STUDIES ON CYTOPHILIC ANTIBODIES

THESIS SUBMITTED TO THE AUSTRALIAN NATIONAL UNIVERSITY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

LIEW FOO YEW

Department of Microbiology,
The John Curtin School of Medical Research.

TO MY FATHER
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMS</td>
<td>Anti-macrophage serum</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>B cell</td>
<td>Bursa equivalent or thymus independent lymphocyte</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C.Ab</td>
<td>Cytophilic antibody</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
</tr>
<tr>
<td>FIA</td>
<td>Freund's incomplete adjuvant</td>
</tr>
<tr>
<td>PIN</td>
<td>Flagellin</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal exudate cells</td>
</tr>
<tr>
<td>PFC</td>
<td>Plaque forming cell</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>POL</td>
<td>Polymerised flagellin</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>T cell</td>
<td>Thymus dependent lymphocyte</td>
</tr>
</tbody>
</table>

1 gram = $10^3$ mg = $10^6$ μg = $10^9$ ng = $10^{12}$ pg = $10^{15}$ fg.
STATEMENT

Some of the experiments described in papers III-VI were carried out jointly with Dr. C.R. Parish. The remainder of the work described in this thesis was carried out by the candidate.

Liew Foo Yew
ACKNOWLEDGEMENTS

I am grateful to my supervisor, Professor G.L. Ada, for his invaluable guidance, advice and constant encouragement throughout the course of this work. I am also indebted to Dr. C.R. Parish with whom I had the pleasure of undertaking collaborative projects, and whose influence is to be found in many aspects of this thesis.

My sincere thanks also go to Mr. R. Tha Lha for excellent technical assistance; Mr. K. Clark for technical advice; Mr. R. Hill for the histological preparations; members of the staff of the Animal Breeding Establishment for the supply and care of the animals; staffs of the Photography Department for the photographic materials; Miss J. McCalman for reading the manuscript; and Miss R. Veoten for collating the thesis.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMS</td>
<td>Anti-macrophage serum</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>B Cell</td>
<td>Bursa equivalent or thymus independent lymphocyte</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C.Ab</td>
<td>Cytophilic antibody</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
</tr>
<tr>
<td>FIA</td>
<td>Freund's incomplete adjuvant</td>
</tr>
<tr>
<td>FIN</td>
<td>Flagellin</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal exudate cells</td>
</tr>
<tr>
<td>PFC</td>
<td>Plaque forming cell</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>POL</td>
<td>Polymerised flagellin</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>T cell</td>
<td>Thymus dependent lymphocyte</td>
</tr>
</tbody>
</table>

1 gram = $10^3$ mg = $10^6$ µg = $10^9$ ng = $10^{12}$ pg = $10^{15}$ fg.
TABLE OF CONTENTS

Statement ................................................................. (i)
Acknowledgement ...........................................................(ii)
Abbreviation .................................................................(iii)

PART A

INTRODUCTORY REVIEW

"The Cytophilic Antibodies"

I. INTRODUCTION ......................................................... 1
II. THE MACROPHAGE CYTOPHILIC ANTIBODY ................. 9
   1. The macrophages ............................................... 9
   2. Methods for detecting macrophage cytophilic antibody .............................................. 17
   3. Production of macrophage cytophilic antibody ......................................................... 21
   4. Properties of macrophage cytophilic antibody ............................................................ 27
   5. The binding of cytophilic antibody to macrophages .................................................... 35
   6. Possible biological role of macrophage cytophilic antibody ......................................... 40
III. ANTIBODIES CYTOPHILIC FOR CELLS OTHER THAN MACROPHAGES ................................. 50
    1. Lymphocytes ....................................................... 50
    2. Basophils and Kurloff cells ................................. 56
    3. Lymphoid follicles and Dendritic cells . 57

Page
PART B

ORIGINAl WORK

"Some Chemical and Biological Properties of Macrophage Cytophilic Antibody"

PAPER I

The detection and production of macrophage cytophilic antibody to different antigens in different laboratory animals ............................................. 76
References ......................................................... 94

PAPER II

The binding of the Fc fragment of guinea-pig cytophilic antibody to peritoneal macrophages ........ 96
References ........................................................... 111

PAPER III

Suppression of antibody formation and concomitant enhancement of cell-mediated immunity by passive antibody .................................................. 113
References ........................................................... 133
PAPER IV
The effect of different classes of passive antibody on humoral and cell-mediated immunity .................. 135
References ........................................................................ 158

PAPER V
The effect of macrophage cytophilic antibody on humoral and cell-mediated immunity .................. 160
References ........................................................................ 181

PART C
CONCLUDING DISCUSSION

I. INTRODUCTION ......................................................... 183
II. THE PRODUCTION OF MACROPHAGE CYTOPHILIC ANTIBODY ............................................. 183
III. THE BINDING OF CYTOPHILIC ANTIBODY TO MACROPHAGES ............................................ 185
IV. THE ROLE OF MACROPHAGE CYTOPHILIC ANTIBODY IN THE IMMUNE RESPONSE ........... 188
V. CONCLUSIONS ......................................................... 190
REFERENCES ................................................................... 193
PART D
APPENDIX
PAPER VI

Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin .............. 195
References ........................................... 214
PART A

INTRODUCTORY REVIEW

"THE CYTOPHILIC ANTIBODIES"

It has been known for centuries that a person who survived an initial attack of a disease, such as plague or smallpox, was usually able to resist a second attack of the same disease, but a general understanding of this phenomenon, called immunity, awaited the discovery that microorganisms were the causative agents of infectious disease. A key step in this understanding was taken in 1901 by Paul von Behring and Shibasaburo Kitasato, who showed that an animal could be made resistant to tetanus by an injection of the blood serum obtained from an animal that had survived the disease and hence had developed immunity to it. Thus, immunity to tetanus seemed to be a function of a substance or substances in the blood. These substances were named antibodies.

The term antibody implies no more than a molecule which reacts with antigen. With the possible exception of "natural antibody", antibodies are characteristically formed as a specific response to the introduction into the body of materials which are recognised as foreign by the body. Their characteristic property is to combine, under physiological conditions, with the material in response to which they were formed. They are globular proteins and are therefore commonly known as immunoglobulins.
I. INTRODUCTION

It has been known for centuries that a person who survived an initial attack of a disease, such as plague or smallpox, was usually able to resist a second attack of the same disease, but a general understanding of this phenomenon, called immunity, had to await the discovery that microorganisms were the causative agents of infectious disease. A key step in this understanding was taken in 1890 by Emil von Behring and Shibasaburo Kitasato, who showed that an animal could be made resistant to tetanus by an injection of the blood serum obtained from an animal that had survived the disease and hence had developed immunity to it. Thus, immunity to tetanus seemed to be a function of a substance or substances in the blood. These substances were named antibodies.

The term antibody implies no more than a molecule which reacts with antigen. With the possible exception of "natural antibody", antibodies are characteristically formed as a specific response to the introduction into the body of materials which are recognised as foreign by the body. Their characteristic property is to combine, under physiological conditions, with the material in response to which they were formed. They are globular proteins and are therefore commonly known as immunoglobulins.
The aim of this thesis is to investigate the properties and function of a special class of antibody - the cytophilic antibody. Cytophilic antibody is unique in that it has an affinity for certain cell types. Before embarking on a more detailed discussion of cytophilic antibody, it is necessary to consider briefly the structure and biological function of antibody in general.

