself, plasma cells are found in large numbers and these synthesise and secrete antibody. It is thought that differentiation of lymphocytes into plasma cells is dependent upon precursor lymphocytes becoming fixed in the tissues of the node and it has been demonstrated that at least some of the blast cells and large lymphocytes in the lymphatics undergo transformation into classical plasma cells after entering lymphatic tissues (Birbeck and Hall, 1967).

It has been shown that small lymphocytes are the precursor cells of the antibody-synthesising blast cells and plasma cells (Ellis, Gowans and Howard, 1969) and the most definitive evidence has shown that stimulated small lymphocytes develop by division and differentiation into antibody-forming cells (Ellis and Gowans, 1973).

A large proportion of those cells present in the lymph issuing from a stimulated lymph node during an immune response have been shown to secrete antibody (Hall and Morris, 1963; Cunningham, Smith and Mercer, 1966; Hay, 1971; Hay, Murphy, Morris and Bessis, 1972). The free floating cells of lymph that are involved in antibody synthesis and secretion have been identified as small, medium and large lymphocytes, blast cells and plasmablasts (Cunningham, Smith and Mercer, 1966; Hummeler, Harris, Tomassini, Hechtel and Farber, 1966; Cunningham, 1968; Hay, Murphy, Morris and Bessis, 1972; Murphy, Hay, Morris and Bessis, 1972); although the blast cell is the most characteristic antibody-forming cell in the efferent lymph (Hall and Morris, 1963; Hay, Murphy, Morris and Bessis, 1972). The proportion of blast cells secreting antibody in the efferent lymph at the peak of the primary immune response has been estimated as 1:2 (Cunningham, Smith and Mercer, 1966), 1 in 10 (Hay, 1971), whilst the proportion of
cells containing antibody at the peak of the secondary immune response has been estimated at 3:4 (Hay, Murphy, Morris and Bessis, 1972).

Small and large lymphocytes have also been identified as antibody-forming cells in spleen and lymph nodes (Vazquez, 1961; Attardi, Cohn, Horibata and Lennox, 1964; Cunningham, 1968; Leduc, Avrameas and Bouteille, 1968; Avrameas and Leduc, 1970).

The Establishment of Systemic Immunity

If the efferent duct of the popliteal lymph node of the sheep is cannulated prior to antigenic stimulation, and if antigen is delivered directly to the node, a localised immune response results which is confined to the regional lymph node (Hall and Morris, 1962). When the popliteal lymph node is antigenically stimulated with the efferent lymphatic intact, much more antibody is produced than in the regional lymph node (Morris, 1966). This finding indicated that the free floating lymph-borne cells are important in some way in the development of systemic immunity (Morris, 1966; Hall, Morris, Moreno and Bessis, 1967).

It may be that stimulated immunocompetent cells and not antigen are capable of sequentially colonising other lymph nodes along the route of lymphatic drainage distal to the regional lymph node, and initiating cellular proliferation and antibody synthesis at these sites and in other lymphatic tissues by virtue of the proliferating cells seeding out into the blood compartment. Thus, in this way, the initial immune response in the regional lymph node may be amplified many times (Morris, 1966; Hall, Morris, Moreno and Bessis, 1967). A similar process has been postulated to occur in mice when an antigenic challenge localises in the spleen (Anderson and Dresser, 1972). Antibody-forming cells and their precursors migrate from the spleen to other lymphoid organs where antibody
production continues for a greater duration than in the spleen. This suggests that the spleen is not the major source of continued serum antibody production in mice but rather that it is the initiating organ in terms of immune reactivity to blood-borne antigens.

Antigenic Memory

It has been suggested that the small lymphocyte may be the cell responsible for antigenic memory and it has been shown that circulating lymphocytes are capable of transferring secondary reactivity to irradiated animals (Gowans and McGregor, 1965). Recent evidence (Ellis and Gowans, 1973) has confirmed this and established that an antigenically primed population of long lived small lymphocytes divide and differentiate into plasma cells after further contact with specific antigen. Thus small lymphocytes are directly implicated as the carriers of antigenic memory.

Secondary reactivity in lymphoid cells exists in both fixed and migratory components of the immune system (White, 1960; Nossal and Mäkela, 1962; Smith, Cunningham, Lafferty and Morris, 1970; Ellis and Gowans, 1973).

A regional lymph node that has encountered antigen previously, is capable of responding more readily to a second encounter with the same or a cross-reacting antigen than are other lymph nodes situated elsewhere in the body. This difference in reactivity has been attributed to a population of memory cells resident within the regional lymph node (Smith, Cunningham, Lafferty and Morris, 1970). More distant lymph nodes have secondary reactivity which is less immediate and this can be attributed to the circulating population of memory cells known to be small lymphocytes (Smith, Cunningham, Lafferty and Morris, 1970). It has been proposed (Nossal and Mäkela, 1962) that residential memory can be attributed to a continuously dividing population of large lymphocytes.
although it has been established that recirculating small lymphocytes functioning as memory cells may spend part of their life as sessile cells in lymphoid tissues (Ellis and Gowans, 1973) and could conceivably perform the function of both residential and circulating components of antigenic memory. The anatomical site where the large dividing cells are found may be the germinal centres in lymphoid tissues (Nossal and Mäkela, 1962) and it has been suggested that germinal centres play a specific role in secondary reactivity by focussing antigen on to reactive lymphoid cells and thereby providing centres in which large numbers of plasma cells are produced (White, 1960; Thorbecke, Asofsky, Hochwald and Siskind, 1962; Ward, Johnson and Abell, 1963; Cottier, Odartchenko, Keiser, Hess and Stoner, 1964). Blast cells and lymphocytes may withdraw from antibody production and revert to the normal lymphocyte morphology. These may be the cells in which antigenic memory resides.

The Regulation of Metabolism and Differentiation in Cells

The literature relating to the regulation of the immune response is both diverse and conflicting and has for the most part been confined to the study of cellular and humoral interactions in vitro. Such interactions have been described in terms of the immunological effects which they induce and whilst the regulation and fate of those cells which participate in the immune response have been regarded as unique, certain useful parallels can be drawn with regulatory mechanisms which control, in a general way, the metabolic activity and fate of most cells.

Interactions occur between cells at a variety of levels to determine the expression of the genome and to modify and regulate the production of specific products by cells. Cells in the vegetative region of the amphibian embryo have been shown to possess an inherent capacity for
self-differentiation, whilst cells in the animal region of the embryo are capable of differentiating into a variety of embryonic tissues (Raven, 1959), their ultimate fate being dependent upon their position within the embryo. It has been shown that the differentiation of these cells is dependent in some way upon other cells that underlie or surround them (Brachet, 1950). Although evidence is lacking, it may be that the underlying cells secrete some product which directs the differentiation and decides the ultimate fate of these cells.

Hormonal regulatory mechanisms are characterised by the action of a product of one group of cells upon another group of cells, thus the activity of target cells is regulated by the specific gene product of the hormone-secreting cells. The initial event in all hormone-target cell interactions appears to be facilitated by specific receptors on the target cell membrane, such interactions have been shown to activate the membrane-bound enzyme adenyl cyclase which catalyses the cyclization of ATP to cyclic AMP (Sutherland, Rall and Menon, 1962; Butcher, Robison and Sutherland, 1970). Cyclic AMP has been proposed to function as an intracellular transmitter of information (Butcher, Robison and Sutherland, 1970) acting in some way to derepress that part of the genome to which the cell may have become restricted, or to give impetus to a variety of biochemical events already occurring and which may culminate in functional and morphological changes in the cell. In physiological situations hormones exist in extremely low concentrations and yet produce effects which are highly amplified at the molecular level. In many biological systems the amplification of an initiating event is achieved by multi-step or cascade processes and such is thought to be the case for hormones to exert their effects (Butcher, Robison and Sutherland, 1970). For instance, insulin and growth hormone seem to give rise to increased synthesis of those proteins already synthesised by the target cells (Korner, 1970). Such an
increase in the rate or amount of protein synthesised may result from a variety of factors such as an increase in the availability of messenger RNA, amino-acyl transfer RNA complexes or increased synthesis of ribosomes. The nature of the initial inductive processes, however, remains a mystery.

It is known that certain (B) lymphocytes have immunoglobulins present on their cell surface and these immunoglobulins which are a product of the cell bind antigen specifically. Thus the specificity of the cells towards an antigen is displayed at the cell surface. The lymphocyte once activated by antigen differentiates and divides to provide a population of cells whose function it is to synthesise and secrete antibody, a product which specifically inactivates the antigenic determinants which induced its formation. This system is not unique and an analogous situation exists in those nerve cells that are activated by acetylcholine, these cells which possess specific receptors for acetylcholine synthesise cholinesterase, an enzyme which acts to break down acetylcholine and eliminate the specific inductive stimulus.

Numerous reports in the literature have indicated that antigen specifically induces a second population of (T) lymphocytes to elaborate a product which non-specifically activates those B lymphocytes that have antigen bound to their surface receptors, to differentiate and divide. Thus the antigenic signal to B lymphocytes may be amplified. Whilst the end products of B lymphocyte differentiation and division may be antibody-forming cells the precursor cells themselves synthesise antibody in the form of surface receptors so that it would appear that antigen and the products of T lymphocytes give impetus to the production of antibody rather than cause some derepression of the genome, in other words they act like insulin and growth hormone.
The analogies which can be drawn between regulatory mechanisms in general and the apparently specific immunoregulatory mechanisms are numerous and beyond the scope of this thesis but it is evident that the cells involved in immune reactions are subjected to regulatory mechanisms that are similar to those that act on a wide variety of other types of cells in different organs of the body even though there may be certain specific and characteristic controls which relate only to the immune response.

Cellular Participants in the Immune Response

1. Macrophages and other phagocytic cells - Cells specifically able to phagocytose foreign materials probably represented one of the few effective but nonspecific defence mechanisms available to primitive invertebrates (Bang, 1973). Even today many invertebrates show little differentiation from this defence mechanism.

In attempting to define mammalian macrophages and phagocytic cells on functional and morphological characteristics, considerable difficulty is encountered. The classical definition of the macrophage is a mononuclear cell capable of phagocytosis, but such characteristics are also possessed by reticulum cells, Kupffer cells, blood monocytes and certain cells in peritoneal exudates (Gottlieb and Waldmann, 1972).

Additional features used to classify mononuclear phagocytes are the nature and content of the lysosomes, adherent properties and the presence of receptors for IgM, IgG and complement on the plasma membrane. The primary function of macrophages in particular and the reticuloendothelial system as a whole is to rid the tissues and body fluids of micro-organisms, foreign materials and effete cells and it has been suggested that phagocytic cells possess receptors that are capable of recognising effete cells and denatured self-proteins (Cohn, 1972).
It seems that macrophages have become integrated into the complex immunological apparatus of the vertebrates and that they are capable of several different functions which operate in both cell mediated and humoral immune responses. Macrophages may be crucial in the initial stages of an immune response in order to break down antigen into smaller immunogenic particles (Shortman, Diener, Russell and Armstrong, 1970). Indeed, it has been shown that particulate and aggregated antigens that are easily phagocytosed give rise to better immune responses than antigens that are soluble or poorly phagocytosed (Biro and Garcia, 1965, Frei, Benacerraf and Thorbecke, 1965). Phagocytosis has been regarded as one of the critical events in the induction of an immune response (Frei, Benacerraf and Thorbecke, 1965) although more recent evidence indicates that antigen in certain forms is capable of interacting directly with lymphocytes and stimulating them to produce antibody (Diener and Feldmann, 1972).

The importance of macrophages in the ontogeny of the immune response in mice has been demonstrated by the transplantation of viable parental, syngeneic macrophages into neonatal mice (Braun and Lasky, 1967; Argyris, 1968). This results in enhanced antibody responses when macrophages are presented simultaneously with sheep red blood cells. Non-viable macrophages are not effective in this system. These findings suggest that the newborn mouse possesses immunocompetent lymphocytes but does not have a system for processing antigen. Macrophages are not required in the induction and subsequent steps in the secondary immune response to tetanus toxoid (Ellis, Gowans and Howard, 1969; Ellis and Gowans, 1973) and such disparate findings suggest that macrophages may only be required in the induction and maintenance of immune responses to certain antigens in certain species.
The work of Fishman (1961), has shown antibody production against T2 bacteriophage by lymph node cells in vitro when these cells are incubated with macrophages or RNA derived from macrophages that had previously been incubated with T2 phage. More recent reports, however, have indicated that the immunogenic activity of RNA-antigen complexes is a function of the antigen rather than the RNA content of the complex (Gottlieb and Waldman, 1972).

Lymph node reticular cells have been shown to bind antigen on their surface, a phenomenon which is greatly enhanced by prior immunisation (Miller and Nossal, 1964), and pre-existing natural opsonins (Boyden, 1966). Similarly, localisation of antigen in the cortex of lymph nodes is dependent upon the presence of antibody, but the uptake of antigen by medullary macrophages is not dependent upon the presence of antibody (Nossal, Ada, Austin and Pye, 1965; Lang and Ada, 1967). Plasma cells have been found in lymph nodes clustered around medullary macrophages (Miller and Avrameas, 1971) and lymphocytes are found closely associated with macrophages in afferent lymph. These associations may provide a means for transferring an antigenic stimulus to immunocompetent lymphocytes.

Macrophages have been shown to possess receptors for immunoglobulins, in particular the Fc portion (Berken and Benacerraf, 1966) and lymphocytes with surface immunoglobulins bind to macrophages (Schmidtke and Unanue, 1971).

Macrophages that have been incubated with sheep red blood cells when injected into syngeneic animals produce an immune response whereas macrophages incubated with isologous, anti-SRBC IgG and SRBC evoke only a marginal response under the same conditions. Macrophages incubated with anti-SRBC IgM and SRBC give feeble and delayed immune responses (Pryjma, Ptak, Szybinski and Sarnowicz, 1972). It is envisaged that the complement
C3 receptor of the macrophage is principally involved in causing particles and immune complexes to attach to the cell, whereas IgG acts as an opsonin and facilitates ingestion of immune complexes by macrophages. IgM-antigen complexes appear to remain on the macrophage surface thus being in a position to contact immunocompetent lymphocytes (Mantovani, Rabinovitch and Nussenzweig, 1972). This could account for the enhanced responses that occur when antigens are presented with small amounts of specific IgM antibody. Such a reaction may occur in normal primary immune responses to antigens presented to animals which have IgM antibody normally present. Subsequent antigenic challenge results in a predominance of IgG antibody and the IgG-antigen complexes will be phagocytosed by the macrophages (Mantovani, Rabinovitch and Nussenzweig, 1972).

Recent evidence indicates that macrophages may elaborate substances which act to regulate the immune response. The removal of macrophages from in vitro cultures of mouse spleen cells results in a reduction of the spleen cells capability to give an immune response. The cultures can be restored to normal by adding the supernatant material from cultures of peritoneal exudate cells. The activity in the supernatant can be absorbed out by adding antigen (SRBC), and SRBC recovered from the supernatant are effective in stimulating immune responses in the absence of macrophages or the supernatants from peritoneal exudate cell cultures (Kennedy, Treadwell and Lennox, 1970). Other investigators (Hersh and Harris, 1968; Mitchison, 1969; Feldmann and Palmer, 1971), have also shown macrophages to be capable of enhancing immune responses.

The evidence suggests that macrophages regulate lymphocyte function directly, perhaps having an influence upon lymphocytes that are in close proximity to them. Areas such as the splenic red pulp and
marginal zone surrounding the periarteriolar sheath and lymph node medullary cords, and afferent lymph itself may be sites where the activities of lymphocytes are regulated by macrophages.

2. The integrated function of lymphocytes in the immune response - The participation of two populations of lymphocytes, bone marrow derived lymphocytes (B) and thymus derived lymphocytes (T) in the development and subsequent expression of humoral immune responses to a variety of antigens has been well established in several species (Katz and Benacerraf, 1972), since it was first demonstrated by Claman, Chaperon and Triplett (1966), that T and B lymphocytes collaborated in immune responses in mice. Experimental results suggest that T lymphocytes play a multi-functional role in the development of humoral immunity (Katz and Benacerraf, 1972).

It has been claimed that T lymphocytes, like B lymphocytes, have surface immunoglobulins which function as antigen recognition units (Marchalonis, Cone, Von Boehmer, 1974). The role of T lymphocytes in humoral immunity lies in their regulatory influence on the response of B lymphocytes to antigen. This regulation is mediated by soluble factors released by antigen activated T lymphocytes; such factors may directly or indirectly modulate the activities of B lymphocytes and the subsequent expression of the immune response (Katz and Benacerraf, 1972).

The regulatory effects of T lymphocytes upon antibody responses by B lymphocytes was postulated to be mediated by a soluble non-specific factor by Burnet (1968). Evidence for the existence of a soluble inhibitory factor active in antigenic competition was first indicated by studies in mice (Radovich and Talmage, 1967). They found that an injection of horse red blood cells given several days before
primary immunisation with sheep red blood cells resulted in a greatly suppressed response to the second antigen, whereas simultaneous presentation of the two antigens resulted in a normal immune response to each antigen. These and subsequent experimental findings have shown that the phenomenon of antigenic competition is not a competitive effect but rather a suppressive effect that is induced by the competing antigen (Kerbel and Eidinger, 1971).

This phenomenon has been referred to classically as antigenic competition (Adler, 1964) and was considered evidence for the existence of multipotent immunocompetent cells, a concept at variance with the clonal selection theory (Burnet, 1959). More recent evidence (Möller and Sjöberg, 1970; Waterston, 1970; Gershon and Kondo, 1971a; Gershon and Kondo, 1971b; Möller, 1971, Sjöberg, 1971) has shown that antigenic competition is unlikely to be the result of competition between multipotent cells for antigen, but rather an effect mediated through humoral factors active in cell collaboration during the induction and establishment of an immune response.

Studies involving the transfer of lymphoid cells have shown that the number of antigen reactive cells specific to a given antigen is not diminished in individuals responding to an unrelated antigen (Möller and Sjöberg, 1970) although it has been suggested that there is a diminished population of so-called 'helper T cells' specific for the antigen in individuals responding to an unrelated antigen (Kerbel and Eidinger, 1971). Antigen induced suppression (classical antigenic competition) has been demonstrated to be a thymus dependent phenomenon (Möller, 1971) as sequential administration of sheep red blood cells and horse red blood cells does not give rise to suppressed immune responses to either antigen in thymus deprived mice. The immune response
to an antigen given during an immune response to an unrelated antigen can be suppressed by the passive administration of normal thymocytes. The magnitude of the suppressive effect is directly related to the number of thymocytes transferred (Gershon and Kondo, 1971a).

Substantial evidence has been presented for the existence of a factor produced by T lymphocytes which is capable of replacing T lymphocytes in the induction of in vitro immune responses and enhancing both in vitro and in vivo antibody responses. Supernatants from antigen stimulated T lymphocyte cultures are capable of reconstituting the in vitro immune response in a T lymphocyte depleted population (Gorczynski, Miller and Phillips, 1972, 1973a) and supernatants from allogeneic lymphocyte cultures enable B lymphocytes to respond to sheep red blood cells in vitro (Hartmann, 1971; Feldmann and Basting, 1972; Schimpl and Wecker, 1972). T lymphocytes sensitised to a given erythrocyte antigen, can in its presence, give rise to enhanced immune responses to other non-cross reacting erythrocytes (Hunter, Munro and McConnell, 1972).

Antiserum raised against the T lymphocyte derived enhancing factor has been shown to inhibit both in vitro and in vivo immune responses to erythrocyte antigens (Gorczynski, Miller and Phillips, 1973c). The enhancing factor has been demonstrated to be a product of activated T lymphocytes since anti- treatment of in vitro lymphocyte cultures abolishes the capacity of cells to produce the factor (Gorczynski, Miller and Phillips, 1973a). The enhancing factor is trypsin sensitive and has a molecular weight in the range 150,000 - 300,000 (Gorczynski, Miller and Phillips, 1973b). Other soluble factors derived from T lymphocytes have been shown to exert an inhibitory influence on antibody production (Katz, Paul and Benacerraf, 1973) and it was suggested that the inhibitory and enhancing factors may represent the two ends of a spectrum of T lymphocyte regulatory activity, perhaps exerted by the same T lymphocyte product,
although it has been suggested that populations of T lymphocytes specifically endowed with either a suppressor or a helper function may exist (Baker, Stashak, Amsbaugh, Prescott and Barth, 1970; Okumura and Tada, 1971).

The soluble product(s) of activated T lymphocytes although specifically induced would seem to exert their influence in a non-specific manner. Feldmann and Basten (1972), however, have shown that specifically activated T lymphocytes synthesise and release a factor showing specificity for the antigen which induced its formation. This T lymphocyte factor has been shown to be surface monomeric IgM to which antigen specifically binds and is released from the T lymphocyte surface as an antigen-antibody complex. The immunoglobulin component of the complex is cytophilic for macrophages and complexes binding to the macrophage surface present a lattice of antigenic determinants which are considered to be more effective in contacting and activating B lymphocytes, thereby initiating an immune response (Feldmann, Cone and Marchalonis, 1973).

The phenomenon of tolerance can also be linked to the regulatory influence of T lymphocytes on antibody responses for the transfer of T lymphocytes from tolerant donors has been found to inhibit the normal co-operative response to sheep red blood cells in thymectomised, irradiated and reconstituted mice (Gershon and Kondo, 1970). It could be proposed that such inhibition may be mediated by an inhibitory factor released by activated T lymphocytes. In rats, the transfer of lymphocytes from normal syngeneic donors into rats tolerant to sheep red blood cells fails to abrogate tolerance but the transfer of allogeneic lymphocytes is effective and it has been demonstrated that the antibody-forming cells are predominantly of host origin. It has been suggested that tolerance to sheep red blood cells in rats is due to the
reversible inhibition of specific reactivity in immunocompetent cells (McCullagh, 1970a) and in addition there is some factor in the tolerant rat that prevents the initiation of an immune response in the transferred, normal (non-tolerant) syngeneic lymphocytes (McCullagh, 1970b). The state of tolerance in most animals seems to be a temporary state of unresponsiveness and this observation together with the observation of Gershon and Kondo (1971a; 1971b) that T lymphocytes release a factor which temporarily inactivates immunocompetent B lymphocytes of varying specificity, suggests that tolerance may be mediated by T lymphocyte derived factors. It remains, however, to explain the specificity of the tolerant state.

The general conclusions to be drawn from the extensive literature on T and B lymphocyte collaboration in immune responses is that helper and suppressor effects are mediated through soluble factors of T lymphocyte origin and the extent of the regulatory influence of T lymphocytes is determined by the immunological status of the individual and the nature of the antigenic challenge. The involvement of other cell types such as macrophages, in regulatory functions is complex and as yet, unclear. T lymphocytes have also been claimed to play a regulatory role in cell mediated immune reactions, graft versus host reactions, delayed type hypersensitivity reactions and allograft rejection. The precise role that they play is unknown for it is thought that subpopulations of T lymphocytes may exert different functions in different ways; there is, however, no direct evidence for this or for the existence of multi-potent T lymphocytes capable of participating in a variety of immunological reactions.

The concept of specific T and B lymphocyte populations, whilst established in certain species, has not been established in the sheep
where foetal thymectomy and thoracic duct drainage have been shown to have little effect on either allograft rejection (Cole and Morris, 1971c) or humoral immune responses (Cole and Morris, 1971b). It is postulated that in the sheep subpopulations of lymphocytes not necessarily arising from or subjected to thymic influence, are implicated in the establishment of humoral and cell mediated immunity and regulatory functions may be assigned to subpopulations of lymphocytes in general or to other cell types involved in the immune response.

**Antibody Mediated Suppression**

It is now well established that passively administered antibody can specifically inhibit the immune response (Uhr and Möller, 1968). The mechanisms involved in antibody mediated suppression are as yet poorly understood but it has been claimed that antibody may act either peripherally by rendering antigenic determinants sterically unavailable to immunocompetent cells, or centrally, by reducing or inactivating immunocompetent cells (Rowley and Fitch, 1964). Lymphoid cells from animals treated with suppressive doses of antibody are competent to respond to the corresponding antigen upon transfer to syngeneic recipients (Möller, 1964) and attempts to repeat the work of Rowley and Fitch (1964) have in most cases failed. Whilst it has been demonstrated that antibody by itself does not directly affect immunocompetent cells, it has been shown that antibody and antigen together may exert a central suppressive effect on immunocompetent cells (Diener and Feldmann, 1972). Such findings have provided a link between antibody mediated suppression of immune responsiveness and tolerance induction. Polymeric antigens may attach to and be retained on the surface of the specific immunocompetent cell by the binding of multiple antigenic determinants of the polymer to the antibody molecules present on the immunocompetent cell.
The more antigen that attaches in this fashion then the more likely is tolerance to be induced.

Antibody produced in response to monomeric antigens can link the monomers which can then act as a polymeric antigen. Thus antibody mediated suppression of immune responsiveness can be due to either tolerance induction (central effect) or the alteration of an antigen's immunogenicity (peripheral effect) both mechanisms being dependent upon the structure of the antigen and the relative concentrations of antigen and antibody (Diener and Feldmann, 1972).

Although passively administered antibody can effectively inhibit the primary immune response to antigen, it is considerably more difficult to inhibit the priming process (Uhr and Baumann, 1961a). It is considered that less antigen is required for priming than for initiating a primary antibody response. This is substantiated by the finding that in certain cases antigen-antibody complexes can prime an animal to a greater extent than can antigen alone and small doses of antigen that do not initiate detectable antibody synthesis will nonetheless prime the animal (Salvin and Smith, 1960). These anomalous findings could be explained by the suggestion that passive antibody may permit antigen to interact with and stimulate T lymphocytes, whilst the level of antigen present is insufficient to stimulate B lymphocytes (Gershon and Kondo, 1971). Indeed, it has been demonstrated that when passive antibody is given at doses that suppress antibody formation following the administration of antigen, there is an increase in the number of T lymphocytes (Möller and Greaves, 1971). The work of Uhr and Baumann (1961b) has shown that not all antigenic determinants on an antigen need to be bound by antibody for antibody mediated suppression to occur, although the suppressive effect may be related to the way in
which antigen is presented to the immunocompetent cell or by the fact that the free antigen in the complex may be below the level of the threshold required for initiation of antibody formation. Proliferation of memory cells may, however, occur under these circumstances (Makinodan and Albright, 1962).

Pearlman (1966) has shown that 7S and 19S antibodies are equally effective in their ability to suppress immune responses whilst other investigators have shown that 19S antibody is much less effective than 7S antibody (Möller and Wigzell, 1965; Henry and Jerne, 1968). It has been proposed that the immunogenicity of an antigen is augmented by pre-existing 'natural' specific antibody molecules in the animal (Jerne, 1955; Boyden, 1960; Eisen and Karush, 1964). Such antibody is assumed to bind to the antigen and assist in the localisation of it within the lymphoid tissues where an efficient interaction can occur between antigen and immunocompetent cells. Thus the balance between the enhancing and suppressive effects of specific antibody may be related to the balance that exists between the number of antigenic determinants on an antigen that are bound by antibody and the increased localisation of such antigen-antibody complexes within the lymphoid tissues (Schrader, 1973). These effects may also be dependent upon the affinity and avidity of the antibody for the antigenic determinants present on the antigen.

The ability of specific antibody to suppress an immune response is related to its affinity for the corresponding antigen; high affinity antibody suppresses the immune response more effectively than low affinity antibody. Antibody of high affinity may prevent the continued recruitment and stimulation of additional antibody-synthesising precursor cells (Eisen and Siskind, 1964) by removing the antigen
present in the system. At low levels of available antigen the continued synthesis of high affinity antibody would be favoured if it is assumed that low affinity precursor cells are less sensitive to activation by antigen than high affinity precursor cells (Walker and Siskind, 1968).

Circulating 7S antibody may be capable of terminating 19S antibody synthesis (Sahiar and Schwartz, 1964), and serve not only to eliminate the inducing stimulus and thus limit the extent of the immune response but to direct the maturation of the immune response by exerting a selective pressure towards increased 7S antibody synthesis and high affinity antibody production. The biological significance of antibody as a recognition and effector mechanism in regulating the immune response may be far reaching.

The Aims and Scope of the Thesis

It is the intention of this thesis to examine the kinetics of normal and modified immune responses in an effort to determine those regulatory mechanisms which act to initiate and limit an animal's response to antigenic insult.