**Structure of Immunoglobulins**

Immunoglobulins are known to occur in five major classes which are called IgG, IgM, IgA, IgD and IgE. These classes are quite distinct from one another in several aspects (see Table 1). However, most of the essential properties of immunoglobulins can be considered in terms of a single class, IgG, which at least in man, is the most prevalent class.

IgG is also known as a 7S-immunoglobulin because it has a sedimentation coefficient ($S_{20}$) of 6.6 to 7.0 Svedberg units, which corresponds to a molecular weight of about 150,000. All classes of immunoglobulins have the same unit structure consisting of heavy (Mol. Wt. 50,000 to 70,000) and light (Mol. Wt. 22,000) chains held together by varying numbers of disulfide bonds. Fig. 1. shows a diagrammatic structure of IgG. The chains can be separated by reduction with mercaptoethanol in the presence of urea or some other H-bond disruptive
### TABLE 1

**KNOWN CLASSES OF HUMAN IMMUNOGLOBULIN (Ig)**

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgA</th>
<th>IgD</th>
<th>IgM</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mol. Wt.</td>
<td>150,000</td>
<td>180,000-500,000</td>
<td>150,000</td>
<td>950,000</td>
<td>196,000</td>
</tr>
<tr>
<td>S&lt;sub&gt;20&lt;/sub&gt;, W</td>
<td>6.5-7.0</td>
<td>7.0-17</td>
<td>6.2-6.8</td>
<td>18-20</td>
<td>7.9</td>
</tr>
<tr>
<td>% carbohydrate</td>
<td>2.9</td>
<td>7.5</td>
<td>?</td>
<td>11.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Normal serum concentration (mg/100ml)</td>
<td>800-1,600</td>
<td>140-420</td>
<td>3-40</td>
<td>50-190</td>
<td>0.01-0.14</td>
</tr>
<tr>
<td>Heavy chains:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>class</td>
<td>γ</td>
<td>α</td>
<td>δ</td>
<td>μ</td>
<td>ε</td>
</tr>
<tr>
<td>subclass</td>
<td>γ&lt;sub&gt;1&lt;/sub&gt;, γ&lt;sub&gt;2&lt;/sub&gt;, γ&lt;sub&gt;3&lt;/sub&gt;, γ&lt;sub&gt;4&lt;/sub&gt;</td>
<td>α&lt;sub&gt;1&lt;/sub&gt;, α&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mol. Wt.</td>
<td>53,000</td>
<td>64,000</td>
<td>-</td>
<td>70,000</td>
<td>75,000</td>
</tr>
<tr>
<td>Light chains (M.W. 22,000)</td>
<td>k, λ</td>
<td>k, λ</td>
<td>k, λ</td>
<td>k, λ</td>
<td>k, λ</td>
</tr>
</tbody>
</table>

* data derived from reference 2, 182.
Fig. 1. Diagrammatic representations of the structure of the IgG molecule. In the upper diagram, some features of the chemical structure of the molecule are presented. There are four interchain and twelve intrachain disulphide bonds. The variable portions (amino acid sequence) of the chains are confined to the N-terminal half of the light and heavy chains. The approximate positions of cleavage by pepsin and papain of the heavy chains are shown.

On the lower diagram is shown the nomenclature assigned to the different fragments obtained, in part, by papain digestion. (from reference 22).
agents and subsequent alkylation with iodoacetamide. If prepared under appropriate conditions (1,2), the dissociated chains may retain some of the biological activity of the intact molecule. For example, heavy chains isolated from a specific antibody preparation are capable of binding that antigen, though with a lower binding constant than that of the original molecule (3,4).

Within any species, all classes of antibody have the same light chains. In humans, there are two serologically distinct light chains called k and λ chains. Mice and rabbits have also been shown to have two types of light chains. The heavy chains, which distinguish the classes of antibodies, could be either γ, µ, α, δ or ε according to their origin IgG, IgM, IgA, IgD or IgE, respectively (Table 1).

At least four methods have been described which allow cleavage of immunoglobulins and result in the formation of biologically active fragments. 1. Porter (5) first showed that papain split the rabbit IgG Molecule into two identical 3.5S Fab fragments (fragment antigen binding) and a crystallisable 3.5S Fc fragment (fragment crystallisable). The Fab fragment contained the amino terminal half of the heavy chain (denoted Fd) which was still bound to the light chain through a disulphide link. The Fab fragment retained specific combining ability for antigen and was univalent. The Fc fragment was a dimer of the carboxyl terminal half of the two heavy chains.
2. pepsin cleaved IgG into a 5S (Fab')₂ fragment, which contained both antibody combining sites. The 5S dimer could be subsequently converted into two 3.5S univalent Fab' fragments by reduction of the disulphide bonds between the heavy chains (7). A fragment corresponding to Fc was not recovered after pepsin treatment as this portion of the IgG molecule was split into smaller fragments (6). 3. IgG which had been mildly reduced and aminoethylated could be cleaved by trypsin to yield two 3.5S (Fab)ₜ fragments which were univalent and an (Fc)ₜ fragment, rather similar to that produced by papain treatment (8). 4. Treatment with cyanogen bromide (CNBr) in dilute acid produced a 5S (Fab")₂ fragment, and a 3.5S Fc" fragment (9). The (Fab")₂ fragment, on reduction, yielded two univalent 3.5S Fab" fragments.

The similarity in size of the Fab fragments which result from the action of the three different enzymes and of CNBr indicates that all these cleavages occur in the same region of the heavy chain and suggests that this region is more accessible than others. There is a second feature about this region. The IgG molecule is known to exist in the shape of the letter "Y" with the two light chains and the Fd portion forming the upper arms and the Fc portion the lower segment. Electron micrographs suggest that the binding site for antigen is at the tip of each arm (11). The IgG molecule is flexible at a position near the disulphide bonds linking the heavy chains. This region is called the hinge region. The
hinge region serves not only as a convenient locus for fragmentation but also has a biological function. It provides the remote link between the two binding sites that enable them to be held at a variable distance from each other. Such an arrangement is advantageous to a molecule that acts as a scavenger for macromolecules.

Biologically, one is impressed by the diversity of antibodies and by their specificity. An individual must be capable of synthesising many thousands of distinct IgG molecules, each with a specific binding site directed towards a different antigen. Even a preparation of antibodies against a single antigen may consist of thousands of distinct IgG molecules which have only one thing in common - the ability to combine with a specific antigen. The chemical basis for the diversity of immunoglobulins seems to lie in the differences in the amino acid sequence of the polypeptide chains (12-14). The differences are largely confined to limited regions of the chains (12,15). The combining ability for a particular antigen is usually present in the isolated heavy chain of an antibody (4,16-19), but full expression of the affinity for the antigen is attained only when the specific heavy chain is combined with a light chain derived from an antibody of the same specificity (16-18, 20-21). As the antigenic determinants of several proteins have been shown to consist of as few as 3-5 amino acids, it is supposed that
the antigen binding sites of antibodies might also be small in size and involve only 1-2% of the total amino acids (22). The precise position in either chain of these sites is not known but there is evidence that amino acid sequences in the N-terminal halves of the light and heavy chains are involved.

Biological Properties of Antibody

The earliest recognised biological role of antibody was opsonisation. Though opsonin is a general name for any factor which promotes phagocytosis, it is usually an antibody but may also be a complement or other serum components (183). Opsonising antibody acts by first combining with antigens, then the antigen-antibody complex becomes attached to macrophages because of a change in the conformation of the Fc fragment following combination with antigen.