The sheep popliteal lymph node model first described by Hall and Morris (1962) has certain advantages which can be used to study aspects of immune regulation. Antigen can be quantitatively introduced to the lymph node and the ensuing immune response confined to the node by the prior cannulation of the efferent lymphatic duct (Hall and Morris, 1962). Cellular and humoral events that occur in the lymph node are closely reflected by events occurring in the efferent lymph. Lymph collected quantitatively throughout the immune response enables the temporal sequence of the immune response to be followed and allows various parameters to be studied and measured which are inaccessible
to analysis in the study of systemic immune responses. Systemic immune responses to specific antigen involve the participation of multiple areas of lymphoid tissue, many of which are at different stages of reactivity at any given time. Thus any analysis of systemic immune responses reflects the accumulative state of all reactions involved in the response to the antigen rather than the state of reactivity in any one reaction centre.

Immune responses occurring in regional lymph nodes can be studied by extirpating the lymph node at various times during an immune response but this approach gives no information on the flux of cells entering and leaving the node. A series of animals has to be used to examine a single immune response by this method and the parameters measured are usually plaque-forming cells and circulating antibody titres. While the plaque-forming cell assays give an estimate of the number of antibody-producing cells secreting antibody in the node, the circulating antibody titres will reflect the antibody produced by other lymph nodes as well.

The sheep popliteal lymph node preparation overcomes these disadvantages and enables the entire immune response to be examined under physiological conditions in a single lymph node. With this system it was proposed to determine the temporal sequence of events expressed in the efferent lymph that might relate to some regulatory control operating at the cellular or humoral level.
CHAPTER II

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

3-5 year-old randomly bred Norway or Sprague-Dawley females were used in experiments. The animals were obtained from the colony of the John Curtin School of Medical Research.

RABBIT

Rabbit aorta and portal venous blood cells were obtained from randomly bred rabbits from the animal colony of the John Curtin School of Medical Research. These animals were also used in vaccine antigen against influenza.

CHICKENS

Chicken erythrocytes were obtained from the animal colony of the John Curtin School of Medical Research.

RASA

Rat aorta, liver, and spleen were used as sources of serum, erythrocytes, and thymus and lymphocytes.

CHEMICALS, SOLUTIONS, CULTURE MEDIA AND MEDIANS

Sodium Chloride Solution

A sterile 0.9% saline solution of sodium chloride in distilled water was used and sterilized by autoclaving in this study.
EXPERIMENTAL ANIMALS

Sheep

3-5 year old randomly bred Merino or Merino-Border Leicester virgin and multiparous ewes were used in experiments. The animals were obtained from the colony of the John Curtin School of Medical Research.

Rabbits

Rabbit serum and rabbit red blood cells were obtained from randomly bred rabbits from the animal colony of the John Curtin School of Medical Research. These animals were also used to raise antisera against sheep immunoglobulins.

Chickens

Chicken erythrocytes were obtained from outbred hens from the animal colony of the John Curtin School of Medical Research.

Rats

Hooded x D.A., F1 rats were used a source of serum, erythrocytes, and thoracic duct lymphocytes.

CHEMICALS, SOLUTIONS, CULTURE MEDIA AND BUFFERS

Sodium Chloride Solution

A sterile 0.9% solution of sodium chloride in distilled water was used and is referred to as saline in this thesis.
Phosphate Buffered Saline (PBS) pH 7.4

PBS was made up of the following three sterile solutions which were mixed before use:

(i) \( \text{NaCl} 8.0 \text{g}, \text{KCl} 0.2 \text{g}, \text{Na}_2\text{HPO}_4 1.15 \text{g}, \text{KH}_2\text{PO}_4 0.2 \text{g} \)
    deionised distilled water (DDW) 800ml.

(ii) \( \text{CaCl}_2 0.1 \text{g}, \text{DDW}, 100\text{ml} \).

(iii) \( \text{MgCl}_2 \cdot 6\text{H}_2\text{O} 0.1 \text{g} \) DDW 100ml.

Calcium-Magnesium Saline (CMS)

Sterile CMS was prepared by dissolving 9.0g NaCl, 0.028g CaCl\(_2\) and 0.079g MgCl\(_2\) in 1 litre of DDW.

Mercaptoethanol Solution

A 0.2 Molar solution of 2-Mercaptoethanol (Eastman Organic Chemicals) in saline was prepared immediately before use.

Hank's Balanced Salt Solution (BSS)

Sterile Hank's BSS was prepared by dissolving 8.0g NaCl, 0.4g KCl, 0.1g MgSO\(_4\) \cdot 7H\(_2\)O, 0.14g MgCl\(_2\) \cdot 6H\(_2\)O, 0.69g Na\(_2\)HPO\(_4\), 0.06g KH\(_2\)PO\(_4\), and 1.0g of Glucose in 1 litre of DDW. 5ml of a 0.4% aqueous solution of phenol red was added as a pH indicator.

Eagle's Medium

Eagle's Medium (Eagle, 1959) was prepared by combining the stock concentrate with glutamine, sodium bicarbonate and antibiotic solution in DDW. An alternative medium used was Eagle's Basal Medium G-13 (Gibco Ltd.) an instant tissue culture powder medium with Earles salts and L-glutamine. The pH was adjusted to pH 7.4 with sodium bicarbonate to give a final volume of 1 litre. The media were sterilised by membrane filtration before use.
Tris pH 7.4 (for horse radish peroxidase staining)

10ml of 2 Molar Tris was added to 176ml of DDW and the pH adjusted to 7.4 with 14ml N HCl.

Tris-EDTA-Boric Acid Buffer pH 8.4 (for polyacrylamide gel electrophoresis)

The following chemicals were dissolved in 4 litres of DDW:

Tris 43.1g, EDTA Na₂H₂O, 3.7g., boric acid (crystalline) 22.0g
as described by Peacock et al. (1965).

Acetate Buffers pH 5.0

One litre volumes of 0.03M, 0.1M, 0.2M, 0.5M and 1M acetate buffers were prepared by adding the necessary volume of the following acetic acid sodium acetate solutions:

<table>
<thead>
<tr>
<th>Molarity of buffer</th>
<th>Sodium acetate 1M</th>
<th>Acetic acid 1M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03M</td>
<td>21ml</td>
<td>9ml</td>
</tr>
<tr>
<td>0.1M</td>
<td>70ml</td>
<td>30ml</td>
</tr>
<tr>
<td>0.2M</td>
<td>140ml</td>
<td>60ml</td>
</tr>
<tr>
<td>0.5M</td>
<td>350ml</td>
<td>150ml</td>
</tr>
<tr>
<td>1.0M</td>
<td>700ml</td>
<td>300ml</td>
</tr>
</tbody>
</table>

Dianisidine (for the determination of horse radish peroxidase)

1% Dianisidine was prepared by dissolving 1g in absolute methyl alcohol. This solution was filtered before use and stored at 4°C in a brown bottle. Under these conditions the reagent was stable for 3-4 weeks.
Scintillation Fluid

One litre of scintillation fluid was prepared by dissolving and mixing the following chemicals according to Formula II (Packard Ltd.):

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>100g</td>
</tr>
<tr>
<td>2.5 diphenyloxazole (PPO)</td>
<td>5g</td>
</tr>
<tr>
<td>1.4 Dioxan</td>
<td>720ml</td>
</tr>
<tr>
<td>Toluene</td>
<td>135ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>45ml</td>
</tr>
</tbody>
</table>

STAINS AND FIXATIVES

Leishman Stain

0.15g of Leishman stain (BDH) was dissolved in 100ml of methanol and filtered before use.

Trypan Blue Solution

A 2% solution of Trypan Blue in PBS was used to assay cell viability.

Carbazole Stain (for the detection of horse radish peroxidase)

4mg of 3-amino 9-ethyl carbazole was dissolved in 1ml of dimethylformamide. 19ml of 0.05M acetate buffer pH 5.0 was added to the carbazole solution and thoroughly mixed before adding 4 drops of 3% H$_2$O$_2$ (dispensed from a Pasteur pipette). The activity of the stain was tested by adding 1 drop of horse radish peroxidase solution (0.1mg/ml in PBS) to a drop of the stain. The stain was prepared immediately before use.

3,3'-diaminobenzidine (DAB) for the detection of horse radish peroxidase

5mg of DAB were added to 10ml of 0.1M Tris/HCl pH 7.4 and mixed before adding 1 drop (dispensed from a Pasteur pipette) of H$_2$O$_2$ (Univar).
The activity of the stain was tested by adding 1 drop of horse radish peroxidase solution (0.1mg/ml in PBS) to a drop of the stain.

The stain was prepared immediately before use.

Amido Black Dye Solution (for staining polyacrylamide gels)

2g of amido black were dissolved in 1 litre of methanol:water: acetic acid (5:5:1).

4% Formaldehyde Solution

The solution was prepared by adding 5g of paraformaldehyde to 125ml PBS and heating on a magnetic stirrer until the solution cleared.

The cooled solution was used to fix cell smears and was discarded if not used within 24 hours of preparation.

ANTIBIOTICS

Penicillin-G

The sodium salt of Penicillin-G ('Crystopen', Glaxo Allenburys) was used to control bacterial growth in lymph samples and to dust surgical wounds prior to their closure.

Streptomycin and Neomycin

Streptomycin sulphate (Evans Medical Ltd.) and Neomycin (Penick Co.) were used to prevent bacterial growth in lymph samples.

ANTICOAGULANTS

Alsever's Solution (modified)

Sterile Alsever's solution was prepared by adding 8g sodium citrate (Na₃C₆H₅O₇·2H₂O) 4.2g NaCl, 20.5g glucose and 8ml of 10% citric acid to 1 litre of DDW.

Alsever's solution was used for the collection of blood in a ratio of 1 volume of whole blood to 1 volume of Alsever's solution.
Heparin

Freeze dried mucoid heparin ('Pularin', Evans Medical Ltd.) was used to prevent clotting in cannulae and lymph samples.

ANAESTHETICS

Thiopentone Sodium B.P.

'Intraval' sodium (May and Baker Ltd.) was used as a 5% solution in distilled water for the induction of anaesthesia.

Halothane B.P.

'Fluothane' (I.C.I. Ltd.) inhalation was used for the maintenance of anaesthesia.

RADIOISOTOPES

Thymidine (methyl-tritiated)

Thymidine (Radiochemical Centre, Amersham) with a specific activity of 5Ci/mM and $^{51}$ chromium (C.E.A., France) with an activity of 50-150mCi/mg chromium were used as markers for labelling cells.

ANTISERA

Rabbit anti-sheep immunoglobulin antisera and normal rabbit immunoglobulins were provided by Dr. H. R. P. Miller.

COMPLEMENT

Frozen guinea pig serum (Commonwealth Serum Laboratories) was used as a source of complement. The serum was stored in small vials and used immediately after thawing. Excess serum not used after one thawing was discarded.
PHYTOHAEMAGGLUTININ (PHA)

PHA-P (Wellcome Lot K4402 MR68) was used. 2mg of PHA-P were made up to 2ml with sterile saline immediately prior to use and used at a concentration of 300µg/ml.

PREPARATION OF DEAE ION EXCHANGE COLUMNS

Cellex D (Biorad Laboratories) an anion exchange cellulose with a capacity of 0.72meq./gm was used to isolate blood and lymph glycoproteins.

The cellulose was prepared by suspending and thoroughly washing in 0.5N NaOH followed by several washes in distilled water. The exchanger was converted into the acetate form by treatment with Molar acetate buffer pH 5.0. The cellulose was then washed, suspended and equilibrated with 0.03M acetate buffer pH 5.0 and used to pack glass columns.

POLYACRYLAMIDE GEL COMPOSITION

Cyanogum 41 (Bis-acrylamide) 10.0g.
Tris-EDTA-Boric acid buffer pH 8.4 200.0ml.
T.M.E.D. (N,N,N',N'-Tetramethylene diamine) 0.2ml.
Ammonium persulphate 0.2g.
Polymerisation was induced by chemical catalysis using T.M.E.D. and ammonium persulphate.

GENERAL METHODS

Surgical Methods I. Anaesthesia and preparation of sheep for surgery

Animals were starved of food and water for 24 hours prior to surgery. Anaesthesia was induced by thiopentone sodium B.P. intravenously.
Immediately following induction the animal was intubated with a Magill's endotracheal tube and anaesthesia was maintained with Halothane B.P. and oxygen, administered through the closed circuit of a Boyle's anaesthetic apparatus (British Oxygen Co.). The area around the site of incision was prepared by clipping the wool and thoroughly washing the skin with a 1% solution of chlorhexidine ('Hibitane' I.C.I. Ltd.). All operating procedures were performed under sterile conditions.

Surgical Methods II. Cannulation of the efferent popliteal lymphatic

The technique used was that described by Hall and Morris (1962). An incision was made through the skin and subcutaneous tissues at a point 2-5 cm below the sciatic tuberosity, the incision being extended ventrally for about 10 cm. Bleeding of subcutaneous tissue blood vessels was controlled by electrocautery. The semitendinosus and biceps femoris muscles were separated and retracted to expose the popliteal fossa. Muscular branches of the posterior femoral artery and vein were retracted but were not ligatured and cut. The position of the popliteal lymph node was determined by palpation and the efferent lymph duct was exposed by blunt dissection. The duct could be found adjacent to the femoral vein although its position was extremely variable. A ligature of 3/0 silk was tied around the duct at a point as distant from the node as possible. The duct was lifted carefully to allow it to be cleaned of adherent fat and tissue, and the silk tie secured with artery forceps. The duct was carefully cleaned of adherent fat and connective tissue and traced to its point of origin from the popliteal
lymph node. Branches of the efferent lymph duct, which were frequently found, were ligatured with 2 occluding ties of 3/0 silk. Occasionally, 0.5ml of a 2% solution of Evans blue in saline was injected into the popliteal lymph node drainage area to facilitate the identification of these ducts; up to 7 efferent lymph duct branches have been detected by this method.

A ligature of 3/0 silk was loosely applied about 1cm below the tie which occluded the main efferent lymph duct. An appropriate sized polyvinyl or polyethylene cannula was led through a stab incision made through the skin immediately above the initial incision so as to correctly position the cannula in the same direction as the lymph duct.

The cannula was rinsed with heparin in saline prior to its insertion in the lymph duct, and the distal end of the cannula sealed with artery forceps. This procedure prevented clotting in the cannula and facilitated the initial flow of lymph into the cannula. A small incision was made in the lymph duct with iridectomy scissors and the end of the cannula inserted into the duct. The cannula was secured in the lymph duct by the lower ligature and a further two ligatures subsequently placed around the cannula. The artery forceps occluding the efferent end of the cannula were removed to allow lymph to flow into the cannula. It was sometimes necessary to insert an aneurism needle into the lymph duct so as to break down valves and facilitate insertion of the cannula. The wound was dusted with powdered penicillin (Crystapen) and the muscle retractors removed. At this point it was critical to ensure that lymph flow was not impeded by muscle movements and this was determined by moving the leg and observing any changes in the rate of lymph flow.
Subcutaneous tissues were secured with at least two silk ties and the skin incision closed with Michel clips. A purse string suture secured the cannula externally at the point of the stab incision. A plastic bottle holder was sutured to the skin in front of the incision.

PREPARATION OF ANTIGENS

**Chicken erythrocytes**

Hens were bled from the wing vein using a 22 gauge needle.

Five ml of whole blood was taken into a syringe containing 5ml of Alsever's solution.

The blood was centrifuged and the red cells washed 3 times with PBS, the buffy coat was removed carefully after each wash. Cell suspensions containing from $10^5$ to $4 \times 10^9$ red cells were prepared and each dilution was used as an antigenic stimulus.

**Rabbit erythrocytes**

Rabbits were bled from an ear vein into Alsever's solution or a diluted Heparin solution. The blood was centrifuged and the red cells washed 3 times with PBS, the buffy coat was carefully removed after each wash. 1ml of a cell suspension containing $2 \times 10^9$ red cells in PBS was used as an antigenic stimulus.

**Salmonella munchen heat killed organisms**

Salmonella munchen boiled organisms were used as an antigen.

$3.4 \times 10^9$ organisms were suspended in 1ml of saline for injection.

**Rat lymphocytes**

Rat lymphocytes were obtained by thoracic duct cannulation.

The lymphocytes were washed 3 times with Medium 199 and
and resuspended in sheep lymph immediately prior to use. A 1ml suspension containing $3 \times 10^8$ viable rat lymphocytes was injected.

**Allogeneic lymphocytes**

Lymphocytes were obtained from the efferent lymphatic of a second party sheep. The cells were washed 3 times in Eagle's medium and resuspended to give a final concentration of $3 \times 10^8$ lymphocytes/ml in cell free efferent lymph from the recipient. 1ml ($3 \times 10^8$ viable lymphocytes) was injected.

**Autologous lymphocytes**

Lymphocytes were obtained from efferent popliteal lymph. The cells were centrifuged and resuspended in their own lymph to give a final concentration of $3 \times 10^8$ lymphocytes/ml. 1ml ($3 \times 10^8$ viable lymphocytes) was injected.

**Lysed sheep red blood cells**

Sheep red blood cells were obtained from the jugular vein and collected in an equal volume of Alsever's solution. The blood was centrifuged and the buffy coat removed during 3 washes with PBS.

The pellet of sheep red blood cells from 5ml of whole blood was lysed by adding distilled water to a total volume of 2.5ml. The lysate was thoroughly mixed and injected.

**Horse radish peroxidase**

Horse radish peroxidase (Sigma Grade VI) and DEAE cellulose purified grade II horse radish peroxidase (Moroz, Joubert and Hogg, 1974) were used.
5-10mg of horse radish peroxidase emulsified in 1ml of Freund's complete adjuvant was injected to give a primary immune response and 1-2.5mg of horse radish peroxidase in 0.25-0.5ml of PBS were injected at least 6 weeks later as a secondary challenge.

Erythrocyte antigen-antibody complexes

Antigen-antibody complexes were suspended in PBS to give a final concentration of $2 \times 10^9$ erythrocytes/ml. Complexes were prepared by incubating erythrocytes in heat inactivated (56°/30') antiserum containing specific IgM, IgG or IgM and IgG antibodies.

Colloidal carbon

'Dag 554' colloidal carbon (Acheson Colloids) a product which contains 0.5-1.5µ graphite particles in water at a concentration of 250mg/ml, was diluted to 120mg/ml with distilled water. 0.1ml of the diluted colloid (12mg) was injected.

Latex particles

0.1ml of a distilled water suspension of Polystyrene Latex particles (Dow Chemical Co.) with a diameter of 0.557µ was injected.

ADMINISTRATION OF ANTIGENS

Antigens were injected subcutaneously into the outer aspect of the lower hind leg in the area drained by the popliteal lymph node. Normal lymphocyte transfer (N.L.T.) reactions were initiated by injecting 0.1ml of a suspension containing $1 \times 10^7$ allogeneic lymphocytes intradermally in the wool free skin of the medial aspect of the thigh (Jones and Lafferty, 1969). The increase in skin thickness at the injection site was measured daily with Schnelltaster skin calipers.
MITOMYCIN C TREATMENT OF LYMPHOCYTES

Lymphocytes were collected from the efferent popliteal lymph duct of sheep and the thoracic duct of rats. All collections were made under sterile conditions. Mitomycin C (Sigma) isolated from Streptomyces caespitosus was made up to a concentration of 10µg/ml or 50µg/ml with 10% lamb serum in Eagle's medium immediately prior to use. Lymphocytes were washed 3 times with Eagle's medium and resuspended in 10ml of Eagle's containing 10% lamb serum and 10µg/ml or 50µg/ml of Mitomycin C. The reaction mixture was incubated for 30 minutes at 37°C. Following incubation the reaction mixture was centrifuged and the lymphocytes washed 2 times with Eagle's medium.

MIXED LYMPHOCYTE CULTURES

Lymphocytes were washed with PBS and resuspended in Eagle's medium supplemented with 10% lamb serum. Unidirectional mixed lymphocyte cultures were prepared by mixing equal volumes of the responding lymphocyte population and Mitomycin C treated stimulating cells. Cell cultures were prepared at a concentration of 6 x 10⁶ cells/ml. Each assay consisted of 3 replicate cultures containing 3ml of the mixed lymphocyte population in flat bottomed glass vials with loose fitting caps. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for a period of 4 days. Tritiated thymidine was added to each culture at a concentration of 1µCi/ml, 16-20 hours before harvesting. Following incubation the lymphocytes were washed with PBS and the supernatants discarded. Two drops of 2% bovine serum albumin in
saline were added to each cell pellet and the cells resuspended. 3ml of ice cold 8% trichloroacetic acid was added to the cell suspension and the mixture left to flocculate for 1 hour at 4°C. The precipitate was washed with 3ml of 8% trichloroacetic acid and finally dissolved in 0.5ml of 3.5N ammonium hydroxide. 0.1ml portions of this solution were dried on glass fibre discs and the amount of radioactivity incorporated into the acid insoluble components was estimated by scintillation counting.

**PHYTOHAEMAGGLUTININ (PHA) STIMULATION OF EFFERENT LYMPH LYMPHOCYTES**

2 x 10^8 efferent lymph cells were washed and resuspended in 10ml Eagle's medium pH 7.4 to which 0.3ml (300µg) of PHA-P (Wellcome) in saline had been added. The cell suspension was thoroughly mixed and left at room temperature for 45 minutes.

The activated cells were then washed 3 times with Eagle's medium and resuspended to give a concentration of 2 x 10^6 cells/1.6ml. 1.6ml of the cell suspension was added to each well of a number of multidish disposable tissue culture trays (Linbro FB-16-24-TC) and 0.4ml immune or non-immune lymph, Eagle's medium or various protein fractions were added. Each culture was set up in triplicate.

The cell cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air for a period of 18 hours after which time 0.1ml (3µCi) of tritiated thymidine was added to each culture well. Following the addition of tritiated thymidine the cell cultures were gassed again with 5% CO₂ in air and incubated at 37°C for a further 6 hours.
After a total of 24 hours incubation the cell cultures were transferred to plastic tubes, centrifuged and washed 3 times with Eagle's medium; the supernatants were discarded. Two drops of 2.5% BSA were added to the cell pellet followed by 3 ml of chilled 10% trichloroacetic acid. The precipitate was suspended and stored overnight at 4°C.

The precipitate was washed 3 times with 10% chilled trichloroacetic acid and the supernatants discarded. The washed precipitate was dissolved in 0.5 ml 3.5N ammonium hydroxide and stored overnight at 37°C. 0.1 ml portions of the dissolved precipitates in ammonium hydroxide were added to 12 ml of scintillation fluid in counting vials and thoroughly mixed before transferring to the scintillation counter. The samples to be examined for tritium content were allowed to cool in the scintillation counter before counting proceeded.

SCINTILLATION COUNTING

All scintillation counting was done with a Packard Tri-Carb liquid scintillation spectrometer model 3320. Counts were allowed to accumulate for 10 minutes.

CELL SMEARS

The cell pellet from 1-10 ml of centrifuged efferent lymph was resuspended and washed 3 times with 5-10 ml of 1% bovine serum albumin in Hank's solution. The washed cell pellet was resuspended in an equal volume of 1% bovine serum albumin in Hank's solution and smeared on clean glass slides, air dried and stained.

Leishman stained cell smears were prepared by pipetting 1 ml of Leishman stain onto the slides. The stain was diluted 5 minutes later by the addition of 2.5 ml of distilled water. The slides were
left for a further 5 minutes, rinsed with distilled water, air dried and examined under an oil immersion lens. The cells of each lymph sample were classified according to their morphology and staining characteristics.

**CELL COUNTS**

A model FN Coulter counter (Coulter Counter Electronics) with a 100µ aperture was used for all cell counts. The counter was calibrated and the resultant cell count from a given suspension of cells was compared at intervals with a Haemocytometer count of the same cell suspension. All cell counts were performed with the counter adjusted to the following settings:

<table>
<thead>
<tr>
<th></th>
<th>Attenuation</th>
<th>Amplification Current</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total white cells</td>
<td>4</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Large white cells</td>
<td>4</td>
<td>8</td>
<td>43</td>
</tr>
</tbody>
</table>

The number of large lymphocytes determined from a Coulter counter assay could be closely correlated with the number of basophilic blast cells determined from Leishman stained cell smears.

**PREPARATION OF LIPOPOLYSACCHARIDE (LPS) COATED CHICKEN RED BLOOD CELLS**

Lipopolysaccharide isolated from Salmonella muenchen organisms was used to coat chicken red blood cells for anti-LPS antibody assays. The cells were prepared in the following way:

To 1ml of 0.02M sodium hydroxide was added 16mg of LPS and the mixture allowed to stand overnight. The mixture was centrifuged and the precipitate discarded. The supernatant was neutralised with 0.1ml of 0.2N HCl and 0.1ml of this solution was made up to 5ml with PBS and added to 5ml of a 5% suspension of chicken red
blood cells in PBS. The reaction mixture was incubated for 1 hour at 37°C then centrifuged and the supernatant discarded. The LPS coated chicken red blood cells were washed twice with cold Eagle's medium and resuspended in 5 volumes of Eagle's medium for use in the detection of anti-LPS antibody-forming cells in the plaque-forming cell assay.

A 0.5% suspension of the LPS coated chicken red blood cells in PBS was used in the haemagglutination assays.

PLAQUE ASSAY FOR ANTIBODY-SECRETING CELLS

Cells secreting antibody against chicken, rat and rabbit red blood cells and Salmonella muenchen lipopolysaccharide were assayed by a plaque assay technique (Jerne, Nordin and Henry, 1963; Cunningham and Szenberg, 1968). Plaque assay chambers were made by applying two strips of 'Scotch Brand' double coated tape No.4010, 22mm apart on an alcohol washed slide. A 22mm x 33mm alcohol washed coverslip was applied to the two strips of tape and firmly pressed so as to effectively seal two sides. These assay chambers had a volume of 0.05ml.

Efferent lymph cells were centrifuged and washed once with Alsever's solution and twice with cold Eagle's medium before being resuspended to a concentration of $10^4$-$10^7$ cells/ml with cold Eagle's medium.

Chicken red blood cells that had been coated with Salmonella muenchen LPS, or free chicken, rat or rabbit erythrocytes were prepared fresh by diluting 1 volume of packed erythrocytes with 5 volumes of cold Eagle's medium.

Specific antibody was removed from guinea pig serum by absorbing the serum with target erythrocytes or LPS. The absorbed serum was used as a source of complement. 0.5ml of the lymphoid cell suspension was
added to 0.1ml of target red cell suspension and 0.05ml of absorbed guinea pig serum. The reaction mixture was thoroughly mixed and 0.05ml immediately transferred to the plaque assay slide chamber. Care was taken to ensure that no air bubbles formed within the chamber and that the cells settled in an even monolayer. The sides of the chamber were sealed with vaseline to prevent evaporation and the slides incubated at 37°C for 30 minutes in a humid atmosphere. The chambers were examined at high and lower power magnification and the areas of lysis surrounding a cell of the lymphocytic series were counted. In the case of chicken red blood cells, areas of lysis could also be determined by the presence of the nucleated chicken cell ghosts in the area surrounding the antibody-secreting cell. The number of plaque-forming cells (PFC) per chamber was converted to the number of plaque-forming cells per million lymphoid cells by the following calculation:

The initial 0.5ml of lymphoid cell suspension was diluted to a volume of 0.65ml, therefore the concentration of cells in the reaction mixture = \( \frac{0.5}{0.65} \times 10^6/\text{ml} \). Since the chamber contained 0.05ml, then the number of PFC/ml of reaction mixture = 20 \times \text{the number of PFC/chamber} \quad \text{and PFC/10}^6 \text{ cells} = \frac{20 \times \text{PFC/chamber}}{0.77} \quad \text{i.e., PFC per 10}^6 \text{ lymphoid cells} = \frac{26 \times \text{PFC/chamber}}{\text{Cell concentration/ml} \times 10^6}

Direct PFC assays were performed in all experiments. It should be noted that the plaque-forming cell assay is one which enables an estimate to be made of the total number of cells actually secreting antibody, it does not necessarily reflect the total number of cells synthesising antibody in a given cell population. A distinction is therefore made between antibody-forming cells (AFC) and plaque-forming cells or antibody-secreting cells (PFC or ASC) in this thesis.
TITRATION OF HAEMAGGLUTINATING ANTIBODY

The lymph and serum samples to be assayed were inactivated of complement by incubating at 56°C for 30 minutes. IgM and IgG antibody was distinguished by incubating samples in an equal volume of 0.2M 2-mercaptoethanol solution for 30 minutes at room temperature prior to their titration. IgM antibody was designated as mercaptoethanol sensitive antibody. Samples were plated in doubling dilutions with PBS in Mictotitre 'V' plates (Cooke Engineering Co.) and one drop of a 0.5% suspension of target red cells in PBS was added to each well. The reaction mixtures were incubated at 4°C for 4 hours before reading the results and were then incubated overnight and read again. The antibody titre of a given sample was taken as the last dilution at which agglutination of red cells occurred. Antibody titres were either expressed as log₂ units or as antibody output, a measure calculated from the titre multiplied by the rate of flow of lymph.