The ability to bind complement is another notable property of antibody. Complement is a normal constituent of serum which, in conjunction with antibody, is capable of destroying certain bacteria as well as animal cells, notably erythrocytes. Detailed studies showed that the antibody combines with antigens at the cell surface and that complement then reacts with the Fc piece in the antigen-antibody complex (184). Complement also binds to antigen-antibody complexes formed in solution. The binding of complement in this way is known as "complement fixation".
It has been known for some time that mixing antigen with an excess of antibody prior to injection can specifically suppress the subsequent production of antibody to the antigen. The possibility that such passively administered antibody can act as a "feedback" mechanism was suggested by the studies of Uhr and Baumann (23,24), who showed that passively administered antibody injected as long as 5 days after the injection of antigen was still capable of inhibiting the antibody response. This particular phenomenon is discussed later in this review.

Apart from the ability to react with antigens, a major property of at least some antibodies is to react with cells. For example, the localisation of antigens on the dendritic cells in the follicles of lymphoid tissue is a function of antibody (25,26).

Recent studies indicate that normal lymph node cells (28,29) and normal spleen lymphocytes (30) can also passively adsorb antibody to an appreciable extent. This, again, will be discussed in more detail later.

The most striking example of the reaction of antibodies with cells is the attachment of cytophilic antibodies to macrophages. In 1960, Boyden and Sorkin first drew attention to the fact that normal rabbit spleen cells which had been preincubated in vitro with rabbit anti-HSA antiserum, and then washed, were capable of taking up relatively large amounts of $^{131}$I-labelled HSA (31). The serum components responsible for this effect...
were found to have properties expected of antibody and were therefore called cytophilic antibodies \((32,33)\). The cell population in a spleen is highly heterogeneous, including lymphocytes, macrophages, plasma cells and others, and it is possible that these cytophilic antibodies were attached to a number of different cell types. That antibodies exist which were specifically cytophilic for macrophages was first indicated by the work of Boyden \((32,34)\) and subsequently confirmed in large part by others. There is now a large amount of data concerning the properties of cytophilic antibody. This is especially so concerning its physical and chemical properties. However, so far, there is very little knowledge of the biological role it may play in the immune system.

In the review which follows, the cytophilic antibodies are, for convenience, considered in two separate parts. In the first, antibody cytophilic for macrophages is discussed. In the second part, our knowledge on the cytophilic antibody for other cell types is reviewed. The emphasis, however, is on the macrophage cytophilic antibody. This is because 1. most of the reports on cytophilic antibody so far have been predominantly on macrophage cytophilic antibody; 2. the original work to be described in this thesis deals only with macrophage cytophilic antibody. The term macrophage cytophilic antibody is used throughout this thesis to denote the antibody cytophilic for macrophages, and the term lymphocyte cytophilic antibody denotes the antibody cytophilic for lymphocytes.
II. ANTIBODIES CYTOPHILIC FOR MACROPHAGES

1. THE MACROPHAGES

Phagocytosis was first reported by Haeckel in 1862 to describe the ingestion of indigo by the blood-cells of Tethys, a gastropod mollusc (35). Later, Panum (1874) and Roser (1881) suggested that the ameboid phagocytic cells in higher animals might have the function of destroying bacteria (37,38). It was Metchnikoff, however, who was responsible for the concept that phagocytic cells formed a defence mechanism within the metazoan body (39,40). Metchnikoff called these phagocytic cells "Macrophages", i.e., "big eater" because they are capable of ingesting large particles, such as erythrocytes, spermatozoa, protozoa, etc. The development of vital dyes (41) which are preferentially ingested by macrophages facilitated the studies of the distribution of macrophages throughout the body. These studies eventually led to the concept of the reticuloendothelial system in which macrophages were a major cell type (42-44). This system of cells has a certain role in inflammation, phagocytosis, resistance to infection and the handling of cholesterol. Since the time of Metchnikoff, a very large amount of work has been carried out on macrophages, and with the recent application of sophisticated and quantitative techniques, there has been a steady increase in our knowledge of the origin, histology, biochemistry and the immunological properties
of macrophages. In this section, a brief review on some aspects of macrophages is presented. Nelson's recently published book (45) is far more detailed and comprehensive.

The distribution and origin of macrophages

The term macrophage is used to describe cells of diverse forms, both as regards their anatomical situation and their physiological states. The characteristics of this family of cells are: 1. The capacity, latent or manifest, for phagocytosis. 2. The presence of large numbers of lysozomes containing acid hydrolases. 3. The presence of microvilli and ruffles or hyaloplasmic veils on at least part of the cell plasma membrane (45). The last characteristic is, perhaps, less definitive than the first two as cells may have to be cultured to demonstrate this. The term macrophage usually includes the large phagocytic mononuclear cells in the serous cavity and pulmonary alveoli; the phagocytic cells lining the sinusoids of liver, spleen and lymph nodes; the monocytes of the blood and perhaps the microglia of central nervous tissue. Some authors consider the reticular cells should not be regarded as macrophages because, although they can ingest particulate materials, they are not highly phagocytic (52). For similar reasons, the dendritic cells in the follicles of the spleen and lymph nodes are not considered as macrophages, although they can retain
antigens at the surface of their dendritic cytoplasmic extensions. Table 2 summarises the distribution of macrophages in mammals. The development by Roser (62) of techniques for following the fate of mouse peritoneal macrophages which have ingested radioactive colloidal gold (Au\(^{198}\) or the longer lived isotope Au\(^{195}\)) has given results of considerable interest. Peritoneal macrophages labelled with Au\(^{198}\) were injected intravenously into syngenic mice and their fate was followed. Radioactivity was localised initially in the lungs, then released slowly until after 8 hours only 10-15% remained in the lungs. The radioactivity, presumably representing live macrophages, then accumulated largely in the liver and to a lesser extent in the spleen. Accumulation in other tissues was minimal. Alveolar macrophages behaved similarly to peritoneal macrophages except that a lower proportion of the injected cells reached the spleen.

There is now abundant evidence that the primitive precursors of macrophage stem cells are mainly located in the bone marrow (46-51). The earliest recognisable precursor cells of macrophages in the bone marrow are the promonocytes. These cells give rise to the monocytes (53). Monocytes in the circulation constitute a mobile pool of incompletely differentiated cells on their way from their sites of origin to the tissues. Since the early studies by Florey and others (54,55) it has been established that under both normal and pathological conditions macrophages
<table>
<thead>
<tr>
<th>Gross anatomical site</th>
<th>Localisation of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Kupffer cells lining hepatic sinusoids</td>
</tr>
<tr>
<td>Spleen</td>
<td>Red pulp, especially lining sinusoids; within lymphoid of white pulp</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>Lining circular and medullary sinuses; elsewhere in medulla; within lymphoid follicles of cortex</td>
</tr>
<tr>
<td>Thymus</td>
<td>Scattered; possibly within Hassall's corpuscles in some species</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Lining sinusoids; probably also scattered</td>
</tr>
<tr>
<td>Lung and pleura</td>
<td>Alveoli; pleural fluid; milk spots</td>
</tr>
<tr>
<td>Peritoneum</td>
<td>Peritoneal fluid; milk spots</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Choroid plexus; ? microglia</td>
</tr>
<tr>
<td>Blood</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Joints</td>
<td>Synovial fluid; Type A synovial lining cells</td>
</tr>
<tr>
<td>Subcutaneous tissue, uterus, intestine, ovary, testes</td>
<td>Connective tissue generally</td>
</tr>
<tr>
<td>All sites</td>
<td>Inflammatory exudates</td>
</tr>
</tbody>
</table>

Adapted from reference 45.
of connective tissues (histocytes) and of serous cavity (e.g. peritoneal macrophages) are derived from peripheral blood monocytes. Additional evidence that tissue macrophages originate from circulating precursor cells has been obtained from ontogenic studies, which show that macrophages are not found in tissues before vascularisation occurs (56). The blood-borne cells giving rise to macrophages in inflammatory exudates are also most likely to be monocytes (55,57). Pulmonary alveolar macrophages, on the other hand, could arise both locally and from blood-borne monocytes (48,58). The monocytic origin of the macrophages in the liver (Kupffer cells) has been established recently (59,60). In spleen, lymph nodes and bone marrow, the free macrophages are derived from monocytes. The fixed macrophages in these organs are probably also of monocytic origin, but definitive proof of this has not yet been given.