ESTIMATION OF CYTOTOXIC ANTIBODY

The cytotoxicity of lymph samples was determined by measuring the amount of radioactivity released from ⁵¹ chromium labelled lymphocytes (Boyle, 1968). Lymphocytes from the efferent popliteal lymph were washed with PBS and resuspended at a concentration of

\[ 2 \times 10^8 \text{ cells/ml} \] in PBS containing 10% lamb serum. 50µCi of ⁵¹ chromium was added to every ml of the cell suspension and the mixture incubated at 37°C in an atmosphere of 5% CO₂ in air for 1 hour. Following incubation the cell suspension was centrifuged, the supernatant discarded and the cell pellet washed with 3 changes of PBS containing 5% lamb serum. The cells were resuspended to a concentration of \[ 1 \times 10^8 \] lymphocytes/ml and 0.1ml of the cell suspension was added to 0.025ml of a 1 in 2 dilution of guinea pig
complement and 0.25ml of a 1 in 2 dilution of lymph. Lymph and complement were diluted with calcium-magnesium saline. The reaction mixture was incubated at 37°C for 1 hour in an atmosphere of 5% CO₂ in air. Following incubation the samples were centrifuged and the supernatant and the cell pellet placed in separate counting tubes. Counts were carried out with a gamma counter (Packard Instrument Co.) and the percentage of ⁵¹ chromium released was calculated as the number of counts in the supernatant divided by the sum of the number of counts in the supernatant and cell pellet.

THE DETECTION OF INTRACELLULAR ANTIBODY TO HORSE RADISH PEROXIDASE

Cells producing antibody against horse radish peroxidase (HRP) were demonstrated by a histochemical technique which involved the reaction of the enzyme with its substrate in the presence of 3, 3'-diaminobenzidine (DAB). The methods of Avrameas and Lespinats (1967), Avrameas (1970) were followed. Cells isolated from lymph node medulla and efferent lymph were centrifuged and washed twice with Hank's medium containing 1% BSA. Cell smears were fixed in 4% formaldehyde in PBS for 15 minutes and washed 3 times with PBS. A solution of HRP in PBS (0.1mg/ml) was carefully layered onto the smears. The antibody-specific antigen reaction was allowed to proceed for 30 minutes in a humid atmosphere at room temperature. The cell smears were washed 3 times with PBS, flooded with the DAB-H₂O₂ reagent (previously described) and allowed to stand for 15 minutes at room temperature after which time they were thoroughly washed in distilled water and then dried in air. Control smears were flooded with DAB-H₂O₂ reagent but were not incubated with HRP.
Cells containing a dark brown precipitate were assessed as positive cells containing HRP specific antibody. The number of antibody-forming cells was assessed by subtracting the number of positively stained cells observed in the controls from those observed in cell smears reacted with HRP.

THE DETECTION OF IMMUNOGLOBULIN PRODUCING CELLS IN HRP RESPONSES

Cell smears were prepared and fixed as described in the method for the detection of intracellular antibody to horse radish peroxidase. Four drops (dispensed from a Pasteur pipette) of a 1 in 10 or 1 in 20 dilution of rabbit anti-sheep immunoglobulin antiserum (conjugated to horse radish peroxidase), in PBS, were carefully layered across the smears and then left for 90 minutes at room temperature in a humid atmosphere. Control smears were incubated for 90 minutes with normal rabbit immunoglobulin conjugated with HRP. Following incubation the smears were washed 3 times with PBS, flooded with the Carbazole-\(\text{H}_2\text{O}_2\) reagent (previously described) and washed thoroughly with distilled water after an incubation time of 5 minutes. Cells containing a reddish-brown precipitate were assessed as positive cells containing immunoglobulin.

The number of immunoglobulin-forming cells was assessed by subtracting the number of positively stained cells observed in the controls from those observed in the smears incubated with rabbit anti-sheep immunoglobulin.

The number of 'nonspecific' immunoglobulin-producing cells in any given cell population was calculated by subtracting the number of antibody-forming cells (DAB stained) from the number of immunoglobulin-producing cells (Carbazole stained).
THE ENZYMATIC ASSAY OF HORSE RADISH PEROXIDASE IN LYMPH

The method employed was based on that published in the Worthington Biochemical Corporation Handbook. The procedure is dependent upon the liberation of oxygen from hydrogen peroxide by peroxidase in the presence of 2-dianisidine. Dianisidine is oxidised to produce a brown oxidation product with a spectral absorption maximum at 460nm. The rate of formation of this product is directly proportional to the amount of enzyme, measured over a period of 3 minutes at 30 second intervals at a wavelength of 460nm.

The instrument used in this study was the Pye-Unicam SP8000 double beam spectrophotometer equipped with program controller and flat bed recorder.

Method - 2.9ml of 0.05M phosphate buffer pH 5.0 was pipetted into a quartz cuvette followed by 0.05-0.1ml of lymph. 0.025ml of 1% 2-dianisidine was added and thoroughly mixed. The cuvette was placed in the sample beam of the spectrophotometer set at a fixed wavelength of 460nm, and 0.03ml of 0.3% H₂O₂ was added to start the reaction. The rate of increase in optical density was recorded at 30 second intervals over a period of 3 minutes, against a blank containing buffer, peroxidase and 2-dianisidine placed in the reference beam of the spectrophotometer. The method was standardised using Sigma grade-VI horse radish peroxidase, amounts of 1-2 nanograms of enzyme were found to give reproducible rates of reaction.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEIN FRACTIONS FROM LYMPH AND PLASMA SAMPLES

The apparatus used was the E-C vertical gel electrophoresis system (E-C Apparatus Co. U.S.A.) fitted with water cooled plates. Electrophoretic separations were carried out using a polyacrylamide
gel made from 95% acrylamide and 5% bis-acrylamide (Cyanogum 41) in Tris-EDTA-Boric acid buffer pH 8.4.

A 3mm thick gel slab (17cm x 12cm) was cast between water cooled perspex plates using ammonium persulphate as a chemical catalyst. The formed gel slab was electrophoresed for 30 minutes at 300 volts to remove unreacted persulphate and monomers before the samples were applied to the gel.

150-300µg of protein in 40% sucrose, or 5-20µl volumes of lymph or serum were pipetted under the buffer into the preformed sample slots in the gel slab. Bromophenol Blue was often used to monitor the progress of electrophoresis, this dye was also bound to albumin. Protein separation was carried out at 300v., giving a current of 80mA, for 2-3 hours by which time the free Bromophenol Blue had migrated off the gel slab.

After electrophoresis, the slab was removed and stained with a solution of 0.2% amido black for 20-30 minutes at room temperature. Excess dye was removed by washing with a solution of methanol:water:acetic acid (5:5:1).

PREPARATION OF GLYCOPROTEIN FRACTIONS FROM SHEEP BLOOD AND LYMPH

The method followed was based on that originally described by Steinbuch and Quentin (1961).

One litre of sheep blood plasma was diluted with DDW to a volume of 2½ litres and the pH adjusted to pH 5.0 with glacial acetic acid. The resultant precipitate was removed by centrifugation and the supernatant applied to the head of a 25g (dry weight) Cellex-D column that had previously been equilibrated with 0.03M acetate buffer pH 5.0.
When all the supernatant had passed through the column, acetate buffers (pH 5.0) of increasing molarity were used to elute components bound to the cellulose anion exchanger. The column was finally washed with 1M NaCl.

The point at which no further protein was eluted from the column by a particular buffer was determined by adding 10% TCA to a portion of the eluate or by examining the eluate at 280nm in a spectrophotometer (Hitachi).

The eluted fractions were annotated thus:

<table>
<thead>
<tr>
<th>Eluting buffer pH 5.0</th>
<th>Eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03M acetate</td>
<td>non-bound fraction</td>
</tr>
<tr>
<td>0.1M acetate</td>
<td>Fraction A</td>
</tr>
<tr>
<td>0.2M acetate</td>
<td>Fraction B</td>
</tr>
<tr>
<td>0.5M acetate</td>
<td>Fraction C</td>
</tr>
<tr>
<td>1M NaCl</td>
<td>Fraction D</td>
</tr>
</tbody>
</table>

Eluates were adjusted to pH 7.4 with 0.1N NaOH and dialysed against saline before being concentrated by pressure dialysis. The concentrated eluates were freeze dried.

Lymph plasma samples were processed in an identical manner with adjustments being made to correct for the smaller volumes used.

**HISTOLOGY OF LYMPH NODES**

Conventional 6µ sections were cut from lymph nodes that had been fixed in formol saline and embedded in paraffin blocks. The sections were stained with hematoxylin-eosin or by a modified Dominici stain.
Sections of lymph nodes were stained with a modified Dominici stain (Litt, 1963; Litt, 1967). Thin paraffin sections of the node were stained for 5 minutes in a mixture of 0.5% Orange-G and 0.5% Eosin-Y after which they were rinsed in 0.1M phosphate buffer pH 6.2, and stained for 5-30 seconds in 0.3% Toluidine Blue 0. All stains were made up with 0.1M phosphate buffer pH 6.2.
CHAPTER III

THE NORMAL IMMUNE RESPONSE TO CHICKEN RED BLOOD CELLS
The immunologically responsive lymphoid organs represented by the lymph nodes, spleen, tonsils, Peyers patches and appendix all possess structures such as lymphoid follicles and germinal centres (Keuning, 1972) even though there are differences in their gross anatomy and the routes by which antigens or foreign materials enter these organs. There is a continual passage of lymphoid cells from the blood through the lymphoid organs and into the lymphatic system, thus antigen localising in these organs may be brought into contact with immunocompetent lymphocytes, and an immune response initiated at the site at which the antigen is localised.

The majority of investigators have studied immune responses in the spleen because this organ is readily stimulated by antigens injected intravenously. There are a number of disadvantages in using the spleen to study immune responses: Antigen injected intravenously to induce an immune response in the spleen may stimulate other lymphoid organs and as cell proliferation cannot be measured directly, splenomegaly and the number of antibody-secreting cells in the spleen have to be used as the indices of the cellular response to the antigen. In addition, systemic antibody titres may be influenced by the production of antibody in other sites of the body.

This chapter deals with the cellular and humoral changes in the efferent lymph which follow stimulation of the popliteal node with chicken red blood cells. The idea of these experiments was to establish as precisely as possible the kinetics of the normal primary and
secondary immune response to this antigen so as to enable further experiments to be done to investigate regulatory mechanisms which might be likely to operate in the control of the immune response to this antigen.

RESULTS

The Immediate Effect of Chicken Red Blood Cells on the Cellular Content of Efferent Lymph Draining the Regional Lymph Node

Haemoglobin was observed in the efferent popliteal lymph following the subcutaneous injection into the lower hind leg of $2 \times 10^9$ chicken red blood cells. Occasionally it appeared within seconds of injecting the red cells but in most cases there was usually an interval of 30 minutes between when the cells were injected and the haemoglobin appeared in the lymph. Whilst entire chicken red blood cells were observed in the afferent lymph draining the injection site, none appeared in the efferent lymph although soluble antigens such as horse radish peroxidase could be detected in the efferent lymph for some considerable time after its injection. A histological study of the popliteal lymph node 20 minutes after the injection of chicken red blood cells revealed entire chicken red blood cells localised in the sub-capsular sinus, cortex and medullary areas of the lymph node (Figures III-1 and III-2). A proportion of those red cells localised in the medullary region had been phagocytosed by macrophages.

The output of lymphocytes in the efferent lymph in the first 30 minutes after the injection of antigen, rose to almost twice the prestimulation level (Figure III-3). This was immediately followed by an acute but transient fall in the cell output. The cell output then
FIGURE III - 1

Chicken red blood cells present in the popliteal lymph node 20 minutes after subcutaneous injection into the lower leg.

A. Chicken red blood cells localised in the subcapsular sinus, cortex and medullary regions.

Stain Dominici. Magnification X60.

B. Chicken red blood cells localised in the cortex.

Stain Dominici. Magnification X700.
FIGURE III - 2

Chicken red blood cells in an afferent lymphatic vessel. Chicken red blood cells were injected subcutaneously into the lower leg 20 minutes before the popliteal lymph node was excised.

Stain Dominici. Magnification X1,000.
The immediate and subsequent effects of antigen administration upon the cell output and lymph flow rate from the regional popliteal lymph node. Upper figure: $1 \times 10^9$ chicken red blood cells administered. Lower figure: $2 \times 10^9$ chicken red blood cells administered.
rose gradually and had returned to prestimulation levels within about 12 hours. The changes in the rate of lymph flow could not be correlated directly with the changes in the cell output. Throughout this time the proportion of small, medium and large lymphocytes in the lymph did not change.

The cell output in the efferent lymph continued to rise during the next 80 hours and there was an increase in the numbers of large blast cells appearing in the lymph. Further experiments showed that these events also occurred after the injection of allogeneic lymphocytes, xenogeneic lymphocytes and antibody coated and free xenogeneic erythrocytes; they did not occur after the injection of autologous lymphocytes or saline. It would appear that these initial events are induced by antigenic or foreign materials although there did not appear to be any relationship between the dose of antigen, its immunogenicity and the extent of these changes.

The Primary Immune Response to $2 \times 10^9$ Chicken Red Blood Cells

1. **Cellular response** - Within the population of cells appearing in the efferent lymph there appeared increasing numbers of blast cells (Figure III-4) and cells undergoing mitosis. Blast cells first appeared in the efferent lymph some 20-30 hours after the antigen was injected and their numbers increased in a logarithmic fashion for the next 50-60 hours; after this time their numbers declined and reached prestimulation levels some 200 hours after the injection of antigen. Very few plasma cells appeared in efferent lymph at any time during the primary response.

At the peak of the cellular response in the efferent lymph, blast cells represented up to 25% of the total cells although there was considerable variation in the total cell outputs of immune responses observed in different sheep. Although the total output of cells in the
Figure III - 4

Normal primary immune response to $2 \times 10^9$ chicken red blood cells.
efferent lymph differed from one sheep to another, the output of blast cells was fairly constant.

The extent of the histological changes induced by antigen in the responding lymph node can be seen in Figure III-5. The responding lymph node was removed 240 hours after giving the antigen and shows clear differentiation between the cortex and medulla and extensive formation of germinal centres in the cortex.

2. **Plaque-forming cell response** - Plaque-forming cells first appeared in the efferent lymph around 60 hours after the antigen was injected; maximum numbers of plaque-forming cells coincided with the maximum numbers of total cells and of blast cells. The rapid increase in the numbers of plaque-forming cells to a maximum at around 80 hours was followed by a gradual decline so that by 230 hours none could be detected in the efferent lymph.

The size of plaques formed by antibody-secreting cells can be measured by counting the number of target cells lysed, provided the effector and target cells exist in a tightly packed and even monolayer. This measure of plaque-forming cell activity gives an indication of the secretion and perhaps the rate of formation of IgM antibody whilst not accounting for antibody affinity. The number of target cells lysed by the plaque-forming cells was counted throughout a primary immune response to chicken red blood cells. The assay of plaque size involved the counting of nuclei in lysed chicken red blood cell 'ghosts'. Figure III-6 shows the results of such an experiment. The number of lysed target cells in the average size plaque was estimated to be 34. The plaque-forming cells which appeared early in the response gave rise to smaller than average plaques whilst 50% of those expressed at the peak of the immune response were larger than average. During the decline phase of the
Gross structure of the popliteal lymph node.

A. Popliteal lymph node from a normal healthy ewe that had not been intentionally exposed to antigen.

Stain Hematoxylin and eosin. Magnification X12.

B. The contralateral lymph node of the same ewe 240 hours after the subcutaneous injection of $2 \times 10^9$ chicken red blood cells into the lower leg.

Stain Hematoxylin and eosin. Magnification X12.
The expression of plaque-forming cells during a primary immune response to $2 \times 10^9$ chicken red blood cells. Size and cellular distribution of plaque-forming cells with respect to time after antigen administration.
response there was a progressive increase in the number of small plaques and at 170 hours after the injection of antigen all plaques were smaller than average.

The cell type responsible for plaque formation in the initial stages of the response was the blast cell, although an increasing number of large, medium and small lymphocytes gave rise to plaques as the immune response progressed (Figure III-6). During the course of these observations it was noted that many of the blast cells secreting antibody were highly mobile and actively extended and retracted pseudopods (Figure III-7); a few of the plaque-forming cells were observed in mitosis. Figures III-7 to III-9 demonstrate that a variety of cell types give rise to plaque formation and that there is some variation in the size of plaque formed.

Whilst the blast cell was the major cell type secreting antibody not all blast cells secreted detectable specific antibody. These cells may have been secreting specific IgG antibody or a non-specific immunoglobulin that was not detected by the assay. Some dead cells were also found to produce plaques but they were few in number and it could not be ascertained if they had died before or after producing a plaque.

Popliteal lymph nodes that were removed 120 hours after a primary injection of $2 \times 10^9$ chicken red blood cells were teased apart in Eagle's medium. Cells that were isolated by passing the lymph node suspension through glass wool columns were examined in plaque assays and it was found that the cells which produced plaques were mostly plasma cells and large blast cells.

The mean total number of plaque-forming cells that appeared in the lymph during primary immune responses to $2 \times 10^9$ chicken red blood cells in four sheep was $1.97 \times 10^7 \pm 1.17 \times 10^6$. 
FIGURE III - 7

The morphology of antibody-secreting cells (ASC/PFC) formed during a primary immune response to $2 \times 10^9$ chicken red blood cells.

A and B. Large blast lymphocytes that were observed to actively extend and retract pseudopods. These cells secreted antibody which lysed chicken red blood cells. Lysed red cell 'ghosts' can be seen and the lytic area is well defined around the centrally situated lymphocyte.

Nomarski interference optics. Magnification X600.
FIGURE III - 8

The morphology of antibody-secreting cells (ASC/PFC) formed during a primary immune response to \(2 \times 10^9\) chicken red blood cells.

A. Large dividing lymphocyte that was observed to be actively engaged in plaque formation. This lymphocyte subsequently completed its division.

B. Plaque formed by a dead lymphoid cell which had lysed resulting in the release of stored antibody.

Nomarski interference optics. Magnification X600.
FIGURE III - 9

The morphology of antibody-secreting cells (ASC/PFC) formed during a primary immune response to $2 \times 10^9$ chicken red blood cells.

A. A large blast lymphocyte which has produced a small plaque.

B. A small blast lymphocyte which has produced a large plaque.

Nomarski interference optics. Magnification X600.
3. Antibody response - IgM haemagglutinating antibodies first appeared in the efferent lymph around 45 hours after the injection of antigen, slightly before the first appearance of plaque-forming cells in the efferent lymph (Figure III-4). The output of IgM antibody in the efferent lymph continued to rise to reach a maximum by 80 hours, a time which coincided with the peak of the cell output and the plaque-forming cell output in the efferent lymph. The output of IgM antibody then fell so that none could be detected in the efferent lymph by 160 hours although plaque-forming cells were still present albeit in small numbers. IgG haemagglutinating antibodies were not detected in the efferent lymph until 120 hours after the injection of antigen (Figure III-4). The output of IgG haemagglutinating antibody in the efferent lymph was low throughout the immune response and could not be detected after 230 hours.

The Effect of Antigen Dose on the Primary Response

Chicken red blood cells were injected into different sheep over the following range of doses: $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $2 \times 10^9$ and $4 \times 10^9$ red cells. Immune responses elicited by doses of less than $10^9$ chicken red blood cells were feeble in terms of total and blast cell output, plaque-forming cell output and antibody output in the efferent lymph draining the regional lymph node; the primary responses to $2 \times 10^9$ chicken red blood cells were not significantly different from responses elicited by $4 \times 10^9$ red cells. Figure III-10 shows the total numbers of plaque-forming cells produced in the response to each antigen dose.

With increasing dose of antigen, the ratio of the number of plaque-forming cells produced in response to a given number of chicken red blood cells decreased, until a dose of $1 \times 10^9$ red cells was exceeded (Figure III-10). This would imply that although there is an increase in the overall magnitude of the immune response with increasing antigen dose, the efficiency of the immune response was less at higher antigen doses.
The dependence of plaque-forming cell production upon antigen dose. Plaque-forming cells are expressed as:

I. Total plaque-forming cells produced in response to a given antigen dose.

II. Plaque-forming cells produced per unit chicken red blood cell.
The output of plaque-forming cells in the efferent lymph increased in a logarithmic manner and the rate of increase was found to be dependent upon the antigen dose (Figure III-11).

The Secondary Immune Response to $2 \times 10^9$ Chicken Red Blood Cells

Chicken red blood cells ($2 \times 10^9$) were injected 8 weeks after a primary challenge with the same dose of antigen. The injection of antigen at any time before 8 weeks resulted in a feeble secondary immune response. The total cell output in the efferent lymph was variable in the secondary immune response but the blast cell response was vigorous and well defined. The total output of plaque-forming cells was higher than in the primary immune response (Figure III-12) and the mean output of two experiments was $3 \times 10^7 \pm 7.99 \times 10^5$ plaque-forming cells. Blast cells and plaque-forming cells appeared in the lymph at an earlier time than in the primary immune response and this could be correlated with an early appearance of IgM antibody. Both IgM and IgG haemagglutinating antibodies were detected in the lymph at an earlier time than in the primary immune response. IgM haemagglutinating antibodies were first detected in the lymph 35 hours after the injection of antigen. The maximum output of IgM antibody occurred around 70 hours after the injection of antigen whereafter the output declined and none could be detected after 150 hours. IgG haemagglutinating antibodies were first detected in the lymph around 70 hours after antigen injection and their continued appearance gave rise to a very high output in the lymph; antibodies were still present in the lymph long after the cellular response had finished.
The effect of antigen dose upon the kinetics of plaque-forming cell output.
Secondary immune response to $2 \times 10^9$ chicken red blood cells. The challenging dose of chicken red blood cells was injected 8 weeks after a primary dose of $2 \times 10^9$ chicken red blood cells.
DISCUSSION

The effectiveness of an immune response against pathogenic bacteria is dependent upon the rate of proliferation and differentiation of immunocompetent cells in relation to the rate of bacterial proliferation, thus a high degree of infection can occur if the immune response is too small or occurs too late (Fahey, Buell and Sox, 1971). As the number of cells that are available in the body to recognise and react against an antigenic determinant is low, some mechanism involving the proliferation of reactive cells or the induction of other initially unresponsive cells would enable a more effective response to be initiated. Theoretically, organisms with a diverse antigenic character would stimulate greater numbers of reactive cells and thus lead to the more rapid elimination of the organism.

The lymph node can be visualised as a reaction centre in which antigen becomes localised. This causes lymphocytes from the recirculating lymphocyte population to be recruited into the node and thus an immune response is induced where the antigen is localised. Due to the low numbers of specifically reactive lymphocytes which exist for any given antigenic determinant and if there is a requirement for interactions between cells to occur in order to regulate the immune response, the induction of an immune response in the vascular compartment might be an inefficient and uncontrolled affair. The lymph node, however, provides a structured microenvironment in which immune reactivity can be expressed and controlled. Antigens localised in the tissues may induce the formation of a granuloma and when this occurs many of the cellular relationships that are present in a lymph node develop in the granuloma.
Antigen has been shown to become localised in the lymph node at three different sites (Nossal, Abbot and Mitchell, 1968). Most of the antigen is phagocytosed and catabolised by macrophages, particularly those lining the sinuses, whilst a small portion of it is trapped without being phagocytosed, in the outer cortical regions on the surface of the dendritic reticular cells and in the centre of lymphoid follicles. The extent to which antigen localises within the lymphoid follicles has been shown to be increased by the presence of specific antibody (Nossal, Ada, Austin and Fye, 1965; Humphrey and Frank, 1967; Lang and Ada, 1967).

Significance of the Immediate Effects of Antigen

It was shown that antigen rapidly became localised in the regional lymph node and induced an immediate but short lived increase in the number of lymphocytes appearing in the efferent lymph draining the node. These events were followed by an acute but transient fall in the number of lymphocytes prior to a recruitment phase during which the cell output from the node increased. Similar events have been investigated and described by Hall and Morris (1963; 1965a; 1965b). These changes in the cell output in the lymph were only induced by foreign and antigenic materials which would suggest that the lymph node represents the initial level at which self and non-self recognition occurs.

Burnet (1963) has suggested that immunologically competent cells may be made tolerant by high concentrations of antigen, thus some mechanism may operate to prevent this from happening during the inductive phase of the response. It has been suggested that the acute fall in the lymphocyte population of efferent lymph may be due to a reduction in the number of cells recirculating from the blood. If this occurred it would prevent competent cells from encountering high concentrations of antigen present early on in the node which may render these cells tolerant (Hall and Morris, 1965a).
The experiments reported in this chapter also suggest that antigenic and foreign but non-immunogenic materials reduce the numbers of lymphocytes recirculating from the blood to the lymph node and cause the expulsion of lymphocytes from the node. Antigens such as chicken red blood cells are rapidly phagocytosed within the lymph node and it is probable that lymphocyte recirculation is restored once the concentration of antigen is reduced to an immunogenic level. Although little is known about the rate of degradation of antigens in lymph nodes Trevella and Morris (1970) have shown that the half-life of flagellin in the popliteal lymph node of the sheep is about $5\frac{1}{2}$ hours. In this case, concentrations of antigen which may render immunocompetent cells tolerant would be rapidly reduced. The recirculating lymphocytes specific for the antigen that is present in the lymph node will have an opportunity to be activated by the antigen and induced to respond immunologically. Whether such events occur in other lymphoid organs such as the spleen, is not known.

**Cellular Events in the Primary Immune Response**

Subsequent to the phase of antigen induced recruitment, blast cells and plaque-forming cells appeared in the efferent lymph and their numbers increased to a maximum some 80 hours after the injection of antigen. The decline in the number of blast cells and of plaque-forming cells was more gradual than the rate at which they appeared and could be due either to the elimination of the antigen or to the reduction of some other inductive stimulus or to some suppressive influence. Factors which may control the cellular events of a humoral immune response are examined in subsequent chapters.
Humoral Events in the Primary Response

The level of IgM haemagglutinating antibody in the efferent lymph correlated closely with the number of plaque-forming cells in the lymph. IgM antibody was first detected some 45 hours after injecting the antigen whereas IgG haemagglutinating antibody was not detected until 120 hours. This could be due to the relative insensitivity of the haemagglutin assay for IgG antibody. Antibody feedback inhibition and enhancement effects have been reviewed by Uhr and Møller (1968) and are examined further in subsequent chapters.

The Plaque-Forming Cell Response

There is a higher proportion of plaque-forming cells in the efferent lymph draining a regional lymph node that is responding maximally to antigen than in the lymph node itself (Cunningham, Smith and Mercer, 1966; Hummeler, Harris, Harris and Farber, 1972). Thus assays of plaque-forming cells in the efferent lymph give an estimate of the events which are occurring within the lymph node.

The plaque-forming cells which appeared in the efferent lymph in response to a priming dose of $2 \times 10^9$ chicken red blood cells were comprised of large blast cells and small, medium and large lymphocytes. Similar observations have been reported for antibody-forming cells in responses to Salmonella lipopolysaccharide (Cunningham, Smith and Mercer, 1966) and horse radish peroxidase (Hay, Murphy, Morris and Bessis, 1972).

The size of plaques formed by individual plaque-forming cells varied considerably, an observation which has also been reported for the plaque-forming cells in the spleen (Meyer, Bourgarit and Bussard, 1972). This may be related to the maturity of the cell, to the time in the immune response at which the cell was isolated (Hay, Murphy, Morris and Bessis,
1972) or to the particular phase of the cell's life cycle (Fahey, Buell and Sox, 1971).

Few plaque-forming cells observed in the primary immune response appeared to be in mitosis although there was always a few that were. This would appear to contradict the findings of Fahey, Buell and Sox (1971) who suggested that antibody-forming cells only synthesised IgM or IgG antibody during the late G1 and early S phases of the mitotic cycle and that mature antibody-forming cells are arrested in a functional G1 condition.

The results presented in this chapter suggest that free-floating plaque-forming cells are capable of releasing antibody during the G and S phases of their cell cycle. Possibly they release preformed antibody during division and at this time antibody synthesis may cease. The results also suggest that division in plaque-forming cells is asynchronous within a given cell population.

Plaque-forming cells have been claimed to arise by cell transformation without division (Baker and Landy, 1967; Claflin and Smithies, 1967; Malaviya and Tannenberg, 1967; Tannenberg, 1967; Perkins, Sado and Makinodan, 1969; Sulitzeanu, Marbrook and Haskill, 1973), while other workers (Dutton and Mishell, 1967; Koros, Mazur and Mowery, 1968; Rowley, Fitch, Mosier, Solliday, Coppeloson and Brown, 1968; Nossal and Ada, 1971; Hüning, Schimpl and Wecker, 1974) have shown that they are derived from proliferating precursor cells stimulated by antigen. These opposite results complicate the study of plaque-forming cell kinetics.

The results presented in this chapter suggest that the doubling time of plaque-forming cells decreases as the dose of antigen is increased and this finding supports that of Campbell and Kind (1969). The significance of this is not known and the effect might not necessarily be
reflected in the antibody-forming cell population for it may be related
to a facilitation in the secretion of antibody by cells that were
synthesising antibody but not releasing it. The expression of antibody-
forming cells as plaque-forming cells is examined in a subsequent chapter.

The Control of the Immune Response

The experiments reported and discussed in this chapter suggest
that there are several levels at which the immune response may be sub-
jected to controlling mechanisms which would be amenable to study:

1. Lymph node control, immediate effects of antigen - An immediate
reduction of the recirculating lymphocyte population and the purging of
lymphocytes from the lymph node seems to occur when an antigen impinges
on a lymph node. This may eliminate tolerogenic levels of antigen by
allowing phagocytosis to proceed in the absence of the immunocompetent
lymphocytes. Lymphocyte recirculation through the node could then be
re-established subsequently thus enabling the immunogenic concentrations
of antigen localised within the lymph node to select out and specifically
activate those lymphocytes traversing the node that are competent for the
antigen.

2. Suppression and expression of the proliferation of immunocompetent
cells - The differentiation of these cells into antibody-forming and
secreting cells may represent levels at which modulation of the immune
response can occur. Modulation may be achieved by direct interaction
between cells or by the elaboration of humoral mediators; in both cases
a microenvironment such as that offered by the lymph node would enable
such control mechanisms to be efficiently expressed.
CHAPTER IV

ANTIBODY MEDIATED SUPPRESSION OF

THE IMMUNE RESPONSE

...
INTRODUCTION

It is now well established that specific antibody injected into animals can either suppress or enhance an immune response to antigen (Uhr and Möller, 1968). The suppressive action of specific antibody diminishes as the interval between antigen and antibody administration is increased (Wigzell, 1966).

The mechanism by which specific antibody exerts its suppressive action is largely unknown. One idea is that specific antibody reduces the number of immunocompetent cells (Rowley and Fitch, 1964) or B lymphocytes (Feldmann and Diener, 1970; Sinclair and Chan, 1971) capable of responding to the antigen. Other investigators (Ryder and Schwartz, 1969; Pryjma, Ptak, Szybiński and Sarnowicz, 1972; Abrahams, Phillips and Miller, 1973) have suggested that the suppressive effect is exerted at the level of the macrophage. Another theory of antibody mediated suppression suggests that antibody competes with immunocompetent cells for antigen and the effect is one of antigen depletion rather than cellular depletion or inactivation (Uhr and Baumann, 1961a, 1961b; Möller and Wigzell, 1965).

Previous investigations of antibody mediated effects have, in the main, been concerned with alterations in systemic or in vitro immunity; the investigations presented in this chapter involve the injection of antigen–antibody complexes into the region drained by the popliteal lymph node after cannulating the efferent lymph duct. In these experiments the cellular and humoral events which follow in the node have been monitored in the lymph in the absence of any systemic reactions. The aim of the experiments reported in this chapter was to examine the immunogenicity of chicken red blood cells subsequent to their exposure to an excess of
specific IgM, IgG, or IgH plus IgG antibody in order to determine the mechanism through which specific antibody affects the immune response.

**RESULTS**

Antigen-antibody complexes were prepared in conditions of antibody excess by incubating $2 \times 10^9$ chicken red blood cells in heat inactivated lymph containing specific anti-chicken red blood cell IgM, IgG, or IgM plus IgG antibody. The antibody used to prepare the complexes was isolated from the efferent popliteal lymph issuing from a popliteal lymph node that had been primed with chicken red blood cells.

Washed antigen-antibody complexes containing $2 \times 10^9$ chicken red blood cells were injected subcutaneously into the lateral aspect of the lower hind limb into the area drained by the popliteal lymph node. The efferent lymph duct of the popliteal lymph node had been cannulated at least two days before the antigen-antibody complexes were injected. The immune response elicited by the antigen-antibody complexes was followed by measuring the cellular changes, the output of plaque-forming cells and the antibody output in the efferent lymph draining the popliteal lymph node.

1. **Primary Immune Responses**

   The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM antibody - The overall immune response was severely depressed and there was little change in the output of total cells or in the numbers of blast cells appearing in the efferent lymph (figure IV-1). The mean total number of plaque-forming cells that appeared in the lymph during the course of these immune responses in three individual sheep was $1.54 \times 10^6 \pm 1.45 \times 10^6$, less than 10% of the number of plaque-forming cells that were produced in the normal primary immune response to $2 \times 10^9$
Primary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgM antibody.
untreated chicken red blood cells (Table IV-1). Both IgM and IgG haemagglutinating antibody outputs in the lymph were depressed below normal levels. IgM haemagglutinating antibodies were first detected in the lymph around 95 hours after the injection of antigen-antibody complexes compared with 45 hours when chicken red cells alone were injected. IgG haemagglutinating antibodies were first detected in the lymph around 120 hours after the injection of the antigen-antibody complexes, or about the same time as when free chicken red cells were injected.

The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgG antibody - The total cell output in the lymph was reduced (Figure IV-2) although not to the same extent as when IgM-chicken red cell complexes were injected. The number of blast cells that appeared in the lymph was low and comparable to the number of blast cells that appeared in the responses to IgM-chicken red cell complexes. The mean total number of plaque-forming cells that appeared in the lymph during the course of an immune response in three individual sheep amounted to $2.62 \times 10^6 \pm 1.06 \times 10^6$, approximately 13% of the number of plaque-forming cells that were produced in normal immune responses to free chicken red cells (Table IV-1). Both IgM and IgG haemagglutinating antibody outputs in the lymph were depressed below normal levels. These antibodies were first detected in the lymph at around the same time as observed in normal immune responses to free chicken red cells.

The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM plus IgG antibody - The total cell output in the lymph was low (Figure IV-3) and similar to that observed in responses to IgG-chicken red cell complexes. The output of blast cells in the lymph was comparable to that observed in the normal immune response to free chicken red cells.
Primary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgG antibody.
Primary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgM and IgG antibody.
 TABLE IV-1

<table>
<thead>
<tr>
<th>Antigen-antibody complex</th>
<th>Total CRBC specific plaque-forming cells per response</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10^9 CRBC + specific IgM</td>
<td>1.48 x 10^5, 3.50 x 10^4, 4.45 x 10^6</td>
<td>1.54 x 10^6 ± 1.45 x 10^6</td>
</tr>
<tr>
<td>2 x 10^9 CRBC + specific IgG</td>
<td>9.50 x 10^5, 2.06 x 10^6, 6.73 x 10^7</td>
<td>2.62 x 10^6 ± 2.06 x 10^6</td>
</tr>
<tr>
<td>2 x 10^9 CRBC + specific IgM + IgG</td>
<td>3.45 x 10^7, 3.30 x 10^7, 4.42 x 10^7</td>
<td>3.72 x 10^7 ± 3.5 x 10^6</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 x 10^9 CRBC + non-specific IgM</td>
<td>2.04 x 10^7</td>
<td></td>
</tr>
<tr>
<td>2 x 10^9 CRBC + non-specific IgG</td>
<td>2.24 x 10^7</td>
<td></td>
</tr>
<tr>
<td>2 x 10^9 CRBC + non-specific IgM + IgG</td>
<td>1.30 x 10^7</td>
<td></td>
</tr>
<tr>
<td>2 x 10^9 CRBC</td>
<td>2.22 x 10^7, 1.38 x 10^7, 2.36 x 10^7, 1.94 x 10^7</td>
<td>1.97 x 10^7 ± 2.17 x 10^6</td>
</tr>
</tbody>
</table>

The total output of plaque-forming cells in the lymph following the injection of antigen-antibody complexes, antigen/non-specific immunoglobulin, and free antigen in primary immune responses to chicken red blood cells.

CRBC = chicken red blood cells
The mean total number of plaque-forming cells that appeared in the lymph during the course of an immune response in three individual sheep was 3.72 x 10^7 ± 3.5 x 10^6, about twice the total number of plaque-forming cells which were produced in immune responses to free chicken red cells (Table IV-1). The output of both IgM and IgG haemagglutinating antibody in the lymph was less than normal. IgM haemagglutinating antibodies were first detected around 75 hours after the injection of antigen-antibody complexes. IgG haemagglutinating antibodies were first detected around 110 hours after the injection of antigen-antibody complexes, at around the same time as they appeared in responses to free chicken red cells.

The response to 2 x 10^9 chicken red blood cells injected with homologous non-specific immunoglobulin - Control experiments in which chicken red blood cells were injected with non-specific IgM, or IgG or IgM plus IgG, demonstrated that non-specific immunoglobulins had no effect upon the primary immune response to chicken red blood cells. Cell outputs, antibody outputs and total plaque-forming cell outputs (Table IV-1) were essentially the same as in primary immune responses to chicken red blood cells injected alone.

2. Secondary Immune Responses

The response to 2 x 10^9 chicken red blood cells complexed with specific IgM antibody - Popliteal lymph nodes were primed with 2 x 10^9 chicken red blood cells eight weeks prior to challenge with antigen complexed with IgM antibody. The secondary immune responses that followed the injection of antigen-antibody complexes were significantly depressed and one experiment is shown in Figure IV-4. Whilst the total cell output in the lymph was comparable to that observed in the normal secondary response to chicken red cells, the output of blast cells was low.
Secondary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgM antibody.

The popliteal lymph node was primed with $2 \times 10^9$ chicken red blood cells 8 weeks prior to injection of the antigen-antibody complex.
The mean total number of plaque-forming cells that appeared in the lymph during the course of immune responses in three individual sheep was $2.04 \times 10^6 \pm 8.06 \times 10^5$, about $5\%$ of the number of plaque-forming cells that were produced in normal secondary responses to chicken red cells (Table IV-2). IgM haemagglutinating antibodies first appeared in the lymph at around 60 hours after the injection of the complexes, whereas IgG haemagglutinating antibodies were first detected at around the same time as they first appeared in normal secondary responses to chicken red cells. The output of both IgM and IgG haemagglutinating antibody in the lymph was significantly reduced.

The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgG antibody - Popliteal lymph nodes were primed with $2 \times 10^9$ chicken red blood cells eight weeks prior to challenge with antigen complexed with IgG antibody.

The total cell output in the lymph was not significantly different from that in a normal secondary response whereas the blast cell output was reduced (Figure IV-5). The mean total number of plaque-forming cells that appeared in the lymph during the course of an immune response in three individual sheep was $2.63 \times 10^6 \pm 1.39 \times 10^6$, less than $10\%$ of the number of plaque-forming cells that were produced in normal secondary responses to chicken red cells (Table IV-2). IgM haemagglutinating antibodies were first detected late in the immune response whereas IgG haemagglutinating antibodies were detected at around the same time as they appeared in the lymph during the course of a normal secondary response to chicken red cells. The output of both IgM and IgG haemagglutinating antibody throughout the response was low.
Secondary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgG antibody.

The popliteal lymph node was primed with $2 \times 10^9$ chicken red blood cells 8 weeks prior to injection of the antigen-antibody complex.
Secondary immune response to $2 \times 10^9$ chicken red blood cells complexed with specific primary IgM and IgG antibody.

The popliteal lymph node was primed with $2 \times 10^9$ chicken red blood cells 8 weeks prior to injection of the antigen-antibody complex.
<table>
<thead>
<tr>
<th>Antigen-antibody complex</th>
<th>Total CRBC specific plaque-forming cells per response</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^9$ CRBC + specific IgM</td>
<td>$1.50 \times 10^6$ $3.63 \times 10^6$ $1.00 \times 10^6$</td>
<td>$2.04 \times 10^6 \pm 8.06 \times 10^5$</td>
</tr>
<tr>
<td>$2 \times 10^9$ CRBC + specific IgG</td>
<td>$1.40 \times 10^6$ $5.40 \times 10^6$ $1.10 \times 10^6$</td>
<td>$2.63 \times 10^6 \pm 1.39 \times 10^6$</td>
</tr>
<tr>
<td>$2 \times 10^9$ CRBC + specific IgM + IgG</td>
<td>$3.17 \times 10^7$ $3.06 \times 10^7$</td>
<td>$3.11 \times 10^7 \pm 5.5 \times 10^5$</td>
</tr>
<tr>
<td>Control</td>
<td>$3.05 \times 10^7$ $3.21 \times 10^7$</td>
<td>$3.13 \times 10^7 \pm 7.99 \times 10^5$</td>
</tr>
</tbody>
</table>

The total output of plaque-forming cells in the lymph following the injection of antigen-antibody complexes or free antigen in secondary immune responses to chicken red blood cells.

CRBC = chicken red blood cells
The response to $2 \times 10^9$ chicken red blood cells complexed with specific IgM plus IgG antibody - Popliteal lymph nodes were primed with $2 \times 10^9$ chicken red blood cells eight weeks prior to challenge with antigen complexed with IgM plus IgG antibody.

The total cell output and the output of blast cells in the lymph were not significantly different from a normal secondary response to chicken red blood cells (Figure IV-6). The mean total number of plaque-forming cells that appeared in the lymph during the course of an immune response was $3.11 \times 10^7 \pm 5.5 \times 10^5$, a figure not significantly different from a normal secondary response to chicken red cells (Table IV-2). The output of IgM and IgG haemagglutinating antibody in the lymph was similar to the normal secondary response. The time at which these antibodies were first detected in the lymph was also similar to the normal secondary response.

**DISCUSSION**

The results of these experiments demonstrated that specific IgM or IgG antibody isolated during a primary immune response to chicken red blood cells exerted a similar suppressive influence upon both primary and secondary immune responses when complexed to the specific antigen. These findings contrast to those of Uhr and Baumann (1961b).

The features of all the suppressed responses were similar; outputs of blast cells were low as were the antibody outputs and the outputs of plaque-forming cells (Table IV-1 and IV-2). The initial rate of increase in the number of plaque-forming cells that appeared in the lymph was significantly lower than in immune responses to the free antigen. In direct contrast to the suppressive effects of specific antibody, it was found that mixtures of specific IgM and IgG antibody enhanced primary immune responses and had little effect upon secondary immune responses.
The Suppressive Effect of Specific Antibody

Specific IgM or IgG antibody had similar suppressive effects upon the immune response. Previous investigations (Finkelstein and Uhr, 1964) showed that 7S antibody was more effective than 19S antibody in suppressing immune responses; Pearlman (1966) on the other hand demonstrated that they were equivalent in their suppressive capacity. The discrepancy between these findings may be due to differences in antibody affinity. The antibodies used in this study were isolated early in the response from lymph draining a single lymph node primed with antigen; in these circumstances IgM and IgG antibodies would be expected to have the same order of affinity.

Sinclair and Chan (1971) have suggested that antigen-antibody complexes may exert their suppressive effects by inactivating antigen reactive cells by way of a signal transmitted through the Fe portion of the antibody component of the complex. This suggestion would not seem likely in view of the findings of Uhr and Baumann (1961b) which demonstrated that while immune complexes suppress the primary response, the animal is nevertheless primed and subsequently gives a vigorous secondary response when challenged even though no detectable antibody was produced in the primary response. The Fe portion of the antibody molecule does not appear to play a role in antibody-mediated suppression according to the experiments of Chang, Schneck, Brody, Deutsch and Siskind (1969) and Uhr (1968). The results of experiments reported in Chapter VI will provide evidence against antigen-antibody complexes acting to suppress immune responses by way of cell inactivation.

While passively administered antibody may prevent antibody formation by immunocompetent B lymphocytes it may not prevent T lymphocytes from becoming activated (Gershon and Kondo, 1971b; Möller and
Greaves, 1971). Doses of free antigen which are insufficient to stimulate antibody formation are nevertheless capable of priming an animal (Salvin and Smith, 1960); in these circumstances T lymphocytes and not B lymphocytes may be activated.

The ability of specific antibody to suppress immune responses is directly related to its affinity for the antigen, thus high affinity antibody has a greater suppressive effect than low affinity antibody and acts at lower concentrations (Eisen and Siskind, 1964). In the normal immune response circulating antibody probably serves to bring about a progressive increase in the affinity of the antibody produced and at the same time limits the immune response by reducing the availability of free antigen to immunocompetent cells (Walker and Siskind, 1968; Bullock and Rittenberg, 1970).

The removal of specific antibody from the serum of immunised rabbits by means of immunoadsorbents (Graf and Uhr, 1969), or exchange transfusion (Bystryn, Graf and Uhr 1970), results in an immediate and specific rise in circulating antibody levels when the adsorbed or exchanged serum is transfused into the rabbit. This has been explained on the basis that immune complexes dissociate to give rise to more free antigen which in turn stimulates immunocompetent lymphocytes to synthesise and release more antibody.

The Enhancing Effect of Specific Antibody

Antigen-antibody complexes prepared with excess specific IgM and IgG antibody were shown to enhance primary immune responses and to have the same effect as free antigen on secondary immune responses. Similar enhancing effects of antigen-antibody complexes have been observed by Osato (1972) who showed that soluble crystalline bacterial α-amylase
antigen-antibody complexes produce a detectable primary immune response in mice whilst the free antigen does not.

Several investigators have demonstrated the enhancing effect of low concentrations of specific IgM or IgG antibody upon primary and secondary responses to antigen (Pearlman, 1967; Henry and Jerne, 1968; Dennert, 1971) and suggested the effect is related to alterations in the efficiency with which antigen is taken up by lymphoid tissues (Dennert, 1971; Schrader, 1973).

In the experiments reported in this chapter, both antigen and antigen-antibody complexes were administered to a single lymph node and it is known that practically all the antigen is localised at this site (Chapter III). Increased uptake of antigen by lymphoid tissues as reported by other investigators would not play a role in the experiments reported in this chapter.

The results presented in this chapter would suggest that antibody-mediated suppression of the immune response is likely to occur at the peripheral level and that there is a competition for antigen between specific antibody and specific immunocompetent cells. The mechanism through which the enhancing effect is exerted remains unknown.
CHAPTER V

ANTIGENIC COMPETITION

The phenomenon of antigenic competition was first observed by Michaelis (1902, 1905) and has been utilized in the elucidation of the immune response as an antigen of multiplicity. Aleksandrov and the American school of another antigen or antigen-antibody reaction (Chapman, 1961). The American school hypothesized that the reaction could be explained by the destruction of antigenic competition by one-way competition. The first general proposition is that the reaction of an immunologically specific effect. Antigen may compete for antibody-reactive sites (Kiermer, 1938; Allfrey, 1951, and Steffen, 1954). An antigen may cross-react with two important somatic reactions may be contributed to the antibody-mediated necrosis. The second general proposition is that the antigenic competition of non-specifically present antigens, then, the reaction is due to the nonspecific components and other antigens, involving the immune response itself and making linear, direct, or indirect reactions.

It is unlikely that any one of the general propositions that may explain the immunity of antigenic competition, as it has been described, has reached such a point where it is not one of the integral parts of the immune system. However, if an antibody-antigen reaction is represented with a linear model rather than with an antigen-antibody reaction (Kiermer and Chapman, 1957), the antibody and antigen (1951) have indicated that the affinity of antibody production is enhanced by antibody-stimulated mechanisms operating in antigenic competition.
**I N T R O D U C T I O N**

The phenomenon of antigenic competition was first observed by Michaelis (1902; 1904) and has been defined as the inhibition of the immune response to one antigen or antigenic determinant by the administration of another antigen or antigenic determinant (Taussig, 1972). The numerous hypotheses that have been advanced to explain the mechanism of antigenic competition fall into two main categories. The first general proposition is that the phenomenon is an immunologically specific effect. Antigen may compete for antigen-reactive cells (Schechter, 1968; Albright, Omer and Deitchman, 1970) or antigens may cross-react so that apparent competition may be due to tolerance or antibody-mediated suppression. The second general proposition explains the phenomenon as an immunologically non-specific effect, that is, antigens compete for some limiting factor such as space or nutrients (Adler, 1964), or for other non-specific components such as macrophages, involved in the immune response (Brody and Siskind, 1969; Schrader and Feldmann, 1973).

It is unlikely that competition for antigen-reactive cells could explain the results of antigenic competition as it has been demonstrated that the competition is most pronounced when there is a time interval between the administration of the two competing antigens (Radovich and Talmage, 1967; Möller and Sjöberg, 1970) and that the effects of antigenic competition are more pronounced in irradiated mice repopulated with large numbers rather than small numbers of spleen cells (Radovich and Talmage, 1967). As Brody and Siskind (1969) have shown that the affinity of antibody produced in animals undergoing antigenic competition is unaltered, this effectively rules out tolerance or antibody-mediated mechanisms operating in antigenic competition.
Möller and Sjöberg (1970) have shown that the number of antigen-reactive cells is not reduced in animals responding to two competing antigens and this finding argues against the concept that competition occurs between antigens for multipotent antigen-reactive cells. It has been suggested that antigenic competition is in fact not a competitive effect at all (Radovich and Talmage, 1967; Möller and Sjöberg, 1970; Pross, Novak and Eidinger, 1971) and that a more accurate term would be non-specific antigen induced suppression (Kerbel and Eidinger, 1971).

In direct contrast to the suppressive effects of one antigen upon another, it has been shown that the immune response to one antigen may have an enhancing effect on the immune response to another unrelated antigen administered subsequently (Rubin and Coons, 1971; Wu and Cinader, 1971). The suppressive and enhancing effects of antigen may therefore be interrelated. Gershon and Kondo (1971a) showed that antigenic competition is thymus dependent and competitive effects may be elicited with very small amounts of the unrelated antigen (Gershon and Kondo, 1971b). The current consensus of opinion appears to be that the ability of antigen to suppress or enhance the immune response to another antigen is controlled in some way by the thymus.

The aim of this chapter is to examine the effects of injecting a competing antigen at different times during the immune response to a related or unrelated antigen and to determine the modifications that occur in the immune response that follows in a single lymph node.
RESULTS

The antigens used in these experiments were Salmonella muenchen organisms, chicken red blood cells and rabbit red blood cells. These antigens were administered in the following doses: Salmonella muenchen 3.4 x 10^9 heat killed organisms in 1 ml saline, chicken red blood cells 1 x 10^9 cells in 1 ml PBS, and rabbit red blood cells 2 x 10^9 cells in 1 ml PBS.

Following the injection of Salmonella or rabbit red blood cells, 2 x 10^9 chicken red blood cells was injected at one of the following times during the response to the initiating antigen; 0 hours, 24 hours, 48 hours, 120 hours, 260 hours. Where 1 x 10^9 chicken red blood cells was used as the initiating antigenic dose, a further 1 x 10^9 chicken red blood cells was injected subsequently at one of these times. At least two days before injecting any antigen, the efferent lymphatic duct of the popliteal lymph node was cannulated and the animal allowed to recover from the surgical procedure. All antigens were injected subcutaneously in the outer aspect of the lower hind limb, the drainage area of the popliteal lymph node.

The immune response to the chicken red blood cells was monitored by measuring the output of lymphocytes and blast cells, the output of plaque-forming cells and the output of antibody in the popliteal efferent lymph. The modified immune responses resulting from these experimental procedures were compared with the normal immune response that follows a single injection of chicken red blood cells.

Where rabbit and chicken red blood cells were injected as competing antigens then the immune response to both antigens was measured and each was compared to the normal immune response to a single injection of the antigen.
1. The Primary Immune Response to Chicken and Rabbit Red Blood Cells

The immune response that followed a primary injection of $2 \times 10^9$ chicken red blood cells has been described in Chapter III; the cellular and humoral changes that occurred in the lymph are shown in Figure III-4. The mean total number of plaque-forming cells that appeared in the lymph in four individual experiments was $1.97 \times 10^7 \pm 2.17 \times 10^6$.

The cellular changes that occurred in the lymph following a primary injection of $2 \times 10^9$ rabbit red blood cells were much less pronounced than those that occurred after a similar injection of $2 \times 10^9$ chicken red blood cells (Figure V-1). Total and blast cell outputs in the lymph were low and the blast cell output did not increase until around 70 hours after injection of the red cells. Plaque-forming cells were first observed in the lymph around 50 hours, but their numbers in the lymph remained low. Over the course of the response a total of $1.3 \times 10^7$ plaque-forming cells appeared in the lymph (Table V-1). IgM and IgG haemagglutinating antibody outputs were low.

2. The Modification of the Immune Response to Chicken or Rabbit Red Blood Cells by Rabbit Red Blood Cells Injected Before or at the Same Time as the Chicken Red Blood Cells

(a) Chicken and rabbit red blood cells injected together - Total cell and blast cell outputs in the lymph were similar to those observed in the control response to chicken red cells alone and greater than those observed in the control response to rabbit red cells alone (Figure V-2). Plaque-forming cell outputs to both antigens were greater than those observed in the control responses and their output was maximal at the same time as when the antigens were injected alone. The total
Normal primary immune response to $2 \times 10^9$ rabbit red blood cells.
Primary immune response to $2 \times 10^9$ chicken and $2 \times 10^9$ rabbit red blood cells administered concurrently.
output of specific plaque-forming cells in the lymph was $3 \times 10^7$ and $1.8 \times 10^7$ for chicken and rabbit red blood cells respectively. These outputs were 50% higher than those observed in the control responses when each antigen was injected alone (Table V-1). The output of IgM haemagglutinating antibody that was specific for chicken red cells was similar to that observed in the control response to chicken red cells whilst the output of IgG haemagglutinating antibody was greater. The outputs of both IgM and IgG haemagglutinating antibodies that were specific for rabbit red cells was greater than that observed in the control response to rabbit red cells alone.

(b) Chicken red cells injected 24 hours after rabbit red cells -
Total cell and blast cell outputs were significantly reduced below normal (Figure V-3). Plaque-forming cell outputs in response to both antigens were reduced and a total of $9 \times 10^6$ plaque forming-cells that were specific for chicken red cells and $2 \times 10^6$ plaque-forming cells that were specific for rabbit red cells appeared in the lymph (Table V-1). The total output of specific plaque-forming cells was 45% of the control value for chicken red cells and 15% of the control value for rabbit red cells. The plaque-forming cell responses for both antigens occurred at the same time as in the control responses when the antigens were injected alone. The output of IgM and IgG haemagglutinating antibodies, specific for either chicken or rabbit red cells, was lower than in the control responses.

(c) Chicken red cells injected 48 hours after rabbit red cells -
The cellular response to both antigens was significantly enhanced when compared to control responses (Figure V-4). The blast cell output in the lymph was significantly higher than normal and reached a maximum of
Primary immune response to chicken and rabbit red blood cells.  
$2 \times 10^9$ rabbit red blood cells administered 24 hours prior to $2 \times 10^9$ chicken red blood cells.
Primary immune response to chicken and rabbit red blood cells. 2 x 10^9 rabbit red blood cells administered 48 hours prior to 2 x 10^9 chicken red blood cells.
6 x 10^7 cells per hour. The peak output of plaque-forming cells specific for chicken or rabbit red cells occurred at around the same time as in control responses to chicken red cells alone. The peak output of plaque-forming cells that were specific for rabbit red cells occurred later than in control responses to rabbit red cells. A total of 8.3 x 10^7 plaque-forming cells against chicken red cells and 5.7 x 10^7 plaque-forming cells against rabbit red cells appeared in the lymph (Table V-1). The total output of plaque-forming cells was increased four-fold above the control responses to either antigen injected alone. The output of IgM and IgG haemagglutinating antibodies specific for rabbit red cells was lower than in the control response. The output of IgM haemagglutinating antibody specific for chicken red cells was lower than in the control response; the output of IgG haemagglutinating antibody was not significantly different.

(d) Chicken red cells injected 260 hours after rabbit red cells

Total cell and blast cell outputs in the lymph were depressed and the immune response was short-lived. Plaque-forming cells that were specific for chicken red cells appeared at around the same time as in control responses but their numbers were less and the period during which they were present in the lymph was significantly reduced (Figure V-5). Very few plaque-forming cells appeared against rabbit red cells. The total output of plaque-forming cells against chicken red cells was 1.9 x 10^6, about 10% of the output in the control responses (Table V-1). The output of IgM and IgG haemagglutinating antibodies that were specific for either chicken or rabbit red cells was lower than in the control responses.