Morphology of Macrophages

A major impression gained from studying cells included in the family of macrophages, is their great variation in morphology. The sizes range from cells not much larger than the small lymphocytes (Diameter <10µ) (e.g. marrow and blood monocytes) to cells with a volume 10 times greater or more (e.g. cells present in the peritoneal cavity after an injection of thioglycollate). Nuclear shape is variable, ranging from oval to vermiform,
and nuclear area varies from more than 1/2 to less than 1/10 of the overall cell area. The numbers of some of the cytoplasmic organelles such as granules, Golgi apparatus, vesicles and vacuoles, polyribosomes and fat deposit are extremely variable.

The morphology of macrophages such as those found in the peritoneal cavity of guinea-pigs, viewed under the light microscope is described. The peritoneal cavity is a common source of macrophages. The detailed structure of peritoneal and other macrophages have been described by Nelson (45) and Hirsch et al (53). A brief description now follows.

In normal guinea-pigs, peritoneal macrophages vary considerably in size (diameter 12-30µ). The nucleus may be round, indented or almost bilobed; sometimes two or more nuclei are present. The nuclear chromatin is usually reticular with an inconspicuous nucleolus. The cytoplasm usually contains vacuoles, including fat vacuoles, and ingested materials. Adjacent to the indented region of the nucleus is the centrisomal region which contains the centrioles, the Golgi apparatus and a few mitochondria. In smears stained with conventional haematological stains, the cytoplasm appears faintly basophilic. Living macrophages rapidly take up a large amount of lithium carmine and acid aniline dyes such as pyrrhol blue, isamin blue and trypan blue. The staining is cytoplasmic and is not, in the case of trypan blue, to be confused with the
nuclear staining characteristic of dead cells. Neutral red (a basic dye) is also rapidly taken up by living macrophages and results in a characteristic rosette of red granules, usually most marked in the perinuclear area, but also seen throughout the cytoplasm.

When freshly isolated and cultured at 37° in a suitable glass (or plastic) chamber, in the presence of serum proteins (e.g. foetal calf serum), macrophages adhere to and in due course spread out on the glass by means of cytoplasmic processes. Phase contrast microscopy shows that the processes are motile and the advancing edges are rounded and have undulating membrane. The undulating membrane has been referred to as being composed of hyaloplasmic veils or ruffles (71). The ruffles may be of great importance in pinocytosis and phagocytosis (72).

Functional Identification of Macrophages

The criteria mentioned so far for the identification of macrophages are morphology and the capacity for phagocytosis. These, in fact, prove rather inadequate in a situation where precise identification between macrophages and other cells (notably lymphocytes) is required. For example, although phagocytosis is a notable property of macrophages, it is not exclusive to them. Unstimulated lymphocytes phagocytose poorly, but after a brief period in an inflammatory site, their ability to phagocytose becomes very pronounced (61). Some lymphocytes have been shown to ingest colloidal gold (62) and
erythrocytes (63). Several reports (64,65) indicated that splenic plaque forming cells which appeared after the injection of foreign erythrocytes, were phagocytic. The occurrence and frequency of lysosomes is also not an unambiguous criterion for differentiating between lymphocytes and monocytes, since such structures can be found in both types of cells, their frequency being related to the functional state of the cell (66).

The ability to adhere to glass surface has also been used to identify macrophages (48,50). However, there are reports (67) that lymphocytes can also adhere to glass. In addition, Johnson and Garvin (68) found that, whereas under certain conditions few normal blood lymphocytes would stick to a glass bead column, almost all of the blood lymphocytes from patients suffering from lymphocytosis or from a recent infection, would remain in the column. Thus, the ability of a cell to adhere to glass surface, though a common and useful characteristic of macrophages, is not strictly exclusive to macrophages.

Using red cells sensitized with an IgG anti-Rh0 antibody, Huber et al (69) demonstrated that more than 90% human peripheral blood monocytes, hepatic macrophages and splenic macrophages possessed receptors for IgG. Such receptors were present on less than 1% of normal lymphocytes, or on lymphocytes prestimulated with phytomitogens. They proposed that this receptor could be regarded as an immunological marker for identification of macrophages.
However, in view of the recent findings (105, 167) that there also exist antibodies cytophilic for lymphocytes, the general applicability of the marker is therefore doubtful.

Unanue (70) has recently developed highly specific antisera in rabbits to mouse macrophages, and proposed that the anti-macrophage serum (AMS) could be used for the identification of macrophages. He found that properly absorbed AMS readily distinguished between macrophages and transformed lymphocytes which had other properties similar to macrophages. AMS promises to be a valuable tool for studying the origin and development of macrophages.

Summary

In this section, the distribution, origin and morphology of macrophages have been discussed. The term macrophage is used to describe a family of cells with extensive capacity for phagocytosis, usually with large numbers of lysozomes and frequent microvilli. Macrophages are distributed throughout the body but the most common source of macrophages is from the peritoneal cavity. The primitive precursors of macrophages, the promonocytes, are mainly located in the bone marrow. The promonocytes give rise to monocytes which are found in the circulation. The monocytes in turn develop into macrophages in the tissues. There is at present no completely satisfactory means of distinguishing between monocytes and lymphocytes,
though specific anti-macrophage serum promises to be a useful reagent for this purpose.

2. METHODS FOR DETECTING MACROPHAGE CYTOPHILIC ANTIBODY

Cytophilic antibodies have been defined as globulin components of antiserum (of known specificity) which become attached in vitro to certain cells in such a way that these cells are subsequently capable of specifically absorbing antigens (32,33). Thus, techniques used for the detection of cytophilic antibodies have been based on the ability of these agents to confer on cells the capacity to take up increased amounts of antigen. In this section, the methods used for detecting macrophage cytophilic antibody are discussed.

**Rosette Test**

The most commonly used technique for detecting cytophilic antibody is the rosette test first used by Boyden (34) for demonstrating macrophage cytophilic antibody to SRBC (sheep red blood cell). The method involves the following steps: 1. Incubation of a cell suspension containing macrophages in a well-type chamber at 37° so that a monolayer of macrophages is formed on the bottom of the well. 2. Washing to remove cells that have not attached to glass. 3. Incubation of the adherent cells in the wells with serial dilutions of serum which may contain cytophilic antibody. 4. Washing to remove free serum so that the only antibody remaining is attached to
macrophages. 5. Adding washed SRBC to the well and incubating. 6. Washing to remove unattached SRBC. 7. Examination under the phase contrast microscope for rosettes. The results are recorded as a graded response (0 to +++ or ++++) or as the average number of erythrocytes adherent to macrophages.