The results indicated that where suppressive or enhancing effects were exerted on the primary immune response to chicken red cells, similar effects were operative on the response to rabbit red cells.
Primary immune response to chicken and rabbit red blood cells
2 x 10^9 rabbit red blood cells administered 260 hours prior to 2 x 10^9 chicken red blood cells.
Figure V-6 shows the similar total plaque-forming cell responses to each of the antigens.

3. The Modification of the Immune Response to Chicken Red Blood Cells when Salmonella muenchen Heat Killed Organisms were Injected Before or at the Same Time as Chicken Red Blood Cells

Salmonella muenchen heat killed organisms were injected at the same time or 24, 48, 120 or 260 hours prior to giving chicken red cells. A comparison of the output of plaque-forming cells specific for chicken red cells in these responses revealed that whether enhancement or suppression occurred depended on the time that chicken red cells were given in relation to the Salmonella organisms. The time relationships between the injection of the antigens and the enhancing or suppressing effect on the plaque-forming cell response were identical to those found in the experiments with rabbit and chicken red cells (Table V-1).

Enhanced outputs of plaque-forming cells were obtained when Salmonella organisms were administered together with or 48 hours prior to giving the chicken red cells (Figure V-7). The output of plaque-forming cells in the lymph was suppressed when Salmonella organisms were injected 24, 120 or 260 hours prior to giving the chicken red cells. Other parameters used to measure the vigour of the immune response such as antibody output and the output of total cells and blast cells in the lymph, correlated well with the extent of enhancement or suppression of the plaque-forming cell response. The overall degree of enhancement or suppression was greater in these responses than in the rabbit red cell, chicken red cell experiments.
Total number of plaque-forming cells obtained in primary immune responses in which chicken red blood cells were administered at various times after rabbit red blood cells.
Total number of plaque-forming cells obtained in primary immune responses in which chicken red blood cells were administered after one of the following antigens:

I. $3.4 \times 10^9$ Salmonella muenchen heat killed organisms.
II. $1 \times 10^9$ chicken red blood cells.
III. $2 \times 10^9$ rabbit red blood cells.

Plaque-forming cells that were directed against chicken red blood cells are shown.
## TABLE V-1

<table>
<thead>
<tr>
<th>First antigen</th>
<th>Second antigen</th>
<th>Time of CRBC administration (hours)</th>
<th>PFC type assayed</th>
<th>Total PFC per response x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4x10^9 Salmonella</td>
<td>2x10^9 CRBC</td>
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<td>CRBC</td>
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<td>CRBC</td>
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<td>CRBC</td>
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<td>CRBC</td>
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</tr>
<tr>
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<td>RRBC</td>
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</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2x10^9 CRBC</td>
<td>RRBC</td>
<td>13</td>
<td></td>
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</tr>
</tbody>
</table>

Total plaque-forming cell outputs in antigen suppressed and antigen enhanced immune responses.

PFC = plaque-forming cells
CRBC = chicken red blood cells
RRBC = rabbit red blood cells
4. The Modification of the Immune Response to Chicken Red Blood Cells when a Similar Dose of Chicken Red Blood Cells was Injected at the Same Time or After the Initial Antigenic Challenge

Two injections of $1 \times 10^9$ chicken red cells were given simultaneously or 24, 39, 48, 120 or 260 hours apart. The output of plaque-forming cells in the lymph was found to be enhanced when the two doses of antigen were administered 39 or 48 hours apart, and suppressed when administered 24, 120 or 260 hours apart (Table V-1). The output of total cells and of blast cells, the antibody output and the output of plaque-forming cells changed together being either suppressed or enhanced. The relationship between the time of administration of the second dose of antigen and the subsequent enhancement or suppression of the immune response was identical to that found in the experiments with chicken red cells and rabbit red cells and in the experiments with chicken red cells and Salmonella muenchen organisms (Table V-1, Figure V-7).

Under normal circumstances the kinetics of the primary immune response to chicken red cells, rabbit red cells or Salmonella muenchen organisms in the sheep are similar and the peaks of the responses occur at around 80-90 hours after the injection of antigen. The results presented in this chapter suggested that the proliferative stimulus given to lymphoid cells by antigen can in some way influence the immune response to a subsequently administered related or unrelated antigen; in turn, the immune response to the initiating antigen itself is modified. The influence of an immune response to one antigen on the immune response to another antigen must be exerted non-specifically. The antigens used in this study did not give cross-reacting plaque-forming cells or cross-reacting antibody.
DISCUSSION

There are many conflicting reports in the literature on the consequences of administering two antigens together or sequentially. The experiments reported in this chapter would seem to be the first to examine the effects of sequential administration of dissimilar antigens in a single lymph node. The results suggest that the way in which the immune response to the second antigen is expressed depends on the immune status of the lymph node when the second antigen is administered; thus during the early proliferative phase of an immune response circumstances are conducive to the enhancement of an immune response to an identical or unrelated antigen; during the latter stages of an immune response, circumstances exist which tend to depress the immune response to an identical or unrelated antigen.

Enhanced Immune Responses

The enhancement phenomenon was related to the extent of the immune response elicited by the initiating antigen, thus Salmonella muenchen which produced a strong immune response by itself enhanced chicken red cell immune responses to a greater extent than the weaker antigen, rabbit red cells. When rabbit red cells or Salmonella organisms were injected 48 hours before giving chicken red cells then the rate of increase in the number of plaque-forming cells that appeared in the lymph was greatly enhanced. This suggested that more cells were being induced to make antibody rather than being rendered tolerant by the antigen. It has been suggested by Katz (1972) that the balance between an antigen's capacity to stimulate antibody formation or to induce tolerance is exerted through thymus derived lymphocytes. Tolerance induction appears to be induced specifically and more readily in T lymphocytes than in B lymphocytes (Katz, 1972).
T lymphocytes specifically activated by antigen appear to elaborate a non-specific factor that activates B lymphocytes (Katz, 1972; Watson, 1973). B lymphocytes have been said to require two signals, a specific antigenic signal and some non-specific signal derived from T lymphocytes before they are stimulated to proliferate and produce antibody. The non-specific signal elaborated by specifically activated T lymphocytes is claimed to increase the threshold level of B lymphocytes to antigen doses that would otherwise render them tolerant. If this is the case and specific T lymphocytes are limited in number (Campbell, 1972) then a high dose of antigen would induce some degree of tolerance in the small population of specific T lymphocytes; as a consequence B lymphocytes would receive only a specific (antigenic) signal which would render them tolerant. Lower doses of antigen would induce specific T lymphocytes to elaborate signal 2 thus B lymphocytes would be provided with both specific and non-specific signals. When immunogenic doses of two non-cross-reacting antigens are injected together then T lymphocytes will be increasingly activated and two populations of specific T lymphocytes will be induced to elaborate the non-specific signal 2. The immune response to both antigens will be enhanced as a result of the increased level of signal 2, that is, the tolerance threshold of the B lymphocytes to the specific signals from the two antigens will be increased and therefore more specific B lymphocytes will proliferate giving rise to increased populations of antibody-forming cells. When the second antigen is administered 48 hours after the first antigen, it acts in an environment in which production of the non-specific signal 2 has already been stimulated; thus greater cell proliferation will occur.

There is direct evidence that in cultures of mouse spleen cells depleted of T lymphocytes, a soluble product obtained from cultures of
antigen activated T lymphocytes can restore normal immune responsiveness in the depleted spleen cell population and enhance responsiveness to the same or a different antigen in normal spleen cell cultures (Rubin and Coons, 1971; Rubin and Coons, 1972; Gorczynski, Miller and Phillips, 1973a, 1973b; Waldmann, Munro and Hunter, 1973; Watson, 1973).

The increased total output of plaque-forming cells could not be correlated directly with the increases in the total cell and blast cell outputs that occurred in the enhanced immune responses. Evidence to be presented in Chapter VII will suggest that although more antibody-forming cells are produced in the enhanced responses, a further factor is required to induce these cells to secrete antibody and it is the production of this factor which enables a greater number of antibody-forming cells to secrete the antibody they form.

The significant enhancement of responses to chicken red cells which followed the concurrent or prior administration of Salmonella muenchen organisms could be explained by the ability of its LPS component to activate a specific population of T lymphocytes (Nakano, Uchiyama and Saito, 1973; Armerding and Katz, 1974). There is, however, some dispute that LPS can activate T lymphocytes (Andersson, Sjöberg and Möller, 1972; Sjöberg, Andersson and Möller, 1972; Louis, Chiller and Weigle, 1973; Watson, Trenkner and Cohn, 1973) and for the most part LPS is considered to be a B lymphocyte mitogen. The enhancing effect of Salmonella muenchen LPS may be due to its ability to stimulate B lymphocytes directly in a non-specific manner, that is it acts non-specifically as signal 2 (Watson, Trenkner and Cohn, 1973) and specifically as signal 1.
Suppressed Immune Responses

When two unrelated or identical antigens were administered 24 hours apart there was a significant suppression of the immune response to both antigens. Total cell and blast cell output was depressed and as a consequence of this, very few plaque-forming cells were elaborated against either antigen.

Zatz and Goldstein (1973) found that in spleens which had been subjected to antigen 6 hours previously there was a significant depression of DNA synthesis. Whether or not DNA synthesis is depressed in lymph nodes to which antigen has recently been administered is not known. Soluble mediators which may give rise to the depression of DNA synthesis have not been detected (Möller and Kashiwagi, 1972) and the phenomenon remains unexplained. It may be that the injection of a second antigen 24 hours after the first antigen was given further depresses the ability of lymph node cells to synthesise DNA although there is no evidence to substantiate this.

When two unrelated or identical antigens were administered at least 120 hours apart then the immune response to the second antigen was severely depressed; total cell and blast cell output and the output of plaque-forming cells were low. It has been shown that T lymphocytes are capable of elaborating a soluble suppressor material (Watson, 1973) and that the serum of normal mice contains a factor which suppresses immune responses to sheep red blood cells by spleen cells of mice in vitro (Veit and Michael, 1973). This factor increases in concentration after immunisation and has been shown to exert its effect in the early stages of an immune response (Veit and Michael, 1973). Other investigators have suggested that antigen may induce the activation of two types of T lymphocyte (Baker, Stashak, Amsbaugh, Prescott and Barth,
1970; Gershon, Cohen, Hencin and Liebhaber, 1972); one type are "helper" cells which act in the early stages of an immune response and provide signal 2 to B lymphocytes, and the second type are "suppressor" cells which suppress the ability of B lymphocytes to respond to antigen, presumably in the latter stages of an immune response. This helper and suppressor activity could be provided by the same T lymphocyte population (Katz, 1972) and indeed may be effected by the same product. The experiments reported in this chapter do not determine which, if any, of these hypotheses is correct.

There is now a considerable body of evidence to suggest that cell mediated immune reactions are capable of modulating humoral immune responses, thus Rowland, Edwards, Hurd and Summer (1971) have shown that in mice bearing mammary adenocarcinomas the immune response to sheep red blood cells is enhanced in the regional lymph nodes and spleen during the phase of tumour growth but suppressed during advanced malignancy. Miller, Mackaness and Lagrange (1973) have shown that the immune response to sheep red blood cells is considerably enhanced when this antigen is administered during the proliferative phase of an immune response to BCG. To achieve this enhancement, BCG and sheep red blood cells must both be administered in the drainage area of the one lymph node. BCG induces a predominantly T lymphocyte response in lymph nodes and the enhancement of the sheep red blood cell response has been correlated with an increase in the activity of helper T lymphocytes (Mackaness, Auclair and Lagrange, 1973). Antigens such as BCG which primarily induce cell-mediated immunity, can exert strong modulating influences on other cell-mediated reactions such as those against poorly immunogenic murine mastocytomas (Hawrylko and Mackaness, 1973a).
The maximum reaction against an irradiated mastocytoma occurs when the mastocytoma is injected into recipients in the proliferative phase of a BCG response (Hawrylko and Mackaness, 1973b). Thus cell-mediated immune reactions can modulate both types of immune reaction. The enhancing and suppressive effects of lymphocyte transfer on humoral immune responses in alogeneic recipients have been well established and were reviewed by Katz (1972). The mechanism by which these effects are exerted is examined in Chapter VIII.

It may be that the phenomenon of antigenic competition merely reflects the physiological regulatory capacity of T lymphocytes to initiate and limit the extent of normal immune responses. It would appear that antibody regulates the immune response by neutralising those antigenic determinants whose structure is complementary to the antibody, thereby increasing the affinity of antibody formed in response to the antigen by increasing competition for the antigen between antibody and those immunocompetent cells which show specificity for the antigen. T lymphocytes appear to regulate the immune response by means of a secreted product(s) whose action is to non-specifically initiate or inhibit the proliferation of those B lymphocytes which have specific antigen bound to the antibody receptors on the cell membrane.
CHAPTER VI

THE EFFECT OF ANTIGEN-ANTIBODY COMPLEXES ON IMMUNE RESPONSES
INTRODUCTION

The immunogenicity of antigen-antibody complexes has been examined by several investigators with conflicting results. Complexes have been shown to be both more immunogenic than free antigen in inducing primary immune responses (Terres and Wolins, 1959; Segre and Kaeberle, 1962; Dennert, 1971; Osato, 1972) or less immunogenic (Uhr and Möller, 1968). Irrespective of whether or not antigen-antibody complexes are more or less immunogenic in a particular experimental situation they are effective in priming the animal so that subsequent antigenic challenge produces a secondary immune response (Uhr and Baumann, 1961; Terres, Habicht and Stoner, 1974). It was shown in Chapter IV that chicken red blood cells complexed with IgM or IgG antibody are poorly immunogenic in the sheep.

The effect of prior introduction of specific or unrelated antigen-antibody complexes on the immune response to chicken red blood cells in the sheep is examined in this chapter.

RESULTS

The Effect of Antigen-Antibody Complexes on the Immune Response to Chicken Red Blood Cells

$2 \times 10^9$ chicken or rabbit red blood cells were incubated with an excess of specific IgM or IgG antibody. The immune complexes were washed and injected subcutaneously into the outer aspect of the lower hind limb 48 hours or 260 hours prior to the injection of $2 \times 10^9$ chicken red blood cells in the same site. The immune responses elicited
by such procedures were monitored in the efferent lymph draining the popliteal lymph node.

1. **Red cell-IgM complexes given 48 hours prior to chicken red blood cells**

The injection of chicken red blood cell-IgM complexes 48 hours before $2 \times 10^9$ free chicken red blood cells produced a blast cell output that was significantly increased over the levels of a normal primary immune response (Figure VI-1). The rate of increase in the number of plaque-forming cells and the total number of plaque-forming cells appearing in the lymph (Table VI-1) was significantly enhanced. The total output of plaque-forming cells in the response was $9.5 \times 10^7$, a five-fold increase over the control responses. IgM and IgG haemagglutinating antibody outputs in the lymph were normal (Figure VI-1).

When rabbit red blood cell-IgM complexes were injected 48 hours before giving the chicken red blood cells the cellular response was similar to that observed in the control responses (Figure VI-2). The output of plaque-forming cells specific for either chicken or rabbit red blood cells and the rate of increase in the number of these plaque-forming cells in the lymph was significantly greater than in the control responses (Figure VI-2 and Table VI-1). The total number of specific plaque-forming cells appearing in the lymph was $1.2 \times 10^8$ for rabbit red blood cells and $5.4 \times 10^7$ for chicken red blood cells. These outputs represented a nine-fold and a three-fold increase respectively over control responses. IgM and IgG haemagglutinating antibody outputs in the lymph were significantly increased over control responses (Figure VI-2).
Primary immune response to chicken red blood cells.

$2 \times 10^9$ chicken red blood cell - IgM complexes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells.
Primary immune response to chicken and rabbit red blood cells. 
2 x 10^9 rabbit red blood cell - IgM complexes administered 48 hours prior to 2 x 10^9 chicken red blood cells.
2. **Red cell-IgG complexes given 48 hours prior to chicken red blood cells**

Chicken red blood cell-IgG complexes injected 48 hours before 2 x 10⁹ chicken red blood cells did not produce any significant differences in the total cell output when compared with the control responses (Figure VI-3). The rate of increase in the number of plaque-forming cells appearing in the lymph was similar to that of control responses but the total output of plaque-forming cells was 6.1 x 10⁷, a three-fold increase over control responses (Table VI-1). IgM and IgG haemagglutinating antibody outputs in the lymph were normal (Figure VI-3).

When rabbit red blood cell-IgG complexes were injected 48 hours before giving the chicken red blood cells, the total cellular response was similar to that observed in the control responses (Figure VI-4). The rate of increase in the number of plaque-forming cells appearing in the lymph was similar to that of control responses. The total number of plaque-forming cells appearing in the lymph was 6.1 x 10⁷ for chicken red blood cells and 1.05 x 10⁸ for rabbit red blood cells (Table VI-1). These outputs represented a three-fold and an eight-fold increase respectively over control responses. IgM and IgG haemagglutinating antibody outputs in the lymph were normal (Figure VI-4).

3. **Red cell-IgM complexes given 260 hours prior to chicken red blood cells**

When chicken red blood cells were complexed with IgM antibody and injected 260 hours before giving the chicken red blood cells, the total cell and blast cell output in the lymph was significantly
Primary immune response to chicken red blood cells.

$2 \times 10^9$ chicken red blood cell - IgG complexes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells.
Primary immune response to chicken and rabbit red blood cells.  
$2 \times 10^9$ rabbit red blood cell - IgG complexes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells.
Primary immune response to chicken red blood cells.
2 x 10^9 chicken red blood cell - IgM complexes administered 260 hours prior to 2 x 10^9 chicken red blood cells.
Primary immune response to chicken red blood cells.

2 x 10^9 chicken red blood cell - IgG complexes administered 260 hours prior to 2 x 10^9 chicken red blood cells.
The effect of specific and non-specific antigen-antibody complexes on the total number of plaque-forming cells appearing in lymph during immune responses to chicken red blood cells injected 48 hours or 260 hours after the complexes.

CRBC = chicken red blood cells
RRBC = rabbit red blood cells
PFC = plaque-forming cells

### TABLE VI-1

<table>
<thead>
<tr>
<th>Antigen-antibody complex</th>
<th>Time of CRBC administration (hours)</th>
<th>PFC type assayed</th>
<th>Total PFC per response x 10⁶</th>
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<td>CRBC - IgM</td>
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<td>95</td>
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<tr>
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<td>54</td>
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<td>CRBC</td>
<td>6.04</td>
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</table>
depressed (Figure VI-5). The rate of increase and the total number of plaque-forming cells appearing in the lymph was significantly lower than in control responses (Table VI-1). The total output of plaque-forming cells was $3.6 \times 10^6$, approximately 20% of the control value. IgM and IgG haemagglutinating antibody outputs in the lymph were very low (Figure VI-5).

4. Red cell-IgG complexes given 260 hours prior to chicken red blood cells

Chicken red blood cell-IgG complexes injected 260 hours before giving free chicken red blood cells, produced an overall immune response that was similar to that elicited by chicken red blood cells injected 260 hours after IgM-antigen complexes. The total cell output was normal but the blast cell response was short-lived and depressed below the normal levels observed in control responses (Figure VI-6). The rate of increase and the total number of plaque-forming cells appearing in the lymph was considerably lower than in control responses. The total output of plaque-forming cells was $6.4 \times 10^6$, this was about 32% of the number appearing in a normal primary immune response to chicken red blood cells (Table VI-1). IgM and IgG haemagglutinating antibody outputs in the lymph were very low (Figure VI-6).

DISCUSSION

The results presented in this chapter demonstrate that antigen-antibody complexes can modify the immune response to subsequently administered antigen. This occurs whether or not the antigen is the same as that in the complex.
Results presented in Chapter IV showed that IgM or IgG antibody-antigen complexes gave a very weak primary immune response in comparison to that elicited by the free antigen. When chicken red blood cells were injected 48 hours after related or unrelated antigen-antibody complexes then the immune response to the free or complexed antigen was significantly enhanced. These findings were similar to those reported in Chapter V where, in studies on antigenic competition, identical or unrelated free antigen injected 48 hours prior to chicken red blood cells were shown to give enhanced immune responses to both the initiating and the subsequently administered antigen.

It has been demonstrated (Uhr and Baumann, 1961) that even though passively administered antibody suppresses the primary immune response it does not prevent the animal from being primed. Other investigators (Stoner and Terres, 1963; Hess, Terres and Stoner, 1965; Osato, 1972) have shown that antigen-antibody complexes may be more immunogenic than free antigen, and that they enhance the immune response to unrelated antigens (Baenkler, Scheiffarth and Loeffholz, 1972) or partially related antigens (Terres, Habicht and Stoner, 1974). Kappler, Hoffmann and Dutton (1971) have suggested that antigen-antibody complexes may suppress the appearance of plaque-forming cells whilst at the same time stimulating specific T lymphocytes. The threshold at which B and T lymphocytes are stimulated by antigen appears to differ, thus T lymphocytes can be activated by low concentrations of antigen which fail to cause the production of antibody (Greaves, Möller and Möller, 1970; Gershon and Kondo, 1971b; Falkoff and Kettman, 1972). The maximum level of T lymphocyte activity has been shown to occur three days after the administration of antigen-antibody complexes (Kettman and Dutton, 1971). If antigen is administered at this time, it will give rise to increased
B lymphocyte activity and an enhanced immune response (Terres, Habicht and Stoner, 1974). The enhancing effects exerted by antigen-antibody complexes upon the immune response to a subsequently administered antigen appears to be a thymus dependent effect in mice for it does not occur in thymectomised mice (Terres, Habicht and Stoner, 1974).

The present results are similar to those reported by Terres, Habicht and Stoner (1974) and suggest that if the effect is dependent upon T lymphocytes then the T lymphocytes are being specifically activated but are influencing B lymphocytes in a non-specific manner. When rabbit red blood cell-antibody complexes were injected 48 hours prior to chicken red blood cells, the immune responses to both erythrocyte antigens were enhanced; this was an anomalous finding as the complex alone did not give rise to any significant level of immunity. Although it appeared that immune complexes have the capacity to activate T lymphocytes to elaborate a product which activates B lymphocytes in a non-specific manner, the amount of this product that was formed was small and this, together with the low concentration of specific antigen, failed to initiate proliferation of B lymphocytes. When a high concentration of free antigen was administered 48 hours later, then more T lymphocytes were activated and these elaborated more of the non-specific product. The high levels of T lymphocyte product may be significant in reducing the threshold at which B lymphocytes can respond to antigen, and in consequence the very low concentrations of antigen in the complex may be sufficient to induce specific B lymphocytes to proliferate. Plaque-forming cells specific for the antigen in the complex appeared at the same time as plaque-forming cells specific for the free antigen administered 48 hours after the complex but their time of appearance was much later than in a normal response to the free antigen.
Antigen-antibody complexes have been shown to stimulate small lymphocytes to divide (Bloch-Shtacher, Hirschhorn and Uhr, 1969) and to augment the uptake of $^3$H thymidine by sensitised lymph node cells (Thorbecke and Siskind, 1973). These findings could be related to those which have demonstrated that T lymphocytes are particularly sensitive to activation by immune complexes (Greaves and Möller, 1970; Gershon and Kondo, 1971b; Falkoff and Kettman, 1972). Experiments in which mice have been made tolerant to a specific antigen have shown that tolerance can be abrogated by specific antigen-antibody complexes but not by the antigen alone (Hemphill, Segre and Myers, 1966; Intini, Segre, Segre and Myers, 1971). This suggests that T lymphocytes may be activated more readily by antigen-antibody complexes than by free antigen.

The ability of antigen-antibody complexes to either enhance or suppress immune responses to free antigen is dependent upon the time that the free antigen is injected in relation to the complex. Suppression of the immune response occurred when the free antigen was administered 260 hours after the complex. The effects of free antigen upon the immune response to a subsequently administered antigen reported in Chapter V, are identical to the effects of antigen-antibody complexes on the immune response to subsequently administered free antigen. The results presented in this chapter support and extend the observations and conclusions drawn in Chapter V and further show that antigenic enhancement and suppression can be exerted by very low concentrations of free antigen in the presence of specific antibody.
CHAPTER VII

MEDIATORS OF HUMORAL IMMUNITY
INTRODUCTION

Regulation of immune reactivity may be effected by the

generation and release of pharmacologically active materials by lympho-
cytes following activation with antigen (Burnet, 1968). Dumonde,
Wolstencroft, Panayi, Matthew, Morley and Howson (1969) demonstrated
that sensitised lymphocytes exposed to the sensitising antigen,
released substances which caused inflammation and damage to cells,
stimulated mitosis in cells and inhibited the migration of macrophages
in vitro. These substances were active in the absence of the cells
which produced them. The generic term, lymphokines, was suggested to
describe these immunologically non-specific substances which were
produced during immunologically specific reactions; it was suggested
that these lymphokines may play a role in the regulation of immune
responses (Dunonde, Wolstencroft, Panayi, Matthew, Morley and Howson,
1969).

Spleen cells and lymph node cells from mice and rabbits were
shown to release a soluble factor when an antigen against which the
animals were immunised was added to the cells in vitro. This factor
significantly increased the production of plaque-forming cells and
antibody against bovine serum albumin or sheep red blood cells when
added in vitro to spleen cells or lymph node cells in the presence of
the specific antigen (Rubin and Coons, 1971; Rosenthal, Stastny and

A soluble factor elaborated by lymphocytes responding to
antigen has been shown to stimulate DNA synthesis in autologous and
allogeneic lymphocytes from sensitised and non-sensitised animals
(Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson, 1969; Maini, Bryceson, Wolstencroft and Dumonde, 1969; Spitler and Lawrence, 1969; Falk, Falk, Möller and Möller, 1970; Smith and Barker, 1972; Hay, 1973; Hay, Lachmann and Trnka, 1973a, 1973b) and may facilitate interaction between T and B lymphocytes (Oates, Bissenden, Maini, Payne and Dumonde, 1972). Thus T lymphocytes activated by antigen may release a factor which stimulates B lymphocytes to divide and subsequently synthesise antibody (Davies, Leuchars, Wallis, Marchant and Elliott, 1967; Gorczynski, Miller and Phillips, 1972; 1973a; 1973b).

Normal mouse serum and serum from immunised mice contain a factor which inhibits immune responses in a non-specific manner (Veit and Michael, 1972; Lee and Paraskevas, 1974). An α-2 glycoprotein fraction from normal bovine or human blood plasma has also been shown to have immunosuppressive properties (Mowbray, 1963; Mannick and Schmid, 1967). This immunosuppression appears to act through T lymphocytes (Menzoian, Glasgow, Cooperband, Schmid, Saporoschetz and Mannick, 1973).

Studies of mechanisms which operate to control the degree and extent of immune reactivity have mostly been concerned with the initial events of immune responses to antigen; regulatory effects which operate on the antibody-forming cells have not been examined extensively. This chapter sets out to investigate whether mitogenic or other factors are elaborated in vivo during an immune response and whether such factors operate to control initial or subsequent events of an immune response to antigen or allogeneic lymphocytes. The effects of these factors on the immune response were also examined in vitro.
R E S U L T S

Isolation of Factors which Influence Immune Responses

The efferent popliteal lymph coming from nodes responding primarily to $2 \times 10^9$ chicken red blood cells was collected throughout the period of the immune response. The cells in the lymph were removed by centrifugation and the supernatant lymph was stored at $-20^\circ C$ until required. Lymph from immunised sheep and blood plasma from non-immunised sheep was fractionated on D.E.A.E. columns equilibrated with 0.03M acetate buffer pH 5.0 according to the method of Steinbuch and Quentin (1961). Four fractions annotated A, B, C, D were obtained by elution with 0.1M, 0.2M and 0.5M acetate buffer pH 5.0 and Molar sodium chloride respectively.

The in vivo effects of whole lymph, of lymph fractions and of blood plasma upon the immune response to $2 \times 10^9$ chicken red blood cells and on the response to the transfer of normal lymphocytes were examined. The in vitro effects of whole lymph and of fractionated lymph were examined by adding them to PHA stimulated cultures of lymphocytes and to populations of lymphocytes forming specific plaques against chicken red blood cells.

The Effect of Lymph upon the Primary Immune Response to Chicken Red Blood Cells

Lymph was collected from 48-96 hours and from 144-168 hours after a primary antigenic stimulus with $2 \times 10^9$ chicken red cells. Lymphocytes were removed from the lymph by centrifugation and the supernatant was heated at $56^\circ C$ for 30 minutes to destroy complement. Specific antibody was absorbed from the lymph at $4^\circ C$ and $37^\circ C$ with chicken red cells. 2.5 ml of lymph was injected subcutaneously into the drainage area
of the popliteal lymph node of another sheep 24 hours prior to the administration of $2 \times 10^9$ chicken red cells to the same site; 2.5 ml of lymph was injected with the antigen and four further injections of 2.5 ml of lymph were given at 24 hour intervals.