This method was later adapted by other workers (73,74,75) to permit the detection of cytophilic antibody to soluble antigens. In the modified rosette method, the presence of cell-bound cytophilic antibody to soluble antigens was detected by coupling the antigens to erythrocytes which act as indicator particles. This method has proved satisfactory with bovine β-lactoglobulin (73), bovine plasma albumin (73), and human serum albumin (76), using bis-diazotised benzidine as conjugating reagent. Haptens such as arsanilic acid and p-aminobenzoic acid, may be coupled directly to red cells (75). The somatic lipopolysaccharides of gram negative bacteria attach themselves spontaneously to erythrocytes (77). Cytophilic antibody to all of these antigens has been detected by means of erythrocytes modified in this way (74,75,78).

Another modification of the original rosette method is the "suspension-centrifugation" technique (73). This is similar to the rosette test except that the cells are maintained in suspension throughout the test. After addition of antigen (red cells or antigen coated red cells), the cells are centrifuged together with the antigen and the
cell pellets resuspended before examination. This method appears to be more sensitive than the well-type rosette method, and is particularly useful when conditions of low temperature are required, as, at these low temperatures, macrophages adhere poorly to glass.

Berken and Benacerraf (74) treated antigen first with antibody, then mixed the complex with macrophages and observed the degree of adherence. This indirect method, as it is known, resulted in a greater uptake of antigen by macrophages. However, it is uncertain whether it is justifiable to use this technique for estimating cytophilic antibody, as it might also detect specific opsonins.

Radioactive Antigen Method

Cytophilic antibody was first detected by Boyden and Sorkin (31), using $^{131}$I-labelled antigen. In this method, a suspension of normal rabbit spleen cells was incubated with dilutions of rabbit anti-HSA antiserum. After thorough washing, the sensitized cells were incubated with $^{131}$I-labelled HSA. At the end of the incubation, the cells were again washed and the cell associated radioactivity counted. This method has also been successfully applied by Blazkovec (79) in demonstrating guinea-pig antibody which was cytophilic for normal guinea-pig lymph node cells and peritoneal exudate cells.
Adherence of Bacteria

Cytophilic antibodies to bacterial antigens have been detected using whole bacteria (78,80). The method is similar to the rosette technique except that, instead of using erythrocytes, motile bacteria are employed as indicator particles.

Adherence of Tumour Cells

Another method for detecting cytophilic antibody is to use tumour cells as the indicator particles. Hoy and Nelson (81) have studied the production by C57Bl/6J mice of cytophilic antibody directed against histocompatibility antigens (H2) of A/J mice, using the A/J tumour, sarcoma I, as a particle which attached to sensitized macrophages.

All the methods for detecting cytophilic antibody so far described in this section are of qualitative nature. However, attempts have been made to quantitate the rosette method for titrating cytophilic antibody in serum. Gowland (75), for instance, adopted the rosette test for an endpoint titration procedure to assay cytophilic antibody. Serial dilutions of serum were made and the cytophilic antibody titre expressed as the titre giving a certain "cytophilic score", this being the number of erythrocytes adherent to 100 macrophages. The procedure, however, is rather time-consuming and is not readily applicable to soluble antigens, as it is the common experience that not
all techniques used for attaching antigen to erythrocytes yield indicator particles suitable for use in the monolayer technique (75). Hence, in a detailed investigation into the properties of cytophilic antibody, the development of a sensitive, more objective and more convenient method for titrating cytophilic antibody seems necessary.

**Summary**

The most commonly used technique for detecting macrophage cytophilic antibody is the rosette test. This technique is most suitable for a cellular antigen, red blood cell, which serves as an indicating particle. Other cells, motile bacteria or cells coated with antigens may also be used as indicator particles. Another method for detecting cytophilic antibody is the use of radioactively labelled antigens. This method is more objective than the rosette method and is especially convenient for soluble antigens.

3. **PRODUCTION OF MACROPHAGE CYTOPHILIC ANTIBODY**

**Guinea-pigs**

Boyden (34) found that cytophilic antibody could be induced in guinea-pigs by a single injection of SRBC in FCA, but not when the antigen was injected with FIA or in saline. Jonas et al (73) reported that guinea-pigs primed with SRBC in FIA did not produce cytophilic antibody even
when a booster dose of SRBC in saline was given. However, the need to inject an antigen in FCA in order to elicit the production of cytophilic antibody has not been systematically investigated for other antigens.

The effect of routes of injection of SRBC in FCA and of booster doses of SRBC on the production for cytophilic antibody in guinea-pigs has been studied (82). Guinea-pigs were injected by different routes (intradermal, footpad, intraperitoneal, subcutaneous) with SRBC in FCA. Animals immunised by the subcutaneous route tended to develop less cytophilic antibody than those immunised by other routes. This was, however, true only for SRBC in guinea-pigs. The cytophilic antibody responses to HSA injected with FCA were almost as high after a subcutaneous injection as after an intradermal injection.

Factors affecting the production of cytophilic antibody in guinea-pig to soluble antigens have not been studied in detail. Blazkovec (79) studied the production of cytophilic antibody in guinea-pig immunised with HSA-rabbit-anti-HSA complexes. A single injection of complexes (containing 5 µg of HSA) either in FCA or FIA into the footpads did not induce the production of either cytophilic antibody or haemagglutinating antibody, for up to 4 weeks after the injection. However, cytophilic antibody was detected after a second intradermal injection of HSA (30 µg in saline), but only in guinea-pigs which had been preinjected with complexes in FCA. Cytophilic antibody
was first detected 5 days and reached a peak 7 days after the second injection of antigen. Peak titres of cytophilic antibody were higher and persisted longer in guinea-pigs which were given a second injection 3 weeks after the first injection than in those given a second injection 1 or 2 weeks after the first injection.

The time course of production of cytophilic antibody to SRBC in guinea-pigs was also studied (75). In guinea-pigs, cytophilic antibody was variably detected between 5 to 10 days after injection of SRBC in FCA. Both cytophilic and haemagglutinating antibodies reached maximal levels at 17-24 days, after which the levels of cytophilic antibody decreased, and could not be detected 3 months after immunisation. These animals, however, responded with a dramatic rise in serum cytophilic antibody after an intradermal injection of SRBC in saline, carried out either at 24 days or at 3 months after injection. By contrast, the intradermal injection of SRBC in saline caused only a moderate increase in haemagglutinating antibody titres.

**Mice**

The factors affecting the production of cytophilic antibody to SRBC in mice have also been investigated in some detail (Table 3). In their studies on the production of cytophilic antibody to SRBC in out-bred Swiss mice, Nelson and Mildenhall (82) made the following observations: 1. Similar titres of cytophilic antibody
TABLE 3

PRODUCTION OF CYTOPHILIC ANTIBODY BY MICE IMMUNISED WITH SHEEP RED BLOOD CELLS

<table>
<thead>
<tr>
<th>Route of immunisation</th>
<th>Adjuvant</th>
<th>Cytophilic antibody titre</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>primary&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>subcutaneous</td>
<td>FCA</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>subcutaneous</td>
<td>FIA</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>subcutaneous</td>
<td>saline</td>
<td>trace</td>
<td>low</td>
</tr>
<tr>
<td>intraperitoneal</td>
<td>FCA</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>intraperitoneal</td>
<td>FIA</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>intraperitoneal</td>
<td>saline</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>intravenous</td>
<td>saline</td>
<td>—</td>
<td>high</td>
</tr>
<tr>
<td>footpad</td>
<td>FCA</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>footpad</td>
<td>saline</td>
<td>high</td>
<td>high</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cytophilic antibody titre before a booster injection of SRBC in saline.