Lymph collected 48 - 96 hours after antigenic challenge had little effect upon the output of total cells or on the output of blast cells (Figure VII-1). The output of plaque-forming cells was significantly increased and a total of $1.17 \times 10^8$ plaque-forming cells appeared in the lymph; this was about six times greater than in control responses. The peak output of plaque-forming cells in the lymph coincided with the peak output of total cells and of blast cells. The output of IgM haemagglutinating antibody in the lymph was significantly reduced; IgG haemagglutinating antibody output was normal but this antibody appeared at an earlier time than in the control response. In a second experiment in which 1 ml instead of 2.5 ml injections of lymph were given, a total of $4 \times 10^7$ plaque-forming cells appeared in the lymph, about twice the number in the control response.

Lymph collected 144-168 hours after antigenic challenge had a significant effect on the primary immune response to chicken red cells. Total cell output and blast cell output was reduced (Figure VII-2) and the total output of plaque-forming cells, $8.58 \times 10^6$, was about half the control value. The output of IgM haemagglutinating antibody in the lymph was less than in the control response, whilst the IgG haemagglutinating antibody output was unchanged. Normal lymph from a sheep that had not been immunised had no effect upon the primary immune response to chicken red cells.
Primary immune response to $2 \times 10^9$ chicken red blood cells. Lymph from another sheep was collected during the period 48 - 96 hours after a primary injection of chicken red blood cells; this lymph was heated at 56°C for 30 minutes to inactivate complement. Specific antibody was absorbed from the lymph by the addition of chicken red blood cells; these cells were subsequently removed from the lymph by centrifugation and 2.5ml of lymph was injected 24 hours before and 0, 24, 48, 72 and 96 hours after the administration of chicken red blood cells.
Primary immune response to $2 \times 10^9$ chicken red blood cells. Lymph from another sheep was collected during the period 144 - 168 hours after a primary injection of chicken red blood cells; this lymph was heated at 56°C for 30 minutes to inactivate complement. Specific antibody was absorbed from the lymph by the addition of chicken red blood cells; these cells were subsequently removed from the lymph by centrifugation and 2.5ml of lymph was injected 24 hours before and 0, 24, 48, 72 and 96 hours after the administration of chicken red blood cells.
These results suggested that factors are elaborated during the course of an immune response which influence either the production of plaque-forming cells or the secretion of antibody by them.

The Evaluation of Antibody-Containing Cells and Antibody-Secreting Cells during a Primary Immune Response to $2 \times 10^9$ Chicken Red Blood Cells

Rabbit serum used as a complement source was found to lyse all the lymphoid cells in the popliteal lymph even if it had been absorbed previously with sheep lymphocytes. This rabbit serum was used as a complement source in conventional plaque-forming cell assays to detect all cells which were synthesising antibody. It was argued that those cells which contained antibody but for some reason were not secreting it would not be measured as antibody-forming cells in tests with guinea pig complement but they would be measured as antibody-forming cells when the cells were lysed with rabbit complement. Not all lysed lymphoid cells produced specific plaques against chicken red cells.

Antibody-forming cells and antibody-secreting cells were assayed throughout an immune response to $2 \times 10^9$ chicken red cells. Figure VII-3 shows that the number of antibody-forming cells was significantly greater than the number of antibody-secreting cells throughout the response. The percentage of antibody-forming cells which actually secreted antibody rose to a maximum around 96 hours into the immune response and thereafter fell rapidly.

These findings provide evidence for the elaboration of factors which influence the secretion of antibody by antibody-forming cells.
Primary immune response to $2 \times 10^9$ chicken red blood cells. The upper figure shows the cellular changes which occurred in the lymph. The centre figure shows the number of plaque-forming cells and the number of antibody-forming cells which appeared in the lymph. The lower figure shows the percentage of antibody-forming cells which were expressed as plaque-forming cells.
The Effect of Lymph on the Secretion of Antibody by Antibody-Forming Cells

Lymph samples were collected from the efferent popliteal lymph of sheep responding to a primary antigenic challenge with $2 \times 10^9$ chicken red cells. Cells were obtained from the popliteal lymph of other sheep responding to a primary antigenic challenge of $2 \times 10^9$ chicken red cells or $2 \times 10^9$ rabbit red cells. These cells were suspended to their original concentration and incubated for 30 minutes at $37^\circ C$ in lymph collected from sheep immunised with chicken red cells or lymph obtained from non-immunised sheep. The cells were then examined for plaque-forming activity.

Cells were incubated with lymph obtained at the peak of the response (96 hours), lymph collected after the peak of the response (168 hours) or lymph collected from non-immunised sheep (0 hours). Cells incubated in the same lymph in which they were collected acted as the controls. The total number of plaque-forming cells was measured after 30 minutes incubation at $37^\circ C$. The results of this experiment are shown in Figure VII-4.

Cells incubated in lymph collected at the peak of the immune response from two individual sheep, increased the number of plaque-forming cells in all samples of cells collected throughout the response; the lymph was particularly effective in increasing the numbers of plaque-forming cells in samples collected during the early phase of the response. Lymph collected after the peak of the response suppressed the number of plaque-forming cells in all samples of cells collected up until the peak of the response. Normal lymph from non-immunised sheep reduced the number of plaque-forming cells during the early phase of the response but increased the number of plaque-forming cells in those samples of cells collected during the late stages of the response.
Primary immune response to $2 \times 10^9$ chicken red blood cells. The upper figure shows the results of incubating plaque-forming cells and antibody-forming cells in lymph obtained from another sheep responding to a primary dose of $2 \times 10^9$ chicken red blood cells. The lower figure shows the normal plaque-forming cell response.
These results suggested that some factor which increased the secretion of antibody by antibody-forming cells was elaborated during the early phase of the immune response and this factor reached a maximum level in the lymph at the same time as the cell output in the lymph was maximum. Lymph collected after the peak of the immune response reduced the number of plaque-forming cells to a greater extent than did lymph from non-immunised sheep suggesting the presence of some factor which inhibited the secretion of antibody by some of the antibody-forming cells.

The factor which increased the number of plaque-forming cells appeared to act non-specifically for lymph obtained at the peak of an immune response to chicken red cells caused an increase in the number of plaque-forming cells in populations of cells obtained from sheep immunised with rabbit red cells. When cells collected from the efferent lymph of a sheep responding to chicken red cells were incubated in lymph collected at the peak of responses to allogeneic lymphocytes, Salmonella organisms or rabbit red blood cells, the number of plaque-forming cells was increased.

In the experiment in which antibody-forming cells and antibody-secreting cells were evaluated (Figure VII-3) it was shown that the number of antibody-secreting cells in lymph was always less than the number of antibody-forming cells and that between 40-96 hours after the administration of antigen the number of antibody-secreting cells increased relative to the number of antibody-forming cells. It appeared that lymph obtained at the peak of an immune response contained a factor which enhanced the secretion of antibody by lymphoid cells to the same or a different antigen and this finding was further substantiated by the result that the plaques formed by efferent lymph cells when incubated in lymph obtained at the peak of an immune response, were significantly larger in
diameter than the plaques formed by similar cells incubated in lymph obtained from non-immunised sheep.

In the experiment in which antibody-forming cells and antibody-secreting cells were evaluated (Figure VII-3) it was shown that there was a rapid fall in the number of antibody-secreting cells in the lymph between 100 and 200 hours after the administration of antigen, and in the ratio of antibody-secreting cells to the number of antibody-forming cells.

The Effect of Lymph upon the Uptake of ³H Thymidine in Lymphocytes Stimulated Previously with Phytohaemagglutinin

Samples of efferent lymph were collected from individual sheep responding to a primary antigenic challenge with \(1 \times 10^9\) or \(2 \times 10^9\) chicken red cells, \(3.4 \times 10^9\) Salmonella muenchen heat killed organisms or \(3 \times 10^8\) allogeneic lymphocytes. The samples were treated as described previously and their effect on DNA synthesis in lymphocytes stimulated in vitro with PHA was studied. Cells stimulated with PHA and incubated in lymph obtained from non-immunised animals acted as controls.

Figures VII-5 and VII-6 show the results of these experiments. Lymph collected from sheep at the height of responses to allogeneic lymphocytes or antigen was found to increase the uptake of ³H thymidine in lymphocytes stimulated previously with PHA. The same lymph samples had no effect upon the uptake of ³H thymidine in normal unstimulated lymphocytes.

These results suggested that factor(s) which increase DNA synthesis in PHA stimulated lymphocytes were elaborated during the primary response to the antigens tested and that the factor(s) were present in the lymph in maximum concentrations when the cellular response in the lymph was maximum. The amount of these factor(s)
The effect of heat inactivated and antibody absorbed immune lymph upon $^3$H-thymidine uptake by PHA stimulated lymphocytes in vitro and on the ability of lymphocytes to produce a normal lymphocyte transfer reaction in vivo.

Upper figure: Lymph collected from a sheep responding to a primary dose of $2 \times 10^9$ chicken red blood cells.

Centre figure: Lymph collected from a sheep responding to a primary dose of $1 \times 10^9$ chicken red blood cells.

Lower figure: Lymph collected from a sheep responding to a primary dose of $3.4 \times 10^9$ Salmonella muenchen heat killed organisms.
The effect of heat inactivated and antibody absorbed immune lymph upon the uptake of $^3$H-thymidine by PHA stimulated lymphocytes in vitro. Lymph was collected from a sheep responding to a primary injection of $3 \times 10^8$ viable allogeneic lymphocytes.
produced appeared to be a function of the magnitude of the cellular response in the lymph for during vigorous responses to allogeneic lymphocytes the factor(s) appeared in the lymph at a high concentration.

The Effect of Lymph upon Normal Lymphocyte Transfer Reactions

Factor(s) produced during an immune response to antigen or allogeneic lymphocytes were shown to increase the uptake of $^3$H thymidine in lymphocytes stimulated in vitro by PHA. Further experiments were designed in order to determine whether or not these factor(s) were capable of acting in vivo to increase the response of sheep to allogeneic lymphocytes. Normal allogeneic lymphocytes were suspended to a concentration of $1 \times 10^8$ lymphocytes/ml in lymph collected from sheep responding to a primary challenge of $1 \times 10^9$ or $2 \times 10^9$ chicken red cells or $3.4 \times 10^9$ Salmonella muenchen heat killed organisms. Doses of 0.1 ml were injected intradermally into the wool free skin on the inside of the thigh of recipient sheep. Normal allogeneic lymphocytes suspended in lymph obtained from non-immunised sheep acted as the controls. The increase in skin thickness at the site of injection was measured each day over the next eight days and the maximum skin thickness was recorded. Lymph obtained from the peak of chicken red cell or Salmonella responses when injected with the allogeneic lymphocytes gave maximum increases in skin thickness (Figure VII-5). It was found that the degree to which the NLT response was enhanced by any given sample of lymph correlated with the degree to which $^3$H thymidine uptake was enhanced in PHA stimulated lymphocytes.

The results of these experiments reinforced the idea that factor(s) which may act as mitogens, were elaborated during the primary immune response to antigens and that maximum levels of these factor(s)
occur in the lymph at the same time as the maximum cellular response. The mitogens were shown to exert their effects both in vitro and in vivo.

The Effect of DEAE Fractions from Normal Sheep Blood Plasma on the Immune Response to Chicken Red Blood Cells

Normal sheep blood plasma was separated on DEAE columns with acetate buffer pH 5.0 and fractions designated A, B, C and D were subjected to acrylamide gel electrophoresis. Figure VII-7 shows that each of these fractions possessed bands in the α1 and α2 regions; the only common bands were shared between fractions C and D. Freeze dried fractions were made up to a concentration of 5 mg/ml with sterile saline and 2 ml was injected subcutaneously into the popliteal lymph node drainage area at various times before and after the injection of 2 x 10^9 chicken red cells to the same site. The fractions were injected 24 hours before and 0, 24, 48 and 72 hours after injecting the chicken red cells. In control experiments 1 ml of whole blood plasma was injected 24 hours before and 0, 24, 48 and 72 hours after injecting chicken red cells. Whole blood plasma had no effect on the response to chicken red cells and the cellular and humoral response was not significantly different from normal. Further control experiments were done by injecting the fractions alone. No fraction produced a cellular response in the efferent lymph when administered in the absence of antigen.

1. Primary responses

Fraction A (Figure VII-8) and fraction B caused a significant increase over control values in the total cell output and the blast cell output in the lymph (Table VII-1). The total output of plaque-forming cells was slightly increased (20-40%) above the control level (2.8 x 10^7; fraction A, and 2.4 x 10^7; fraction B, plaque-forming cells). The output
FIGURE VII - 7

Acrylamide gel electrophoresis of DEAE cellulose eluates.

1. Sheep IgM. Albumin, $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ components are also present.

2. Sheep Albumin. $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ components are also present.

3. Fraction A. (0.1M acetate buffer pH5.0 eluate).

4. Fraction B. (0.2M acetate buffer pH5.0 eluate).

5. Fraction C. (0.5M acetate buffer pH5.0 eluate).


7. Whole sheep lymph plasma.

8. Sheep Albumin. $\alpha_1$, $\alpha_2$, $\beta_1$ and $\beta_2$ components are also present.
Primary immune response to $2 \times 10^9$ chicken red blood cells. 10mg doses of Fraction A were injected subcutaneously into the lower leg 24 hours before and 0, 24, 48 and 72 hours after the administration of chicken red blood cells.
Primary immune response to $2 \times 10^9$ chicken red blood cells. 10mg doses of Fraction D were injected subcutaneously into the lower leg 24 hours before and 0, 24, 48 and 72 hours after the administration of chicken red blood cells.
### TABLE VII-1

<table>
<thead>
<tr>
<th>Fraction administered</th>
<th>Total plaque-forming cell output $\times 10^6$</th>
<th>Peak blast cell response Cell output per hour $\times 10^6$</th>
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</thead>
<tbody>
<tr>
<td><strong>Primary responses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>28</td>
<td>86</td>
</tr>
<tr>
<td>Fraction B</td>
<td>24</td>
<td>83</td>
</tr>
<tr>
<td>Fraction C</td>
<td>4.7</td>
<td>28</td>
</tr>
<tr>
<td>Fraction D</td>
<td>5.7</td>
<td>27</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td><strong>Secondary responses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction B</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>Fraction C</td>
<td>8</td>
<td>51</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>46</td>
</tr>
</tbody>
</table>

The Effect of DEAE Fractions of Sheep Blood Plasma on the Immune Response to Chicken Red Blood Cells

Fractions were injected 24 hours before and 0 hours, 24 hours, 48 hours and 72 hours after injecting the chicken red blood cells at the same site.
of IgM and IgG haemagglutinating antibody was not significantly different from the control experiments.

Fraction D (Figure VII-9) and fraction C had little effect on the total cell response or the blast cell response to chicken red cells but the number of plaque-forming cells \((5.7 \times 10^6; \text{fraction D}, 4.7 \times 10^6; \text{fraction C})\) that appeared in the lymph was reduced 20-30% below the levels of the control responses (Table VII-1). The output of IgM haemagglutinating antibody was low whilst the output of IgG haemagglutinating antibody was not significantly different from that of the control responses.

2. Secondary responses

Fraction C significantly reduced the number of plaque-forming cells appearing in the lymph. The total output of \(8 \times 10^6\) plaque-forming cells was 27% of the control responses (Table VII-1). The total cell and the blast cell output in the lymph was unchanged. The outputs of IgM and IgG haemagglutinating antibody were not significantly different from controls.

Fraction B had no significant effect upon the secondary immune response to chicken red cells. The effects of fraction A and fraction D on secondary immune responses was not determined as an insufficient amount of these fractions was available.

The Effect of DEAE Fractions from Normal Sheep Blood Plasma on the Lymphocyte Transfer Reaction

Allogeneic lymphocytes were suspended to a concentration of \(1 \times 10^8\) lymphocytes/ml in a saline solution containing either fraction A or fraction C (5 mg/ml). Doses of 0.1 ml were injected intradermally
FIGURE VII - 10

NLT RESPONSES TO $1 \cdot 10^7$ ALLOGENEIC CELLS

The effect of Fraction A and Fraction C on the normal lymphocyte transfer reaction. Increase in skin thickness with time.
into the wool free skin of the inside thigh of recipient sheep. Control experiments were done with allogeneic lymphocytes injected in saline. The increase in skin thickness at the site of injection was measured each day over the next 7 days (Figure VII-10). Fraction A significantly enhanced the NLT response and produced a maximum increase in skin thickness of 12mm. Fraction C had no significant effect upon the NLT response and the maximum value of 7mm was much the same as given by the control cells.

The Effect of DEAE Fractions from Sheep Lymph and Blood Plasma on the Response of Lymphocytes to PHA

Efferent lymph was collected from a popliteal lymph node responding to a primary antigenic challenge of $2 \times 10^9$ chicken red cells and separated into five collection intervals representing periods before antigenic challenge, and 58-77 hours, 96-106 hours, 120-144 hours and 250-264 hours after antigenic challenge. These lymph samples were fractionated on acetate equilibrated DEAE columns as described previously. Lyophilised fractions designated A, B, C and D were dissolved in Eagle's medium and added to PHA stimulated lymphocyte cultures to a final concentration of 1 mg/ml. Control lymphocyte cultures were done in Eagle's medium alone.

All samples of fraction A increased the uptake of $^3$H thymidine by PHA stimulated lymphocytes; the maximum increases occurred with those samples of fraction A that had been isolated from lymph collected 96-106 hours and 120-144 hours after antigenic challenge at a time when the blast cell response in the lymph was at a maximum (Figure VII-11).

Fractions B and C collected during each time interval had little effect upon the uptake of $^3$H thymidine by PHA stimulated lymphocytes.
The effect of Fraction A upon the uptake of $^3$H-thymidine by PHA stimulated lymphocytes in vitro. Fraction A was isolated from lymph collected at intervals during a primary immune response to $2 \times 10^9$ chicken red blood cells.
Fractions A, B, C and D isolated from normal sheep blood plasma had no significant effect upon the uptake of $^3$H thymidine by PHA stimulated lymphocytes.

**DISCUSSION**

Lymph collected during the period of the immune response when the blast cell output in the lymph was increasing, had little effect upon the blast cell response *in vivo* to a primary challenge with chicken red cells, but increased the total number of plaque-forming cells appearing in the lymph. The same lymph enhanced NLT reactions, gave rise to an increased uptake of $^3$H thymidine in lymphocytes stimulated with PHA *in vitro* and increased the number of plaque-forming cells in *in vitro* cultures.

Lymph collected late in the immune response, after the period of maximum cellular output in the lymph, when injected into sheep responding to a primary challenge with chicken red cells reduced both the blast cell response *in vivo* and the number of plaque-forming cells appearing in the lymph. The same lymph had no significant effect upon NLT reactions or on the uptake of $^3$H thymidine by lymphocytes stimulated with PHA *in vitro* but reduced the number of plaque-forming cells in *in vitro* cultures.

These results indicated that mitogen like factor(s) and factors which influence the secretion of antibody by antibody-forming cells were elaborated during that period of the immune response when the blast cell output in the lymph was increasing. The increased number of plaque-forming cells observed *in vivo* experiments may have been due to the fact that secretion of antibody by the antibody-forming cells was facilitated, leading to the formation of plaques by cells that were not
previously secreting antibody. From the \textit{in vitro} experiments it appeared that a pre-existing population of antibody-forming cells could be induced to secrete their antibody under the influence of some factor(s) and form plaques in the assay system. After the period of maximum cellular output in the lymph, some factor(s) were present in the lymph which inhibited the secretion of antibody by antibody-forming cells, this factor was capable of exerting its effects \textit{in vivo} and \textit{in vitro}. During the course of a primary immune response to chicken red cells, the number of antibody-forming cells that appeared in the lymph was found to exceed the number of antibody-secreting cells. The number of antibody-secreting cells rose to a maximum around the time that the output of cells in the lymph was maximum; after this time the number of antibody-secreting cells in the lymph fell more rapidly than did the number of antibody-forming cells. These results suggested that the secretion of antibody by antibody-forming cells was controlled \textit{in vivo} by factors elaborated during the course of the immune response.

It has been demonstrated that fractions A, B, C and D isolated from blood plasma by DEAE chromatography can modify immune responses; thus fraction C eluted with 0.5M acetate buffer pH 5.0 suppressed graft rejection (Mannick and Schmid, 1967), antibody formation (Mowbray, 1963; Glasgow, Cooperband, Occhino, Schmid and Mannick, 1971) and lymphocyte proliferation induced \textit{in vitro} by mitogens and antigens (Cooperband, Bondevik, Schmid and Mannick, 1968). Mannick and Schmid (1967) have also demonstrated that fraction B eluted from plasma with 0.2 M acetate Buffer pH 5.0 may have a suppressive action on the immune response. Fraction A eluted with 0.03 - 0.1 M acetate buffer pH 5.0 has been shown to increase the uptake of $^3$H thymidine in lymphocytes stimulated with PHA \textit{in vitro} (Chase, 1972).
The results reported in this chapter indicate that in general, fractions A and B have similar enhancing effects while fractions C and D have similar suppressive effects on immune reactivity.

Fractions A and B were found to increase cell proliferation and increase the number of plaque-forming cells in primary immune responses to chicken red cells; fraction B had little effect on secondary immune responses. Fraction A enhanced NLT reactions and stimulated the uptake of $^3$H thymidine by lymphocytes stimulated with PHA whilst it exerted no effect on the number of plaque-forming cells in in vitro cultures. Fractions A and B appeared to be mitogenic in vivo and in vitro and the increased numbers of plaque-forming cells produced in response to these fractions in in vivo primary immune responses, may be related to the production of more antibody-forming cells rather than to any direct effect which promoted the release of antibody from antibody-forming cells. Fractions C and D had no influence upon cell proliferation in primary and secondary immune responses, NLT responses or PHA stimulated lymphocyte cultures. Neither fraction decreased the number of plaque-forming cells in in vitro cultures but they did reduce the number of plaque-forming cells appearing in the lymph in primary and secondary immune responses to chicken red cells. These fractions may inhibit the proliferation of antibody-forming cells or the synthesis of antibody by these cells although they did not appear to inhibit the general cellular response in primary and secondary immune responses.

It has been suggested that fraction C may suppress a variety of functions attributed to T lymphocytes (Menzoian, Glasgow, Cooperband, Schmid, Saporoschetz and Mannick, 1973) and that T lymphocytes may modulate the expression of plaque-forming cells (Gorczynski, 1974). It seemed that fractions C and D inhibited the secretion of antibody by antibody-forming
cells in some indirect way, but it could not be said whether or not there was any decrease in the number of antibody-forming cells; thus no predictions could be made concerning the mechanisms underlying the phenomenon.

The capacity of both lymph and fraction A to stimulate cell proliferation could be correlated with the stage of the primary immune response at which the lymph was collected; thus the mitogenic factor(s) may originate from the blast cells and be represented by fraction A.

Oates, Bissenden, Maini, Payne and Dumonde (1972) suggested that mitogenic factors may mediate lymphocyte transformation by facilitating and regulating the interaction between T and B lymphocytes, whilst Hay, Lachmann and Trnka (1973a) have related the mitogen present in lymph draining the popliteal lymph node of sheep responding to PPD to the factor which permits T and B lymphocytes to collaborate.

In all probability there are many factors elaborated during the course of an immune response which effect interactions between cells and which alter the activities of antibody-forming cells. The regulatory factors examined in this chapter, function in the microenvironment of the lymph node and when they appear in the efferent lymph they may be present at a much lower concentration, but even so their effects on modifying the immune response can be measured readily both in vivo and in vitro.
Studied in which histocompatible cells and lymphs have been transplanted into hosts it has been suggested that the stimuli which leads to cell proliferation and antibody formation by the resistant is provided by surface histocompatibility antigens. Data on HEL and MHC reactions are the result of antigenic stimulation and antigen is to which MLC reactions appear to be different and it is now apparent that antigen alone does not stimulate cell proliferation in this case (Sibly and King, 1967). Schettina and Kline, 1970, and that these reactions are more dependent upon allogenic stimulation type on the response of immune competent lymphocytes in histocompatibility antigens (Lafferty and Lowe, 1969). An analysis of MLC monolayer reactions (Lafferty, Mellor, Schettina and Milby, 1970) demonstrated the presence of immune reactivity. In the absence of antigenic reimmunization and allogenic stimulation, the mixture of lymphocytes were used to stimulate allogenic lymphocytes to which immune cell reactions indicate that high concentrations of the spleen enhanced the capacity of lymphocytes to stimulate although those cells were specific antigenic lymphocytes previously treated with the contaminating antigenic lymphocytes. However, retained their capacity to stimulate syngenic lymphocytes, but when they themselves were unable to mediate the stimulation (Lafferty and Pessert, 1971).

Allogenic lymphocytes have been shown to affect the immune response to an independent antigen when presented simultaneously. The interleukin stimulus is not always by enhancing of suppressor effect on the immune response but served to the production of antigen as measured by the number of antibody secreting cells in the spleen and the titre of antibody-
INTRODUCTION

Studies in which histoincompatible cells and tissues have been transplanted into animals have suggested that the stimulus which leads to cell proliferation and antibody formation in the recipient is provided by surface histocompatibility antigens, that is GVH and HVG reactions are the result of antigenic stimulation. The situation in in vitro MLC reactions appears to be different and it is now evident that antigen alone does not stimulate cell proliferation in this case (Hardy and Ling, 1969; Schellekens and Eijsvoogel, 1970) and that these reactions are more dependent upon allogeneic stimulation than on the response of immunocompetent lymphocytes to histocompatibility antigens (Lafferty and Jones, 1969). An analysis of GVH and MLC reactions (Lafferty, Walker, Scollay and Killby, 1972) led to the postulate that there are two distinct types of immune reactivity involved in these reactions, antigen responsiveness and allogeneic stimulation. Studies in which Mitomycin C treated sheep lymphocytes were used to stimulate allogeneic lymphocytes in mixed lymphocyte reactions indicated that high concentrations of the drug inhibited the capacity of lymphocytes to stimulate although these cells were still antigenic. Lymphocytes previously treated with low concentrations of Mitomycin C, however, retained their capacity to stimulate allogeneic lymphocytes in vitro while they themselves were unable to respond (Scollay, Lafferty, and Poskitt, 1974).

Allogeneic lymphocytes have been shown to affect the immune response to an independent antigen when presented with it. The allogeneic stimulus may exert an enhancing or suppressive effect on the immune response to the independent antigen as measured by the number of antibody-secreting cells in the spleen and the titre of systemic antibody (Katz,
1972), it may eliminate the requirement for carrier specificity in secondary anti-hapten responses (Katz, Paul, Goidl and Benacerraf, 1971; Hamaoka, Osborne and Katz, 1973), or it may terminate a pre-existing state of tolerance to an antigen (McCullagh, 1970a; Nachtigal and Zanbar, 1973).

It has been suggested that the suppressive effect exerted by GVH reactions on antibody synthesis to independent antigens is due to antigenic competition (Möller, 1971; Katz, 1972). The primary immune response of mice was suppressed when antigens were injected 7 days or more after the initiation of a GVH reaction (Lawrence and Simonsen, 1967; Möller, 1971); no suppression resulted when antigens were injected at the same time as the allogeneic cells (Möller, 1971).

Katz (1972) considered that the variability of the enhancing or suppressive effect of allogeneic reactions on the immune response to subsequently administered antigens was due to the magnitude of the GVH reaction as determined by the strength of the histoincompatible antigens, the number of cells transferred, the route of transfer and the time interval between administration of allogeneic cells and the administration of antigen.

Katz (1972) considered two possible ways in which the allogeneic reaction could suppress the immune response. Intense GVH reactions may destroy large numbers of host cells including the reactive lymphocytes, or the GVH reaction may induce the elaboration of non-specific factors, presumably by T lymphocytes, which exert a suppressive influence on B lymphocytes and possibly other T lymphocytes. Katz (1972) thought the second possibility more likely and this was supported by the finding that anti-DNP antibody production in guinea pigs was suppressed when DNP-BGG
was administered 1-3 days after allogeneic cell transfer; when subse-
sequently challenged with DNP-OVA the animals gave a strong immune response
against DNP. An increased response would not have been expected to occur
if the earlier suppression had been due to the reduction in numbers of
specific B lymphocytes (Katz, Paul and Benacerraf, 1971).

It is the purpose of this chapter to investigate the immune
response resulting from the transfer of lymphocytes to allogeneic
recipients and to examine in vivo the reported enhancing and suppressing
effects of reactions against allogeneic lymphocytes on antigenic responses,
in order to determine the temporal relationships of these effects and the
mechanisms through which they operate to control immune responses.

RESULTS

In Vitro Studies of the Effect of Mitomycin C upon Lymphocytes

Mitomycin C has been shown to prevent DNA synthesis and cell
division through the introduction of covalent linkages and the depoly-
erisation of the complementary strands of DNA. (Iyer and Szybalski, 1963).
Exposure of lymphocytes to low concentrations of Mitomycin C does not
impair RNA synthesis whilst exposure to high concentrations of Mitomycin C
inhibits RNA synthesis.

The capacity of lymphocytes to induce cell proliferation in
allogeneic lymphocytes after pretreatment with Mitomycin C at concen-
trations of 10µg/ml or 50µg/ml was assayed in mixed lymphocyte cultures.
It was found that pretreatment of lymphocytes with 50µg/ml Mitomycin C
eliminated their capacity to stimulate \(^3\)H thymidine incorporation in
normal allogeneic lymphocytes whilst pretreatment with 10µg/ml Mitomycin C
did not (Table VIII-1). Lymphocytes pretreated with 50µg/ml Mitomycin C
had no effect upon the capacity of lymphocytes pretreated with 10μg/ml Mitomycin C to stimulate normal allogeneic lymphocytes in mixed lymphocyte cultures (Table VIII-1) thus the failure of lymphocytes treated with 50μg/ml Mitomycin C to stimulate in mixed lymphocyte cultures was not thought to be due to Mitomycin C leaking from the cells and affecting the uptake of ³H thymidine in the normal allogeneic lymphocyte population.

The ability of normal and Mitomycin C treated lymphocytes to stimulate lymphocytes sensitised to allogeneic cells was examined. When the sensitised cells were cultivated together with lymphocytes pretreated with either 10μg/ml or 50μg/ml Mitomycin C, very high levels of ³H thymidine incorporation occurred on the fourth day of cultivation and the degree of stimulation was independent of Mitomycin C dose (Table VIII-2). If the sensitised cells were responding to histoincompatible antigens on the Mitomycin C treated lymphocytes then treatment of these cells with Mitomycin C at the two dose levels tested did not alter their antigenicity.

The capacity of lymphocytes pretreated with 50μg/ml Mitomycin C to absorb specific cytotoxic antibody was compared to that of untreated lymphocytes (Table VIII-3); their ability was found to be unimpaired. These results suggested that the Mitomycin C at the concentrations tested, did not cause any alteration or loss of lymphocyte histocompatible antigens and that the capacity of lymphocytes to stimulate in mixed lymphocyte cultures was dependent upon the presence of histoincompatible antigens and some other factor which may be elaborated by lymphocytes.
### TABLE VIII-1

<table>
<thead>
<tr>
<th>Mixed lymphocyte culture</th>
<th>$^3$H thymidine uptake</th>
<th>Ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cpm/culture</td>
<td>±S.E.</td>
</tr>
<tr>
<td>A+B$_{10}$</td>
<td>2,360</td>
<td>±200</td>
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<tr>
<td>A+B$_{50}$</td>
<td>445</td>
<td>±30</td>
</tr>
<tr>
<td>A+B$<em>{10}$+B$</em>{50}$</td>
<td>2,795</td>
<td>±215</td>
</tr>
<tr>
<td>A</td>
<td>385</td>
<td>±50</td>
</tr>
<tr>
<td>B$_{10}$</td>
<td>260</td>
<td>±40</td>
</tr>
<tr>
<td>B$_{50}$</td>
<td>190</td>
<td>±20</td>
</tr>
</tbody>
</table>

The capacity of sheep lymphocytes to stimulate the proliferation of normal allogeneic lymphocytes in vitro: The effect of Mitomycin C pretreatment.

A = normal lymphocytes from sheep A.

B$_{10}$ = lymphocytes from sheep B pretreated with Mitomycin C at a concentration of 10µg/ml.

B$_{50}$ = lymphocytes from sheep B pretreated with Mitomycin C at a concentration of 50µg/ml.

Ratio = counts per minute in test cultures to mean counts per minute in separate control cultures.
TABLE VIII-2

<table>
<thead>
<tr>
<th>Mixed lymphocyte culture</th>
<th>(^3\text{H\ thymidine uptake})</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/culture ±S.E.</td>
<td></td>
</tr>
<tr>
<td>R+D(_{10})</td>
<td>138,000 ±45,000</td>
<td>400</td>
</tr>
<tr>
<td>R+D(_{50})</td>
<td>155,500 ±40,000</td>
<td>540</td>
</tr>
<tr>
<td>R</td>
<td>520 ±130</td>
<td>-</td>
</tr>
<tr>
<td>D(_{10})</td>
<td>175 ±175</td>
<td>-</td>
</tr>
<tr>
<td>D(_{50})</td>
<td>60 ±10</td>
<td>-</td>
</tr>
</tbody>
</table>

The response of specifically immune lymphocytes to stimulation by Mitomycin C treated donor cells.

R = immune recipient sheep lymphocytes.
D\(_{10}\) = normal donor sheep lymphocytes pretreated with Mitomycin C at a concentration of 10\(\mu\)g/ml.
D\(_{50}\) = normal donor sheep lymphocytes pretreated with Mitomycin C at a concentration of 50\(\mu\)g/ml.
Ratio = ratio of counts per minute in test cultures over mean counts per minute in separate control cultures.
A comparison of the antibody binding capacity of normal and Mitomycin C (50µg/ml) treated sheep lymphocytes.

$^{51}$Cr release following incubation of cells in normal serum was 13% of total uptake.
The Response of the Sheep Popliteal Lymph Node to Allogeneic Lymphocytes

Virgin ewes were used in the following experiments to ensure that as far as possible, primary immune responses would result from the injection of the allogeneic lymphocytes.

The popliteal efferent ducts of both donor and recipient sheep were cannulated at least two days before lymphocytes were collected from the donor and injected into the recipient. The injected lymphocytes were 98% small lymphocytes. In all these experiments, $3 \times 10^8$ viable lymphocytes were injected into the recipient sheep unless otherwise stated. Lymphocytes were injected into the outer aspect of the lower hind limb of the recipient and the subsequent immune response was monitored in the efferent popliteal lymph.

The immune response which resulted from the injection of lymphocytes into an allogeneic recipient was a vigorous one (Figure VIII-1), the cell numbers in the lymph increased to a peak 7 days after the cells were injected; the response declined thereafter to reach prestimulation levels some 15 days after the transfer of lymphocytes.

The immune response to lymphocytes pretreated with 10µg/ml Mitomycin C was quantitatively less than the response to untreated lymphocytes (Figure VIII-2) although it was still quite vigorous and well sustained. The maximum cell output in the lymph occurred 3-5 days after lymphocyte transfer, some four days earlier than the maximum response to untreated lymphocytes.

Lymphocytes treated with 50µg/ml Mitomycin C gave significantly diminished immune responses (Figure VIII-3). Maximum cell output in the lymph occurred 3-5 days after the transfer of lymphocytes and the overall reaction was quite feeble.
Primary immune response to $3 \times 10^8$ viable allogeneic lymphocytes.
Primary immune response to $3 \times 10^8$ allogeneic lymphocytes that were pretreated with Mitomycin C at a concentration of 10µg/ml.
Primary immune response to $3 \times 10^8$ allogeneic lymphocytes that were pretreated with Mitomycin C at a concentration of 50µg/ml.
Primary immune responses to $3 \times 10^8$ rat lymphocytes.

Upper graph shows the response to untreated viable rat lymphocytes.

Lower graph shows the response to rat lymphocytes that were pretreated with Mitomycin C at a concentration of 50µg/ml.
These experiments demonstrated that lymphocytes pretreated with Mitomycin C gave a more rapid but less vigorous immune response when injected into allogeneic recipients than that induced by the untreated lymphocytes.

The Immune Response to Rat Lymphocytes

Figure VIII-4 compares the immune responses to $3 \times 10^8$ rat lymphocytes and $3 \times 10^8$ Mitomycin C (50µg/ml) pretreated rat lymphocytes. The treatment of rat lymphocytes with Mitomycin C had no demonstrable effect upon their immunogenicity; both responses were feeble and quantitatively and qualitatively comparable. The maximum cellular output in the lymph occurred around 3-4 days in both responses; this is a feature commonly shown by xenogeneic antigens.

The in vitro experiments reported in this chapter suggested that Mitomycin C did not cause any alteration or loss of lymphocyte histoincompatible antigens whilst the in vivo experiments demonstrated that pretreatment of lymphocytes with Mitomycin C resulted in a more rapid but diminished immune response when these lymphocytes were injected into allogeneic recipients. These effects were shown to be dependent upon Mitomycin C dose.

The immune response induced by rat lymphocytes was not altered when these lymphocytes were pretreated with 50µg/ml Mitomycin C and it is thought that rat lymphocytes, although antigenic, do not elaborate any factors which might increase the response of sheep lymphocytes to rat antigens.

It was shown in Chapter VII that some factor which enhanced the uptake of $^3$H thymidine in PHA stimulated lymphocytes in vitro, appeared in the lymph draining a popliteal lymph node responding to a primary dose
of allogeneic lymphocytes. If indeed such a factor or factors are elaborated by lymphocytes during the course of an allogeneic response then the introduction of independent antigens some time after the transfer of lymphocytes to an allogeneic recipient, should result in enhancement or suppression of the immune response to the independent antigen, the eventual outcome being dependent upon the time at which the independent antigen is administered in relation to the injection of lymphocytes into the allogeneic recipient. The allogeneic effect may be similar or identical to antigenic competition, a phenomenon discussed in Chapter V.

Further experiments were designed to test this hypothesis and to determine those mechanisms that might operate to control immune responses.

The Effect of Allogeneic Lymphocytes on the Immune Response to $2 \times 10^9$ Chicken Red Blood Cells

1. Chicken red blood cells injected with normal allogeneic lymphocytes - The concurrent administration of allogeneic lymphocytes and chicken red blood cells gave rise to a very vigorous and prolonged immune response (Figure VIII-5) in comparison to the normal primary immune response to chicken red blood cells (Chapter III, Figure III-4). The total cell and blast cell output in the lymph reached maximum levels on day 6-7, while the output of plaque-forming cells that were specific for chicken red blood cells showed two peaks, one at day 3-4 and one at day 6-7. The maximum plaque-forming cell output in the normal response to chicken red blood cells occurs around day 3-4 while the maximum blast cell output in normal allogeneic lymphocyte responses occurs at day 6-7. These results suggested that cells secreting specific antibody against chicken red blood cells appear first in response to the antigen which induced
their formation; as a secondary event further specific antibody-secreting cells appeared due to the non-specific effects of the allogeneic lymphocyte reaction. The immune response produced $3.03 \times 10^8$ plaque-forming cells and this represented a 15-fold enhancement above the levels of a normal primary immune response to chicken red blood cells (Table VIII-4). The output of specific IgM and IgG haemagglutinating antibody in the lymph was significantly greater than in the normal response to chicken red blood cells and continued over a longer period.

2. Chicken red blood cells injected with allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C - The immune response to chicken red blood cells was enhanced (Figure VIII-6) although the numbers of blast cells and plaque-forming cells which appeared in the lymph were significantly less than in the response to chicken red blood cells plus normal allogeneic lymphocytes. There were two peaks in the output of plaque-forming cells, one at day 3-4 and one at day 6. The total output of plaque-forming cells was $7 \times 10^7$ (Table VIII-4), about three and a half times the number produced in control responses to chicken red blood cells. The output of specific IgM and IgG haemagglutinating antibody in the lymph was increased and reached levels comparable to those in the response to chicken red blood cells plus normal allogeneic lymphocytes. The output of both IgM and IgG antibody continued over a long period.

3. Chicken red blood cells injected with allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C - There was some enhancement of the immune response to chicken red blood cells (Figure VIII-7). The output of blast cells was not significantly increased but more plaque-forming cells appeared in the lymph than normal. The total output of plaque-forming cells that were specific for chicken red blood cells was $5.8 \times 10^7$ (Table VIII-4), about three times the number which appeared in
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $2 \times 10^9$ chicken red blood cells and $3 \times 10^8$ allogeneic lymphocytes administered concurrently.

Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $2 \times 10^9$ chicken red blood cells and $3 \times 10^8$ allogeneic lymphocytes administered concurrently. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 10µg/ml.

Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $2 \times 10^9$ chicken red blood cells and $3 \times 10^8$ allogeneic lymphocytes administered concurrently. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 50µg/ml.

Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
the control response. There was now only one peak in the plaque-forming cell output, on day 3. The output of specific IgM and IgG haemagglutinating antibody in the lymph was significantly less than in control responses to chicken red blood cells alone.

4. **Chicken red blood cells injected 24 hours after normal allogeneic lymphocytes** - The output of total cells and blast cells and the plaque-forming cell response in the lymph were significantly enhanced (Figure VIII-8). The total output of plaque-forming cells that were specific for chicken red blood cells was $1.82 \times 10^8$ (Table VIII-4), about nine times the number observed in the control response. There was a single peak in the output of plaque-forming cells, on day 5-6. The output of specific IgM and IgG haemagglutinating antibody in the lymph was significantly greater than in control responses to chicken red blood cells alone and continued over a longer period.

5. **Chicken red blood cells injected 24 hours after allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C** - The total cell and blast cell output in the lymph was significantly less than in the response to chicken red blood cells injected 24 hours after normal allogeneic lymphocytes (Figure VIII-9). The total output of plaque-forming cells that were specific for chicken red blood cells was $3.3 \times 10^7$ (Table VIII-4) just under twice the number which appeared in the control response to chicken red blood cells. A single peak in the plaque-forming cell output occurred on day 6. The output of specific IgM haemagglutinating antibody was low while the output of specific IgG haemagglutinating antibody was significantly increased above control levels and continued to rise throughout the experiment.
6. **Chicken red blood cells injected 24 hours after allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C** - The response to chicken red blood cells was depressed below control levels and there was little change in the output of total cells or blast cells in the lymph (Figure VIII-10). The total output of plaque-forming cells that were specific for chicken red blood cells was $9.8 \times 10^6$ (Table VIII-4) half the number that appeared in the control response to chicken red blood cells alone. The output of specific IgM and IgG haemagglutinating antibody in the lymph was significantly less than in the control response.

7. **Chicken red blood cells injected 48 hours after normal allogeneic lymphocytes** - The output of total cells and blast cells in the lymph was enhanced (Figure VIII-11). The output of plaque-forming cells was maximum on day 6 and the total output of plaque-forming cells that were specific for chicken red blood cells was $4.53 \times 10^8$ (Table VIII-4) about twenty-one times the number which appeared in the control response to chicken red blood cells alone. The output of specific IgM and IgG haemagglutinating antibody in the lymph was significantly increased above the control level and continued over a longer period.

8. **Chicken red blood cells injected 48 hours after allogeneic lymphocytes pretreated with 10µg/ml Mitomycin C** - The maximum output of total cells in the lymph occurred at day 3-4 whereas the maximum output of blast cells occurred at day 6 coinciding with the peak output of plaque-forming cells (Figure VIII-12). The overall immune response was enhanced above control levels although it was not as vigorous as the response initiated by chicken red blood cells injected 48 hours after normal allogeneic lymphocytes. The total output of plaque-forming cells that were specific for chicken red blood cells was $9.2 \times 10^7$ (Table VIII-4) five times the...
Primary immune response to allogeneic lymphocytes and chicken red blood cells. 3 x 10^8 allogeneic lymphocytes administered 24 hours prior to 2 x 10^9 chicken red blood cells. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 24 hours prior to $2 \times 10^9$ chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 10µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells.  $3 \times 10^8$ allogeneic lymphocytes administered 24 hours prior to $2 \times 10^9$ chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 50µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
number observed in the control response to chicken red blood cells alone. The output of specific IgM and IgG haemagglutinating antibody in the lymph was enhanced above control levels but much less so than in responses to chicken red blood cells injected 48 hours after normal allogeneic lymphocytes.

9. Chicken red blood cells injected 48 hours after allogeneic lymphocytes pretreated with 50µg/ml Mitomycin C - The output of total cells and blast cells in the lymph was much less than in the responses to chicken red blood cells injected 48 hours after normal or 10µg/ml Mitomycin C treated allogeneic lymphocytes (Figure VIII-13). The maximum output of plaque-forming cells that were specific for chicken red blood cells occurred on day 6 and the total output of plaque-forming cells was $3.6 \times 10^7$ (Table VIII-4) - almost twice the number observed in the control response to chicken red blood cells alone. The output of specific IgM and IgG haemagglutinating antibody in the lymph was almost completely suppressed.

10. Chicken red blood cells injected 120 hours after allogeneic lymphocytes - At the time the chicken red blood cells were injected (120 hours) the output of blast cells in the lymph was still high. The total output of plaque-forming cells that were specific for chicken red blood cells was $8 \times 10^7$ (Table VIII-4), this was four times the number which appeared in the control response to chicken red blood cells alone. The output of specific IgM and IgG haemagglutinating antibody in the lymph was greater than in the control response.

The injection of chicken red blood cells 120 hours after the injection of Mitomycin C (10µg/ml and 50µg/ml) treated lymphocytes gave rise to feeble immune responses. The total number of plaque-forming
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 10µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 50µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
cells that were specific for chicken red blood cells was $3.5 \times 10^6$ (10µg/ml Mitomycin C treated lymphocytes) and $2.9 \times 10^6$ (50µg/ml Mitomycin C treated lymphocytes) about one-sixth of the number of plaque-forming cells that appeared in the control response to chicken red blood cells alone (Table VIII-4). The output of specific IgM and IgG haemagglutinating antibody in the lymph was less than in the control response.

11. Chicken red blood cells injected 260 hours after allogeneic lymphocytes - By 260 hours the response to the allogeneic lymphocytes was almost over (Figures VIII-1, VIII-2, VIII-3) and the injection of chicken red blood cells at this time gave rise to feeble immune responses (Figure VIII-14). Chicken red blood cells injected 260 hours after the injection of lymphocytes pretreated with either 10µg/ml or 50µg/ml Mitomycin C gave similarly feeble responses (Figures VIII-15 and VIII-16). The output of blast cells was lower than in control responses and the total output of plaque-forming cells that were specific for chicken red blood cells was less than one third of the number that appeared in the control response to chicken red blood cells alone (Table VIII-4).

The output of specific IgM and IgG haemagglutinating antibody in the lymph was low in these responses and no IgG antibody could be detected during the immune response to chicken red blood cells injected 260 hours after the injection of 10µg/ml Mitomycin C pretreated lymphocytes (Figure VIII-15).

The Immune Response to $2 \times 10^9$ Chicken Red Blood Cells Injected 48 Hours after $3 \times 10^8$ Rat Lymphocytes

Normal rat lymphocytes and 50µg/ml Mitomycin C pretreated rat lymphocytes were used to compare the enhancing effects of xenogeneic
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 260 hours prior to $2 \times 10^9$ chicken red blood cells. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. 3 x 10^8 allogeneic lymphocytes administered 260 hours prior to 2 x 10^9 chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 10µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to allogeneic lymphocytes and chicken red blood cells. $3 \times 10^8$ allogeneic lymphocytes administered 260 hours prior to $2 \times 10^9$ chicken red blood cells. Allogeneic lymphocytes were pretreated with Mitomycin C at a concentration of 50µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
lymphocytes and allogeneic lymphocytes on responses to chicken red blood cells.

Rat lymphocytes alone gave a feeble immune response (Figure VIII-4) which was not significantly different from the response induced by 50µg/ml Mitomycin C pretreated rat lymphocytes. The immune response to normal and Mitomycin C pretreated rat lymphocytes enhanced the immune response to chicken red blood cells given 48 hours after the lymphocytes (Figures VIII-17, VIII-18). The output of total cells and blast cells in the lymph was not significantly different from that observed in the response to rat lymphocytes alone. The total output of plaque-forming cells that were specific for chicken red blood cells was $3.3 \times 10^7$ (normal rat lymphocytes) and $3.4 \times 10^7$ (50µg/ml Mitomycin C treated rat lymphocytes), less than a two-fold increase above the numbers that appeared in the control response to chicken red blood cells alone (Table VIII-4). The maximum output of plaque-forming cells occurred on day 6, that is, on the fourth day after the injection of chicken red blood cells. There were few quantitative or qualitative differences between the responses. The output of specific IgM haemagglutinating antibody was comparable to that in the control response to chicken red blood cells alone, whilst the output of IgG haemagglutinating antibody was less than in the control response.

The effect of treating allogeneic lymphocytes with Mitomycin C and the time interval between allogeneic lymphocyte and chicken red blood cell injections upon the subsequent immune response to chicken red blood cells, as measured by the total plaque-forming cell output in the lymph is shown in Figure VIII-19.

The total output of plaque-forming cells in each immune response is presented in Table VIII-4.
Primary immune response to rat lymphocytes and chicken red blood cells. $3 \times 10^8$ rat lymphocytes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
Primary immune response to rat lymphocytes and chicken red blood cells. $3 \times 10^6$ rat lymphocytes administered 48 hours prior to $2 \times 10^9$ chicken red blood cells. Rat lymphocytes were pretreated with Mitomycin C at a concentration of 50µg/ml. Haemagglutinating antibodies and plaque-forming cells that were directed against chicken red blood cells are shown.
The effect of different doses of Mitomycin C and the time interval between allogeneic lymphocyte and chicken red blood cell administration upon the total output of plaque-forming cells in the lymph. Plaque-forming cells that were directed against chicken red blood cells are shown.
<table>
<thead>
<tr>
<th>First immunogen</th>
<th>Time $2 \times 10^9$ CRBC introduced in response to allogeneic lymphocytes (hours)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>$3 \times 10^8$ allogeneic lymphocytes</td>
<td>303</td>
</tr>
<tr>
<td>$3 \times 10^8$ allogeneic lymphocytes 10µg/ml Mitomycin C</td>
<td>69</td>
</tr>
<tr>
<td>$3 \times 10^8$ allogeneic lymphocytes 50µg/ml Mitomycin C</td>
<td>58</td>
</tr>
<tr>
<td>$3 \times 10^8$ rat lymphocytes</td>
<td>33</td>
</tr>
<tr>
<td>$3 \times 10^8$ rat lymphocytes 50µg/ml Mitomycin C</td>
<td>34</td>
</tr>
<tr>
<td>$3 \times 10^8$ autologous lymphocytes</td>
<td>20</td>
</tr>
<tr>
<td>No immunogen</td>
<td>20</td>
</tr>
</tbody>
</table>

The total number of plaque-forming cells ($x \times 10^6$) appearing in the lymph in immune responses to chicken red blood cells injected at different times after the injection of normal or Mitomycin C pretreated allogeneic lymphocytes.
DISCUSSION

Allogeneic lymphocytes when injected with xenogeneic antigens can alter the extent and intensity of the immune response to these antigens. The effect is thus similar to that which occurs with histoincompatible antigens. The time at which the xenogeneic antigen is injected with respect to the time of induction of the allogeneic response determines the outcome of the immune response.

The experimental evidence presented in Chapter VII indicated that mitogenic factors were elaborated during the early phases of primary immune responses and the experimental results presented in this chapter can be explained on this basis. Allogeneic lymphocytes and xenogeneic antigens injected together or within short periods of time of one another probably act synergistically to induce the elaboration of mitogen(s), thus there would be greater cell proliferation in the response to both antigens assuming that these mitogen(s) act in a non-specific way. The increased cell proliferation would result in greater numbers of antibody-forming cells being produced. The evidence presented in Chapter VII suggested that the antibody produced is not secreted unless factor(s) controlling its release from the cells are also present. It was shown that the factor(s) which stimulated antibody secretion appeared in the lymph around the time at which the cell output in the lymph was at a maximum. In this respect it is significant that the two peaks of plaque-forming cell output which occurred when allogeneic lymphocytes were administered concurrently with chicken red blood cells, coincided with the time at which normal chicken red blood cell and allogeneic lymphocyte primary immune responses respectively, were at their maximum. The elaboration of factor(s) which stimulate the secretion of antibody by antibody-forming cells probably occurs at these times.
At the time at which the output of plaque-forming cells reached a second peak (6-7 days after the injection of chicken red blood cells) the secretion of antibody by antibody-forming cells would be suppressed by factor(s) elaborated during the course of the normal primary immune response to chicken red blood cells (discussed in Chapter VII). The normal immune response to allogeneic lymphocytes is at a maximum at around 6-7 days and factor(s) which stimulate secretion of antibody by antibody-forming cells may appear in the lymph at this time. These factors would stimulate the secretion of antibody by cells produced in response to chicken red blood cells.

The increased output of plaque-forming cells in these responses would appear to result, at least in part from the production of an increased number of antibody-forming cells; in turn those factors which stimulate antibody-forming cells to secrete antibody are probably present at higher concentrations and for greater periods of time than in the normal primary immune response to chicken red blood cells.

When allogeneic lymphocytes treated with 50µg/ml Mitomycin C and chicken red blood cells were injected together there was only one peak output of plaque-forming cells in the lymph (Figure VIII-7). The maximum output of blast cells in response to allogeneic lymphocytes treated in this way occurred at about the same time as the blast cells reached their maximum output in the response to chicken red blood cells and this probably explains the single peak in the output of plaque-forming cells.

When chicken red blood cells were injected 24 hours after allogeneic lymphocytes the response to the chicken cells was enhanced although the degree of enhancement was less than that observed when allogeneic lymphocytes and chicken red blood cells were injected together. These results were similar to those obtained when chicken
red blood cells were injected 24 hours after rabbit red blood cells or Salmonella organisms (Chapter V).

There was a significant enhancement of the immune response to chicken red blood cells when this antigen was injected 48 hours after the injection of allogeneic lymphocytes. Chicken red blood cells were injected during the early phase of the immune response to allogeneic lymphocytes at a time when mitogenic factor(s) were being elaborated. The maximum cellular output in response to both allogeneic lymphocytes and chicken red blood cells would occur at about the same time and thus there should be a single peak output of plaque-forming cells; this in fact was the case. The output of plaque-forming cells was greatly enhanced (Figure VIII-11) and again this was probably the result of a greater number of antibody-forming cells being produced through the action of increased amounts of mitogenic factor(s). The increased numbers of antibody-forming cells would be stimulated to secrete more antibody under the influence of an increased level of those factor(s) which non-specifically stimulate the release of antibody from cells synthesising it.

Results obtained from the in vitro experiments suggested that whilst Mitomycin C, at the doses tested, did not cause any alteration or loss of histoincompatible antigens from lymphocytes, it nevertheless inhibited their capacity to divide.

Lymphocytes treated with 10µg/ml Mitomycin C were capable of stimulating the uptake of $^3$H thymidine in mixed lymphocyte cultures whereas treatment of lymphocytes with 50µg/ml Mitomycin C inhibited this ability. These results indicated that some factor(s) which is elaborated by lymphocytes was necessary to induce cell proliferation in other lymphocytes in the presence of histoincompatible antigens.
The *in vivo* experiments demonstrated that the degree of enhancement of chicken red blood cell responses was greatest when chicken red blood cells were injected together with or 48 hours after normal allogeneic lymphocytes and least when chicken red blood cells were injected with or 48 hours after 50μg/ml Mitomycin C treated allogeneic lymphocytes.

The results of both *in vitro* and *in vivo* experiments would suggest that the mitogen is elaborated by lymphocytes and its mode of action is non-specific in that it may stimulate lymphocytes to proliferate in the presence of their specific antigen. It may be that the mitogen is elaborated by T lymphocytes and that this is the non-specific factor involved in the interaction between T and B lymphocytes.

The immune response to chicken red blood cells injected 120 hours after normal allogeneic lymphocytes was significantly enhanced above control levels. In this case chicken red blood cells were injected before the cellular output in the lymph had reached a maximum and at a time when mitogenic factor(s) were probably present in the lymph. The maximum response to chicken red blood cells would be expected to occur around the time that the response to allogeneic lymphocytes is at a maximum and this was the case.

When chicken red blood cells were injected 120 hours after Mitomycin C (10μg/ml or 50μg/ml) treated allogeneic lymphocytes, then the immune response to chicken red blood cells was significantly suppressed. Chicken red blood cells were injected after the maximum cellular response to the allogeneic lymphocytes. Cell output and plaque-forming cell output in the lymph was significantly less than in the control response to chicken red blood cells alone which suggested that some mechanism was operating to suppress the proliferation of antibody-forming cell
precursors. Similar suppressed responses were observed when chicken red blood cells were injected 260 hours after the injection of normal or Mitomycin C treated allogeneic lymphocytes.