<sup>b</sup> Cytophilic antibody titre after a booster injection of SRBC in saline.

FCA = Freund's complete adjuvant
FIA = Freund's incomplete adjuvant
were found in mice injected subcutaneously with SRBC mixed with either FCA or FIA. 2. Cytophilic antibody was not regularly detected after an intraperitoneal injection of the antigen-adjuvant emulsion unless FCA was used. 3. In all cases (i.e., after a subcutaneous or an intraperitoneal injection of the antigen with FCA or FIA), a booster injection of SRBC in saline given one, two or three weeks after the primary injection, resulted in either a sharp rise in the titre of cytophilic antibody in the serum, the reappearance of cytophilic antibody, or even the first appearance of detectable cytophilic antibody. 4. When the initial injection of SRBC in saline was intraperitoneal or subcutaneous, little or no detectable cytophilic antibody was produced, although some appeared in the serum after a booster injection. Tizard (83,84), on the other hand, detected high to moderately high cytophilic antibody titres in the serum of Balb/C mice, following either a single or multiple intraperitoneal injections of SRBC in saline. Berken and Benacerraf (74,85) also found cytophilic antibody in the sera of Swiss-Webster mice previously injected and boosted by an intravenous injection of SRBC. Lojak (86) injected SRBC into the footpads of C57Bl mice and comparable cytophilic antibody levels were obtained independent of whether FCA or saline was used. However, this strain of mice is unusual in that there is always a moderately high level of cytophilic antibody to SRBC in normal, uninjected mice (86,45).
There is only one report on the factors affecting the production by mice of cytophilic antibody to antigens other than SRBC. In this report, Parish (78) observed cytophilic antibody in the sera of mice injected with bovine plasma albumin, with bovine gamma globulin or with E. coli polysaccharide, but gave no details of the injection schedules. Thus, it is not possible to draw general conclusions about cytophilic antibody production in mice. Even using the same antigen, different results have been obtained using different mouse strains.

Other Laboratory Animals

Rabbits have been found to produce cytophilic antibody to SRBC after injection intradermally with SRBC in FCA (76) or after a series of intravenous injection of SRBC in saline (74). However, no systematic investigation either of the effects of adjuvants or of the time course of cytophilic antibody production has yet been made.

Sodomann and Haferkamp obtained macrophage cytophilic antibody in rats injected in the footpads, with BCG-vaccine in FCA (87), or injected intradermally with Streptococci (88). But, again, no systematic study on the production of cytophilic antibody in the rats has yet been reported.

Recently, Kay et al (89) studied the production of macrophage cytophilic antibody in a primate (baboons). The cytophilic antibody to rabbit RBC was produced by injecting
baboons intramuscularly with rabbit RBC in FCA, followed by a second intradermal injection of the antigen in saline 17 days later. Very little cytophilic antibody was produced in baboons unless the second injection was given.

**Man**

Cytophilic antibody as such has not yet been investigated in humans, although there are a number of reports (69,90-92) indicating that there are specific IgG receptor sites on the surfaces of human monocytes and macrophages. Using 125I-labelled proteins, Inchley et al (93) recently demonstrated that human myeloma immunoglobulins became attached to the surface of guinea-pig macrophages at 37°C. The binding was specific and was inhibited by excess unlabelled IgG. It is reasonable to assume that cytophilic antibody exists and can be produced in man as in other vertebrates, but a systematic investigation of the production of cytophilic antibody in man is obviously difficult to carry out.

**Summary**

A general conclusion on the production of macrophage cytophilic antibody in different species is difficult to draw. In guinea-pigs, the incorporation of antigen in adjuvant before injection is necessary for the production of significant amount of cytophilic antibody. Macrophage cytophilic antibody in guinea-pigs is first detected
between 5-10 days after injection of SRBC in FCA and reaches a peak between 17-24 days after injection. Higher titres of macrophage cytophilic antibody are usually obtained when the animals have received a second injection of the antigen.

The factors affecting the production of macrophage cytophilic antibody in the mice are not known. Different mouse strains may give different results to the same antigen. Macrophage cytophilic antibody has also been detected in rabbits, rats, primate and man, but no detailed investigation has yet been done in these species.

4. PROPERTIES OF MACROPHAGE CYTOPHILIC ANTIBODY

Antibody Class

(1) In guinea-pigs

A large amount of data has accumulated indicating that most guinea-pig cytophilic antibodies to sheep red blood cell and to soluble antigens belong to the slow \( \gamma_2 \) globulin fraction of serum. Early evidence was provided by Jonas et al (73) who demonstrated by means of starch block electrophoresis that guinea-pig cytophilic antibody to SRBC was predominantly IgG immunoglobulin. Berken and Benacerraf (74) went on to show that the majority, if not all, guinea-pig cytophilic antibody to SRBC was present in the slow \( \gamma_2 \) globulin fraction of serum. This was later
confirmed by others (75,79,82), using various fractionation techniques. There is, however, one report which points to the contrary. Del Gueveio et al (95) immunised guinea-pigs with killed *Salmonella typhimurium* in FCA and found both \( \gamma_2 \) IgG and IgM to have identical cytophilic activity, when compared on a weight basis.

(2) In mice

There seems to be less agreement among workers concerning the distribution of cytophilic activity among mouse immunoglobulins. In mice, cytophilic antibodies are also found in \( \gamma_2 \) IgG. However, the cytophilic antibodies produced after primary immunisation alone are not necessarily \( \gamma_2 \) IgG. Thus, Parish (78) found that mouse cytophilic antibodies to both bovine plasma albumin and bovine-globulin were almost entirely present in the \( \gamma_2 \) IgG fraction, but cytophilic antibody to *E. coli* polysaccharide was apparently in the IgM fraction. Using SRBC as antigen, Berken and Benacerraf (85), Nelson et al (95) and Brown and Carpenter (94) could not find convincing evidence for the existence of a IgM cytophilic antibody in either early or hyperimmune mouse anti-SRBC sera. In contrast, Tizard (83) found cytophilic antibody to SRBC mainly in the 19S fraction in mice six days after a single intraperitoneal injection of SRBC in saline. But after a booster injection most of the cytophilic antibodies in these mice appeared in the 7S fraction. In addition, Rowley et al (98) have reported
evidence indicating the existence of a mouse 19S cytophilic antibody to *Salmonella typhimurium*. Hoy and Nelson (81) also detected 19S cytophilic antibody in C57Bl/6J mice anti-A/J antiserum, Lojak (86) separated mice anti-SRBC hyperimmune serum by gel exclusion chromatography and found cytophilic activity present mainly in the 7S fraction, though some activity was present in the 19S fraction. It thus appears that, in different situations, mouse cytophilic antibody may be 7S $\gamma_2$ globulins, or 19S globulins, or both.

Nelson and his associates (81,96,97) also found cytophilic activity in the $\alpha_1$-globulin fraction in mouse antisera. The cytophilic activity in these fractions appeared to be unstable to repeated freezing and thawing, but stable to heat (56°, 20 min). In general, it sensitized macrophages only weakly in terms of the subsequent uptake of antigen. It was not shown, however, that the $\alpha_1$-globulins were immunoglobulins. Antibodies, or immunoglobulins, are multichain proteins with a basic structure made up of light and heavy chains linked by noncovalent interaction and disulphide bonds. To qualify as an immunoglobulin, a protein must have to satisfy the above criteria, since the ability for a protein to combine with another substance is too general to be used as the sole basis for classification.

(3) In rabbits

In rabbits, Sorkin (33), and Berken and Benacerraf
identified cytophilic antibody as IgG. However, in a discussion of a paper presented by Boyden (32), Uhr stated that the cytophilic activity of the serum of rabbits injected with $\Phi X$ phage was present in the 19S fraction. No details were given.

(4) In primates

In primates, the immunoglobulin class which has cytophilic activity is not yet determined, but may be related to 7S IgG (89). In man, the cytophilic myeloma immunoglobulin was found (93) to belong to the $\gamma_1$-IgG and not the $\gamma_2$-IgG subclass, as is the case for guinea-pig cytophilic antibody.

Physicochemical Properties

In guinea-pigs, treatment of either whole serum or purified $\gamma_2$IgG with 2-mercaptoethanol alone does not affect cytophilic antibody. In early mouse antisera, on the other hand, treatment of the whole serum with 2-mercaptoethanol sometimes leads to the inactivation of cytophilic activity; this is usually interpreted as indicating the presence of 19S cytophilic antibody in the serum. However, in all cases, reduction of cytophilic antibody with 2-mercaptoethanol followed by alkylation with iodoacetamide consistently diminishes the cytophilic activity, while iodoacetamide alone has no effect.

Peptic digestion of guinea-pig or rabbit 7S $\gamma_2$
antibodies to SRBC destroyed the ability of the antibodies to absorb to macrophages (74). Since pepsin is known to destroy the Fc portion of IgG molecules, it was inferred that the binding of cytophilic antibody to macrophages was a function of the Fc fragment of the molecule. Nussenzweig and Benacerraf (99) also inferred that the different mobilities of guinea-pig \( \gamma_1 \) and \( \gamma_2 \) immunoglobulins were most likely due to the charge differences on the Fc portion of the molecule. Lamm et al (100) and Lamm (101) later extended this observation by amino acid analysis and peptide mapping, and showed that guinea-pig \( \gamma_1 \) and \( \gamma_2 \) immunoglobulins have similar Fab and (Fab')\(_2\) fragments, identical light chains, but dissimilar Fc fragments. It would have been interesting to see if in fact the isolated Fc or heavy chain subunits were capable of binding to macrophages. Recently, Thrasher and Cohen (181) found that chemical modification of the lysine or tryptophan groups of rabbit IgG antibody led to a marked reduction in the binding activity of cytophilic antibody. The procedures used, carbamylation, amidination and benzylation, had no effect on the antigen binding capacity of the antibody.

Evidence that cytophilic antibody is a special class of antibody

In their early experiments on rabbit spleen cell cytophilic antibody to HSA, Boyden and Sorkin (32) showed that absorption of anti-HSA antiserum with sufficient
spleen cells removed all detectable cytophilic activity but resulted in only a slight drop in the level of precipitating antibody in the serum. This was taken as evidence that cytophilic antibody is distinct from most precipitating antibody. Further support for this conclusion was provided by recovering the antibody which had adsorbed to the cells. The technique used was to heat the cells which had adsorbed cytophilic antibody, at 56°, for half an hour, whereupon some of the cytophilic activity was eluted. In such eluates, the ratio of cytophilic activity to precipitating antibody was at least one hundred times greater than in the original serum (32). However, it could also be argued that cytophilic antibody represents only a very small fraction of the total precipitating antibody in serum. In fact, it has been claimed (33) that in rabbit anti-protein sera, cytophilic antibody was responsible for only 0.1-1% of the antigen-combining capacity.

Repeated adsorption of mouse anti-SRBC anti-serum by mouse spleen homogenates (86) or by peritoneal exudate cells (94) removed up to 99% of the cytophilic antibody in the serum but left the titre of both haemagglutinin and haemolysin practically unaffected. The adsorbed antibody, when eluted from the cells at 56° was capable of agglutinating and lysing sheep erythrocytes (94).

Cytophilic antibody has been reported to bind complement (74). However, when individual sera were titrated
for cytophilic activity and complement fixing activity, Nelson and Mildenhall (82) found no correlation between these two activities, and suggested that these two activities were present on different antibody molecules which were produced independently. This may be so; but it is also equally possible that cytophilic antibody binds complement, but that the amount bound in this way is only a small portion of the total bound by all antibodies present.

However, other work would suggest that cytophilic antibody may be produced by a special class of cells: 1. In guinea-pigs, it has been generally observed that cytophilic antibody was produced only in animals injected with antigens in adjuvant. Injections of antigens in saline fail to produce cytophilic antibody despite the fact that such animals usually have comparatively high levels of antibodies with other activities. It is possible that the class of lymphocytes involved in the production of cytophilic antibody needs the additional stimulation caused by adjuvants. 2. Guinea-pigs usually respond to a second injection with a dramatic rise in serum cytophilic antibody titres. In contrast, the effect of a second injection on the production of haemagglutinating and complement fixing antibody titres is much less pronounced (75). This suggests that the memory for the production of cytophilic antibody is different from the memory for the production of antibodies with other activities.
Several reports (78, 83, 84) suggested that macrophage cytophilic antibody is in fact different from opsonin. It has yet to be shown if antigens, especially soluble antigens, remain on the surface of macrophages as antigen-cytophilic antibody-macrophage complexes under in vivo conditions. The importance of this question in relation to the possible role of cytophilic antibody in immune response is discussed in later sections.

Summary

There is strong evidence that, in guinea-pigs, macrophage cytophilic antibodies are found among \( \gamma_2 \) IgG but not among \( \gamma_1 \) IgG or IgM. The distribution of cytophilic antibody among the mouse immunoglobulins, however, is not certain. In different situations, mouse cytophilic antibody may be \( \gamma_2 \) IgG, IgM or both. Cytophilic activity of \( \gamma_2 \) IgG cytophilic antibody is not affected by treatment with 2-mercaptoethanol alone, but it is diminished by reduction with 2-mercaptoethanol followed by alkylation with iodoacetamide. Peptic digestion of guinea-pig or rabbit \( \gamma_2 \) IgG also destroys the ability of the IgG to adsorb to macrophages.

Macrophage cytophilic antibody represents only a small portion of the total antibody in serum and it can be removed from the serum by repeated absorption with macrophages or spleen cells. It is also possible that
macrophage cytophilic antibody represents a special class of antibody whose formation in the body is not necessarily the same as the production of antibodies with other activities.

5. THE BINDING OF CYTOPHILIC ANTIBODY TO MACROPHAGES

Factors Affecting the Attachment of Cytophilic Antibody to Macrophages

Slightly different results have been obtained by workers studying the optimum temperature for the binding of cytophilic antibody to macrophages. Using guinea-pig peritoneal macrophages, Jonas et al (73) found that the degree of sensitization of macrophages was similar whether carried out at 37°, room temperature or at 4°. Berken and Benacerraf (74), on the other hand, found the attachment of cytophilic antibody to macrophages to be greater at 37° than at lower temperatures. The reasons for this discrepancy are not apparent.

When macrophages carrying cytophilic antibodies are incubated at different temperatures, in the absence of serum or antigen, some cytophilic antibodies, as measured by the subsequent uptake of antigen, are lost from the cell surfaces. This loss is more pronounced at 37° than at lower temperatures. In the case of mouse macrophages and mouse serum, this loss is more pronounced with early than with hyperimmune serum (74,76,80). Other factors which
might affect the elution of cytophilic antibody from macrophages have not been studied.