There are many reports in the literature indicating that GVH responses suppress immune responses to unrelated antigens when the antigen is administered at least 6 days after the initiation of the GVH response. Thus GVH responses have been shown to suppress the immune response to the H antigen of Salmonella typhi (Howard and Woodruff, 1961), sheep red blood cells and E. coli LPS (Møller, 1971), Pneumococcal polysaccharide SIII (Byfield, Christie and Howard, 1973) and skin allografts (Lapp and Møller, 1969).

The role of T lymphocytes in regulating the normal immune response to antigen may be two-fold (Katz, 1972); initially they may provide an activating signal to B lymphocytes which otherwise would fail to react to the specific antigen, while some time later T lymphocytes may elaborate another factor(s) which inhibits the proliferation of B lymphocytes even in the presence of specific antigen. These proposed regulatory controls by T lymphocytes acting either to prevent or induce reactions against an antigen are, however, quite hypothetical.

Experiments in Chapter VII have shown that popliteal efferent lymph collected after the maximum cell output occurred in response to a primary dose of chicken red blood cells, contains some factor capable of suppressing the initiation of an immune response when it is injected with antigen; further experiments have shown that the administration of specific antigen, unrelated antigens or allogeneic lymphocytes after the maximum cellular response to a primary dose of chicken red blood cells has occurred, resulted in significantly suppressed immune responses to these antigens.
Experiments in Chapter VII have shown that after the immune response to chicken red blood cells has reached a maximum, some factor(s) which inhibits the secretion of antibody by antibody-forming cells appeared in the lymph.

It is suggested that when chicken red blood cells were injected after the response to allogeneic lymphocytes had reached a maximum, factors were present in the lymph node and lymph which inhibited the proliferation of those lymphocytes specific for chicken red blood cells. Those antibody-forming cells that were produced came under the influence of some factor(s) which inhibited them from secreting their antibody.

In conclusion, the experimental evidence presented in this chapter has shown that the ability of allogeneic lymphocytes to either enhance or suppress the immune response to a subsequently administered antigen is dependent upon the interval between antigen administration and the initiation of the allogeneic response. The intervals between antigen administration and the expression of these effects were similar to those determined in antigenic enhancement and inhibition experiments (Chapter V) and are a function of the state of reactivity in the lymph node to the antigen initially administered.

It is suggested that allogeneic enhancement and inhibition effects are controlled by the same mechanisms as those which control antigenic enhancement and inhibition effects.
CHAPTER IX

THE SYNTHESIS OF NON-SPECIFIC IMMUNOGLOBULINS
DURING THE IMMUNE RESPONSE TO SPECIFIC ANTIGENS
INTRODUCTION

There are reports of several investigations in the early literature which demonstrate that following the injection of an antigen, antibodies with specificities for other antigens are synthesised (Freund, 1953). During the course of certain infectious diseases such as malaria and lobar pneumonia, agglutination titres against typhoid bacilli have been observed to rise in persons previously vaccinated with heat killed typhoid bacilli (Weil and Felix, 1916). Rabbits which have been primed intravenously with killed Shigella dysenteriae organisms and injected with either killed Shigella paradysenteriae or killed Shigella typhosa organisms three weeks later, produced antibodies against Shigella dysenteriae during the course of the second immune response even though there was no known common antigenic determinant between the two types of dysentery bacilli or between the dysentery and typhoid bacilli (Bieling, 1919).

More recently there have been several reports of the concomitant synthesis of specific antibody and non-specific immunoglobulins during the course of immune responses to various antigens. Urbain-Vansanten (1970) showed that the injection of tobacco mosaic virus into rabbits induced the synthesis of both specific and non-specific immunoglobulins. The lymph nodes of rabbits injected with tyrosinase incorporated into an emulsion with Freund's complete adjuvant, produced more immunoglobulin-synthesising cells than specific antibody-producing cells at all phases of the immune response (Antoine and Avrameas, 1973). Furthermore, the first immunoglobulin-synthesising cells detected were hypertrophied plasma cells whilst the first antibody-producing cells were immature plasmacytes or blast cells (Antoine and Avrameas, 1973). Similar
findings have been reported in mice using horse radish peroxidase as an antigen (Miller, Ternynck and Avrameas, 1974). It may be that mechanisms which operate to control the immune response to an antigen, exert some influence upon the activities of cells that are not participating in the specific immune response; such cells may be induced to synthesise and secrete immunoglobulins which will show no specificity for the antigen.

The question of non-specific immunoglobulin synthesis during an immune response was examined to determine whether or not the non-specific immunoglobulins synthesised showed any specificity for antigens which the animal had experienced previously.

RESULTS

The Secondary Immune Response to Horse Radish Peroxidase

Horse radish peroxidase (10 mg Sigma grade VI) emulsified in 1 ml of Freund's complete adjuvant was injected into the drainage area of the popliteal lymph node. At the same time, 10 mg of horse radish peroxidase emulsified in 1 ml of Freund's complete adjuvant or 1 ml of Freund's complete adjuvant alone was injected subcutaneously into the contralateral leg in the popliteal drainage area. Four weeks after the injection of the antigen the efferent lymph ducts of both popliteal lymph nodes were cannulated and several days later 2.5 mg of horse radish peroxidase in 2.5 ml PBS was injected to produce secondary immune responses in the previously primed popliteal nodes (Figures IX-1 and IX-2).

Both the total cell output and the output of blast cells in the efferent lymph were monitored frequently throughout the immune
Secondary immune response to horse radish peroxidase in a popliteal lymph node primed four weeks previously with horse radish peroxidase and Freund's complete adjuvant.
Secondary immune response to horse radish peroxidase in a popliteal lymph node primed four weeks previously with Freund's complete adjuvant.
response and cells containing immunoglobulins or specific antibody were detected by the methods described previously. The number of cells containing non-specific immunoglobulins was calculated by subtracting the number of specific antibody-forming cells from the number of cells containing immunoglobulins.

The secondary immune response to horse radish peroxidase in directly or indirectly primed popliteal lymph nodes was vigorous and large numbers of blast cells appeared in the efferent lymph. Non-specific immunoglobulin-containing cells appeared at the same time as specific antibody-containing cells although their peak output in the lymph occurred earlier.

These experiments suggested that an antigenic stimulus could induce lymphocytes to produce immunoglobulins which have no specificity for the antigen; it may be that as the antigen is processed, determinants are exposed or produced that induce the synthesis of antibodies with new specificities.

Cells appearing in the efferent lymph draining popliteal nodes responding to horse radish peroxidase were assayed for anti-chicken red blood cell and anti-self red blood cell activity by the plaque assay; no plaque-forming cells were detected.

The Primary Immune Response to $3 \times 10^8$ Viable Allogeneic Lymphocytes

Allogeneic lymphocytes were injected subcutaneously into the drainage area of the popliteal lymph node of a sheep which had not been challenged previously with chicken red blood cell antigens. Cellular changes that occurred in the efferent lymph were monitored frequently and the cells assayed for their ability to lyse chicken red blood cells in plaque-forming cell assays.
Primary immune response to allogeneic lymphocytes in a sheep which had no previous experience of chicken red blood cells. The output in the efferent lymph of plaque-forming cells specific for chicken red blood cells.
A vigorous immune response was elicited by the allogeneic lymphocytes (Figure IX-3) and cells secreting antibody specific for chicken red blood cells were detected by the plaque assay. The appearance of these plaque-forming cells correlated closely with the kinetics of the immune response induced by the allogeneic lymphocytes although the number of plaque-forming cells were much fewer than the number of blast cells. A total of \(2.85 \times 10^5\) plaque-forming cells secreting antibody specific for chicken red blood cells was detected in the efferent lymph. Plaque-forming cells were not detected in the efferent lymph prior to the injection of allogeneic lymphocytes and none were found in the population of donor lymphocytes.

This experiment demonstrated that within a population of cells that were synthesising non-specific and specific immunoglobulins there were some cells that were also synthesising and secreting antibody specific for at least one other antigen. The non-specific immunoglobulins synthesised during a response to a specific antigen may be antibodies specific to other antigens which the animal has encountered previously, or to antigens not previously encountered but which cross-react with the specific antigen. Further experiments were designed to test these possibilities.

**The Primary Immune Response to \(3 \times 10^8\) Viable Allogeneic Lymphocytes Injected 5 Weeks after the Injection of \(2 \times 10^9\) Chicken Red Blood Cells**

Chicken red blood cells were injected subcutaneously into the drainage area of the popliteal lymph node. Four weeks later the efferent popliteal lymph duct was cannulated and the cell population assayed for plaque-forming cells directed against chicken red blood cells. No plaque-forming cells were detected at any time during the first week after cannulation. At the end of the first week, that is, five weeks
after injecting the chicken red blood cells, $3 \times 10^8$ allogeneic lymphocytes were injected at the same site. The lymphocytes were obtained from the efferent popliteal lymph of a sheep which had not been previously challenged with chicken red blood cell antigens and there were no plaque-forming cells against chicken red blood cells detectable in this lymphocyte population. Cellular changes in the efferent lymph were monitored frequently and the cells assayed for their ability to form plaques against chicken red blood cells.

The immune response elicited by the allogeneic lymphocytes was feeble (Figure IX-4) when compared to those immune responses demonstrated previously (Figure IX-3 and Chapter VIII); this was probably due to the suppressive influence of the previous challenge with chicken red blood cells some five weeks earlier (a phenomenon discussed in Chapter V). Cells that were secreting antibody specific for chicken red blood cells were first detected 65 hours after the allogeneic lymphocytes were injected and their appearance correlated with the increased output of total cells and of blast cells in the efferent lymph (Figure IX-4). A total of $1.55 \times 10^6$ plaque-forming cells that were specific for chicken red blood cells was detected during the immune response to allogeneic lymphocytes.

This experiment demonstrated that in response to a challenge with allogeneic lymphocytes, cells were produced which secreted antibodies against an antigen which had been encountered previously. Antibody produced in these circumstances would be described as non-specific as its specificity bore no relation to the antigen which induced its formation.

The feeble immune response elicited by allogeneic lymphocytes in the sheep which had encountered chicken red blood cells previously,
Primary immune response to allogeneic lymphocytes in a sheep popliteal lymph node that had been primed with chicken red blood cells five weeks previously. The output in the efferent lymph of plaque-forming cells specific for chicken red blood cells.
gave rise to five times the number of cells secreting antibody against chicken red blood cells than did the vigorous immune response to allogeneic lymphocytes in the sheep which had not encountered chicken red blood cells previously. These results would suggest that cells which were activated or derived from the previous encounter with chicken red blood cells, were in some way influenced by the immune response to allogeneic lymphocytes, to synthesise and secrete antibody against chicken red cells.

The Production of Cells Secreting Antibody Against Rabbit Red Blood Cells following the Injection of $2 \times 10^9$ Chicken Red Blood Cells

An experiment was designed to see if cells secreting antibody against rabbit red blood cells would reappear during the primary immune response to chicken red blood cells in a sheep that had encountered rabbit red blood cells previously. Rabbit red blood cells ($2 \times 10^9$) were injected subcutaneously into the drainage area of the popliteal lymph node and the immune response was monitored in the efferent lymph. The cell output in the efferent lymph had fallen to prestimulation levels 250 hours after the rabbit red cells had been injected and no plaque-forming cells against either rabbit or chicken red blood cells could be detected.

Chicken red blood cells were then injected at the same site, 260 hours after the injection of rabbit red blood cells. The efferent lymph was monitored at frequent intervals for plaque-forming cells against either rabbit or chicken red blood cells. There was no cross-reactivity between anti-rabbit and anti-chicken plaque-forming cells.

Efferent lymph cells that were secreting antibody against rabbit or chicken red blood cells were first observed 50 hours after the
Primary immune response to chicken red blood cells in a sheep popliteal lymph node that had been primed with rabbit red blood cells 280 hours previously. The output in the efferent lymph of plaque-forming cells specific for rabbit or chicken red blood cells.
injection of the chicken red blood cells (Figure IX-5). The increased numbers of specific plaque-forming cells to both rabbit and chicken red blood cells correlated with the increased output of total and blast cells in the efferent lymph and the peak of the plaque-forming cell responses coincided with the peak of both total and blast cell output. The peak numbers of plaque-forming cells against chicken red blood cells were greater than for rabbit red blood cells (Figure IX-5). The total number of plaque-forming cells appearing against chicken red blood cells was $1.9 \times 10^6$. The feeble immune response was due to the suppressive effect of the previous immune response to rabbit red blood cells (a phenomenon discussed in Chapter V). A total of $6.36 \times 10^5$ specific plaque-forming cells against rabbit red blood cells was detected during the immune response to chicken red blood cells. This experiment demonstrated further that an antigen can induce the formation and secretion of antibodies whose specificity is directed against a previously encountered antigen.

**DISCUSSION**

The results of the experiments presented in this chapter demonstrated that at least a proportion of the non-specific immunoglobulin-producing cells which appeared in efferent lymph following a specific antigenic challenge, were synthesising and secreting antibody directed against an antigen which the animal had previously encountered. These findings suggest that anamnestic responses can be elicited by antigens in a non-specific way. This concept implies that the greater the antigenic experience of the animal, and this would be a function of the age of the animal, then the more non-specific immunoglobulin would be synthesised in response to each antigenic challenge. This concept has not been proven as yet.
Pachmann, Killander and Wigzell (1974) showed that in mice immunised with sheep red blood cells, almost all the lymphoid cells of the spleen had an increase in intracellular immunoglobulins some 3-4 days after immunisation. They suggested that the phenomenon may be due to the production of some material which is capable of stimulating many lymphoid cells to synthesise immunoglobulins.

In the primary immune responses examined in this thesis the number of blast cells appearing in the efferent lymph draining the responding popliteal lymph node greatly exceeded the number of specific antibody-forming cells. It could be postulated that those blast cells which displayed no sign of specific antibody production were either immature antibody-forming cell precursors, antibody-forming cells which had secreted all their antibody, immunoglobulin-producing cells whose product showed no specificity for the inducing antigen, or simply non-antibody synthesising blast cells. It would seem most likely on the evidence presented in this chapter and from the observation that many large mature blast cells do not contain or secrete specific antibody at any stage of the immune response that many non-specific antibody-forming cells are formed during an immune response.

The appearance of blast cells that synthesise non-specific immunoglobulins and the kinetics of their expression as immunoglobulin-secreting cells during the immune response to unrelated antigens can be explained on the basis of concepts developed in Chapters V - VIII. Following antigenic challenge an animal develops specific memory cells which exist in various states of activation. The introduction of an unrelated antigen at some later time may give rise to the production of factor(s) which non-specifically stimulates these cells to proliferate and differentiate into antibody-forming cells. Thus memory cells specific for rabbit red blood cells may be induced to proliferate and
differentiate into antibody-forming cells specific for rabbit red blood cells under the influence of an immune response elicited by chicken red blood cells.

In order for at least some antibody-forming cells to be able to secrete their antibody, certain factors which are elaborated during the immune response are required (Chapter VII). The reappearance of plaque-forming cells against rabbit red blood cells paralleled the appearance of plaque-forming cells against chicken red blood cells during the primary immune response to this antigen (Figure IX-5). This would suggest that during the immune response to chicken red blood cells, factors were elaborated which induced those antibody-forming cells which were synthesising antibody against chicken or rabbit red blood cells, to secrete their antibody. Similar mechanisms are proposed to account for the reappearance of plaque-forming cells against chicken red blood cells during the immune response to allogeneic lymphocytes in sheep which had experienced chicken red blood cells previously.
CHAPTER X

SUMMARY AND CONCLUSIONS
The experiments described in this thesis have been designed to examine and elucidate some of the regulatory mechanisms which operate to control the response of an animal to immunogenic materials.

The cellular and humoral events that occur in the efferent lymph draining the popliteal lymph node have been described following the injection of one or more antigens into the drainage area of the node. When the efferent lymphatic duct of the popliteal lymph node had been cannulated prior to the injection of antigen the immune response was confined to the node.

So far as it is known, the experiments described in this thesis are novel in that they have permitted the examination of immune responses localised in single lymph nodes under physiological conditions and in circumstances in which there is no systemic immune response.

Chapter III - The Normal Immune Response to Chicken Red Blood Cells

The experiments in this chapter examined the temporal sequence of events that occurred in the efferent lymph of the popliteal lymph node after primary or secondary challenge with chicken red blood cells. The antigen was injected into a number of sheep and the ensuing immune responses were characterised by measuring the cellular and humoral changes in the efferent lymph. Chicken red blood cells entered the afferent lymph draining the site into which the antigen had been injected and were conveyed to the subcapsular sinus, cortex and medullary sinuses of the regional popliteal lymph node where they were rapidly phagocytosed. The chicken red blood cells were retained by the lymph node and did not appear in the efferent lymph.
The cell output in the efferent lymph rose for the first thirty minutes after the injection of the antigen; this was followed by a fall in cell output and then by a gradual rise to prestimulation levels after some ten to twelve hours. These events did not take place when autologous lymphocytes or saline were injected. The primary immune response to chicken red blood cells was characterised by an increase in the number of blast cells and plaque-forming cells in the efferent popliteal lymph. These cells increased in numbers for some ninety hours after the antigen was injected whereafter they declined to reach prestimulation levels by 200 hours. IgM haemagglutinating antibody was detected in the efferent lymph before IgG haemagglutinating antibody and the output of IgM antibody was greater than the output of IgG antibody. The extent of these changes could be related to antigen dose; a dose of $2 \times 10^9$ chicken red blood cells gave rise to an immune response that was greater than that induced by $1 \times 10^9$ red cells but not significantly different from that induced by $4 \times 10^9$ red cells.

The cells that were secreting antibody were mostly blast cells in the early stages of the primary immune response whereas an increasing number of small and medium-sized lymphocytes secreted antibody later in the response. Many of the antibody-secreting cells were highly mobile and some were in the process of dividing. A significant proportion of the blast cells did not secrete antibody. At the peak of the immune response the antibody-secreting cells gave rise to larger plaques than did cells collected before or after the peak of the response.

It appeared that the secretion of antibody by antibody-forming cells may be controlled by factors elaborated during the course of an immune response.
In the secondary immune response to $2 \times 10^9$ chicken red blood cells larger numbers of blast cells and plaque-forming cells appeared in the efferent lymph. The output of IgM haemagglutinating antibody in the efferent lymph was low whereas the output of IgG haemagglutinating antibody was significantly higher than in the primary immune response.

Chapter IV - Antibody Mediated Suppression of the Immune Response

Specific IgM and IgG antibodies were isolated from the efferent popliteal lymph before the peak of the response to a primary challenge of $2 \times 10^9$ chicken red blood cells. Chicken red blood cells coated with this specific IgM or IgG antibody produced feeble primary and secondary immune responses. Chicken red cells coated with a mixture of specific IgM and IgG antibodies gave rise to enhanced primary immune responses but normal secondary immune responses.

Chicken red blood cells injected with non-specific IgM, IgG or mixtures of IgM and IgG gave normal primary immune responses. These experiments supported the view that specific IgM or specific IgG antibodies suppress primary and secondary immune responses to the same extent when administered with antigen as antigen-antibody complexes. It was considered that the presence of specific antibody on the surface of the red cell blocked the majority of their antigenic determinants and thereby prevented antigenic interaction with the specific recirculating lymphocytes carrying complementary receptors.

The enhanced primary immune responses and normal secondary immune responses elicited by mixtures of specific IgM and IgG antibody-antigen complexes could not be explained.
Chapter V - Antigenic Competition

The injection of two identical or non-cross reacting antigens into the drainage area of the popliteal lymph node resulted in a modified immune response to both antigens. The extent of the modification depended upon the time the second antigen was injected.

When antigens were injected concurrently or 48 hours apart, the immune response to both antigens was significantly greater than when the antigens were injected alone. When the antigens were injected 24 hours apart the immune response to both antigens was significantly less than when the antigens were injected alone. When the second antigen was injected during the later stages of the immune response to the first antigen (120 hours or 260 hours after its injection), then the immune response elicited by the second antigen was feeble.

It was suggested that the expression of the immune response to an antigen depends upon the physiological state of the lymph node at the time the antigen is injected. When antigen was injected together with another antigen or at some time before the peak of the immune response to the first antigen then the mechanisms which control an immune response acted in synergy to enhance the response to both antigens. Conversely, when the second antigen was injected after the response to the first antigen had reached its maximum, the mechanisms which limited the response to the first antigen, in some way prevented the immune response to the second antigen.

The suppressed immune responses which resulted when two antigens were injected 24 hours apart could be explained on the basis of some refractory period existing in the lymph node; this period might be reinforced by the injection of a second antigen at this time. These
modifications of immune responses were thought to be the result of T and B lymphocyte interactions and maybe related to the capacity of T lymphocytes to initiate and limit the extent of the B lymphocytes' response to antigen.

Chapter VI - The Effect of Antigen-Antibody Complexes on Immune Responses

The injection of antigen-antibody complexes 48 hours prior to the injection of chicken red blood cells gave rise to enhanced immune responses when compared to the responses to the free antigen injected alone. The enhanced responses were shown to be independent of the specificity of the antigen in the antigen-antibody complexes thus chicken or rabbit red blood cell antigen-antibody complexes enhanced the immune response to chicken red blood cells injected subsequently. The immune response to the free antigen exerted an enhancing effect upon the immune response against the antigen complexed to the antibody. In all cases the immune response to the complexed antigen was greater when compared to that elicited by an identical dose of the free antigen. When antigen-antibody complexes were injected 260 hours prior to the injection of the free antigen, then the immune response to both the complexed and the free antigen was depressed.

It was proposed that antigen-antibody complexes in some way stimulated specific T lymphocytes to synthesise and secrete factors(s) which non especifically activated or inactivated B lymphocytes that had antigen bound to their surface receptors, thus the injection of antigen-antibody complexes resulted in the stimulation of T but not B lymphocytes. When free antigen was injected 48 hours after the injection of antigen-antibody complexes then more T lymphocytes were stimulated to synthesise
and secrete factor(s) which permitted the activation of specific B lymphocytes. Conversely, 260 hours after the antigen-antibody complexes were injected, T lymphocytes would be synthesising and secreting factors which prevented the activation of those B lymphocytes that had antigen bound to their receptors.

The ability of free antigen or antigen-antibody complexes to enhance or inhibit the immune response to subsequently injected free antigen was found to depend on the time that the free antigen was injected. The mechanism that underlay both these phenomena was thought to be the same.

Chapter VII - Mediators of Humoral Immunity

Efferent popliteal lymph collected during the period 48 - 96 hours after primary challenge with $2 \times 10^9$ chicken red blood cells enhanced the primary immune response to chicken red blood cells in another sheep. The same lymph increased the uptake of $^3$H thymidine in in vitro cultures of lymphocytes that had previously been incubated with PHA, and enhanced normal lymphocyte transfer reactions. Lymph collected early on in the response to Salmonella muenchen organisms also increased the uptake of $^3$H thymidine in lymphocyte-PHA cultures and enhanced normal lymphocyte transfer reactions.

Lymph collected around the time that the primary immune response against chicken or rabbit red blood cells, allogeneic lymphocytes or Salmonella muenchen organisms was at a maximum, increased the secretion of antibody by antibody-forming cells in vitro. Certain fractions isolated from sheep blood serum and lymph plasma by DEAE column chromatography were found to enhance primary immune responses to chicken red blood cells when injected with chicken red blood cells into the popliteal lymph node.
drainage area. This fraction also enhanced normal lymphocyte transfer reactions and $^3$H thymidine uptake in *in vitro* cultures of lymphocytes that had been incubated previously with PHA. A fraction isolated from lymph collected at the peak of a primary immune response to chicken red blood cells was found to increase the uptake of $^3$H thymidine by PHA stimulated lymphocytes to a greater extent than similar fractions isolated from lymph at other times during primary immune responses. These results suggested that mitogens were elaborated during the primary immune response to chicken red blood cells and Salmonella muenchen organisms, and that factors which induced antibody-forming cells to secrete their antibody were elaborated around the time that the primary immune response was at a maximum.

Efferent popliteal lymph collected during the period 144 - 168 hours after primary challenge with $2 \times 10^9$ chicken red blood cells suppressed the primary immune response to chicken red blood cells in another sheep when injected together with chicken red blood cells into the drainage area of the popliteal lymph node. The same lymph had no significant effect upon the uptake of $^3$H thymidine by PHA stimulated lymphocytes, or upon the normal lymphocyte transfer reaction, but inhibited the secretion of antibody by antibody-forming cells *in vitro*.

A further fraction isolated from sheep blood serum and lymph plasma by DEAE chromatography was found to suppress primary immune responses to chicken red blood cells, but had no significant effect upon the uptake of $^3$H thymidine by PHA stimulated lymphocytes or upon normal lymphocyte transfer reactions. These results suggested that factor(s) which inhibited cell proliferation were elaborated during the course of a primary immune response and that factors which inhibited the secretion of antibody by antibody-forming cells were elaborated after the primary immune response had reached its maximum.
Further experiments showed that during the primary immune response to $2 \times 10^9$ chicken red blood cells, the number of antibody-forming cells in the lymph was always greater than the number of antibody-secreting cells; the number of antibody-forming cells that secreted antibody was greatest at the peak of the response and least after the peak of the response.

Chapter VIII - Allogeneic Enhancement and Inhibition of Humoral Immune Responses

Experiments in which allogeneic lymphocytes were cultivated in vitro suggested that whilst lymphocytes treated with 10µg/ml or 50µg/ml Mitomycin C did not lose their surface histocompatibility antigens they were unable to elicit vigorous mixed lymphocyte reactions. It was shown that lymphocytes treated with 10µg/ml Mitomycin C gave more vigorous mixed lymphocyte reactions than did lymphocytes treated with 50µg/ml Mitomycin C.

Experiments in which normal or Mitomycin C treated lymphocytes were injected into the popliteal lymph node drainage area of allogeneic sheep showed that untreated lymphocytes gave rise to vigorous immune responses and that immune responses elicited by lymphocytes treated with 10µg/ml Mitomycin C were less vigorous although greater than those elicited by lymphocytes treated with 50µg/ml Mitomycin C.

These results suggested that lymphocytes elaborated factor(s) which increased the response of lymphocytes to specific antigens.

Mitomycin C treated or untreated allogeneic lymphocytes were injected subcutaneously into the drainage area of the popliteal lymph node concurrently with or 24, 48, 120 or 260 hours prior to injecting chicken red blood cells at the same site. In all cases the immune
response to chicken red blood cells was enhanced when red cells were injected before the response to the allogeneic lymphocytes was at its maximum, and inhibited when chicken red blood cells were injected after the response to the allogeneic lymphocytes had reached its maximum. The enhancement of the response to chicken red blood cells was greatest when untreated allogeneic lymphocytes were injected and least when lymphocytes treated with 50µg/ml Mitomycin C were injected prior to the red cells. It was suggested that lymphocytes reacting to antigens or allogeneic lymphocytes elaborated factors which enhanced the response of other lymphocytes to antigen. During the later part of an allogeneic response lymphocytes elaborated factors which inhibited the response of other lymphocytes to antigen. These results were interpreted in terms of T and B lymphocyte interactions.

The time relationships in respect to antigen spacing and the kinetics of these responses were similar to those demonstrated in antigenic competition experiments (Chapter V). The mechanisms which act to control these immune phenomena were assumed to be the same.

Chapter IX - The Synthesis of Non-Specific Immunoglobulins During the Immune Response to Specific Antigens

"Non-specific" immunoglobulin-forming cells were detected in the efferent lymph of sheep responding to a primary or secondary challenge of antigen. Within this population of immunoglobulin-forming cells there were cells secreting antibody specific for an antigen which was unrelated and did not cross-react with the antigen which induced the immune response. When sheep were injected with antigen five weeks prior to the injection of an unrelated, non-cross-reacting antigen, then cells secreting antibody specific for the first antigen were detected in the efferent lymph.
The number of cells secreting antibody specific for the first antigen was greater in animals that had experienced the first antigen previously.

B lymphocyte memory cells specific for the first antigen may be activated by some factor(s) elaborated by T lymphocytes responding to and specific for the second antigen. This factor(s) would induce memory cells and B lymphocytes (which have antigen bound to their surface receptors) to proliferate and mature into antibody-forming cells.

The expression of this population of antibody-forming cells as antibody-secreting cells closely paralleled the appearance of antibody-secreting cells specific for the second antigen. Thus the population of cells induced to synthesise antibody against the first antigen was controlled in its expression as antibody-secreting cells by factors elaborated during the immune response to the second antigen. Evidence for the existence of such factors was presented in Chapter VII.

The evidence suggested that anamnestic responses could be elicited by non-specific antigenic stimuli.
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