Normal serum and cytophilic antibodies directed against other antigens inhibit the binding of cytophilic antibody to macrophages, as reflected by the subsequent uptake of antigen. The effect is especially marked if hyperimmune serum or 7S $\gamma_2$ fractions from a serum active against another antigen, are used (74). If guinea-pig macrophages which have taken up cytophilic antibody were incubated with normal serum, a "desensitizing" effect occurred, so that the sensitized macrophages now took up less antigen compared to sensitized macrophages incubated in the absence of normal serum. The "desensitizing" effect was slight at room temperature but more marked at $37^\circ$ (76). This type of result would suggest that cytophilic antibodies attached to the surface of the macrophages may be in equilibrium with cytophilic antibodies present in the medium. Normal serum might be expected to contain a pool of cytophilic antibodies of unknown specificity. These would also be in equilibrium with the cytophilic antibody already present on the cell surface. In support of this interpretation is the observation that guinea-pig $\gamma_2$ IgG (which is known to contain cytophilic antibody) is much more effective than $\gamma_1$ IgG (which is known not to contain cytophilic antibody) in inhibiting the uptake of specific cytophilic antibodies by macrophages (74). In addition, the Fc piece isolated
from the cytophilic $\gamma_1$ human myeloma protein, but not the Fc piece from the noncytophilic $\gamma_2$ human myeloma protein, is also capable of inhibiting the uptake of $\gamma_1$ myeloma protein by macrophages (93).

The ability of cytophilic antibodies to attach to macrophages of other species has also been investigated. Table 4 summarises the cross-species sensitization by cytophilic antibodies in different laboratory animals. With few exceptions, most species cross-sensitize readily.

Kossard and Nelson (76) also studied the ability of heterologous normal sera to inhibit the uptake of cytophilic antibodies by macrophages. It was found that normal rabbit serum was inhibitory with all combinations of mouse, guinea-pig and rabbit. Normal guinea-pig serum was inhibitory with all the combinations except mouse antibody on rabbit macrophages. Normal mouse serum was inhibitory only when either the antibody or the cell was from mouse. Normal human serum inhibited the uptake of guinea-pig antibody by guinea-pig macrophages.

These results would suggest that for most species, there are common receptors for cytophilic antibody on macrophages, but this does not exclude that there are receptors for more than one specificity on the one cell.

Receptors for Cytophilic Antibody on Macrophages

Treatment of macrophages with various reagents might be expected to affect their ability to adsorb
cytophilic antibody, and such experiments might lead to information about receptors for cytophilic antibodies on macrophages. Howard and Benacerraf (102) and Davy and Asherson (103) have investigated the effect of enzymes and various reactive chemicals on the uptake of cytophilic antibody by guinea-pig macrophages. From these studies and those of others (76), the following conclusions can be tentatively drawn:

1. Treatment with proteolytic enzymes did not diminish the ability of macrophages to take up cytophilic antibody. In fact, treatment with proteolytic enzymes (trypsin, papain, α-chymotrypsin or ficin) increased the ability of macrophages to take up cytophilic antibody. This suggested that protein, unless it is masked, does not form a functional part of the receptors. The increase in the uptake of cytophilic antibody by proteolytic enzymes treated macrophages could be explained by the assumption that treatment with proteolytic enzymes removes from the macrophage surface substances which mask some of the receptors for cytophilic antibody.

2. Chemicals which inhibit energy transfer in cells (Sodium azide, Sodium fluoride, Dinitrophenol) have no effect on the binding of cytophilic antibody to macrophages. Thus, the uptake of cytophilic antibody by macrophages is a passive process.

3. The receptors for cytophilic antibody are destroyed by agents that react specifically with free SH
groups (Iodoacetamide, β-chloromercurybenzoate). These results suggest that free SH groups are part of the receptor sites.

4. The receptors were inactivated by phospholipase A and chemicals (Iodine, periodate, peracetate, poly-L-lysine, Uranyl ion and Lanthanum) which are known to react with phospholipids. This suggests that the receptors are phospholipids or phospholipoproteins in which the protein moiety is protected.

So far, there is no information on the structure of the receptor, the means by which it is attached to the macrophage surface and the nature of the bond between the cytophilic antibody and the receptor. The fact that cytophilic antibody attached to the macrophage can be eluted by incubation in serum-free media and that the attached antibody readily exchange with cytophilic antibodies of other specificities suggests that the binding between cytophilic antibody and the receptor does not involve a covalent bond.

Summary

The attachment of cytophilic antibody to macrophages can be inhibited by normal serum or, more strongly, by cytophilic antibody of a different specificity. The bond between cytophilic antibody and the macrophages is not strong and is reversible at 37°C or higher temperatures. With a few exceptions, cytophilic antibody from one species is capable of attaching to macrophages of another species.
Similarly, normal serum from one species usually inhibits the binding of cytophilic antibody to macrophages of different species. Little is known of the nature of the receptors for cytophilic antibody on macrophages. The receptors are not affected by proteolytic enzymes and the uptake of cytophilic antibody by macrophages is a passive process, not requiring energy production. That the receptors are destroyed by phospholipase A and also by reagents reacting specifically with free SH groups suggests that the receptors are phospholipid in nature and that free SH groups are part of the receptor sites.

6. POSSIBLE BIOLOGICAL ROLE OF MACROPHAGE CYTOPHILIC ANTIBODY

Although a considerable amount of data on the physical and chemical properties of cytophilic antibody has been accumulated, the biological role of this antibody is, so far, unknown. Several studies suggest that cytophilic antibody might play an important role in certain aspects of the immune response, but the evidence supporting any particular role is largely inconclusive. However, one could argue that the production of a specific type of antibody which attaches to macrophages should have some biological importance. In this section, the possible immunological importance of cytophilic antibody is discussed.

Delayed-Type Hypersensitivity

There is excellent evidence that macrophages act as
effector cells in those immune reactions which characterise delayed hypersensitivity. This has been clearly demonstrated in several systems. McCluskey et al (106) for instance, have shown that blood monocytes are the predominant cells in the exudates of delayed hypersensitivity reactions. Mackaness (107), in addition, found that animals sensitized to bacterial antigens had an increased number of macrophages and a change in the properties of these cells, which developed larger lysosomes, and were better able to kill virulent intracellular organisms. Furthermore, it has been repeatedly demonstrated that the incubation in vitro of antigens with lymphocytes from guinea-pigs with delayed-type hypersensitivity stimulates the synthesis by, and release from, these cells of macromolecular substances, which inhibit the migration of macrophages in vitro (108, 109).

Since macrophage cytophilic antibodies are present in immune animals and become attached to macrophages, it is conceivable that these antibodies, when attached to macrophages, might participate in the reactions which characterise the delayed response. The first observation consistent with this idea came from the work of Boyden (34), who found that guinea-pigs developed delayed hypersensitivity to SRBC only when the animals were injected with the antigen in FCA. This was related to the finding that only guinea-pigs injected in this way produced significant
amount of cytophilic antibody to SRBC. However, later attempts to correlate serum levels of cytophilic antibody with the intensity of the delayed skin reactions were not successful (75, 82, 110, 111). In apparent contrast, there is a correlation between serum levels of cytophilic activity in mice immunised with SRBC and the intensity of the delayed reaction to the antigen after injection into the footpads (112). The cytophilic activity in the serum, however, was not found in the immunoglobulin fraction but in the α₁ globulin fractions (96).

In guinea-pigs, local passive transfer of delayed-type skin reaction to SRBC has been achieved by means of normal peritoneal cells sensitized in vitro with cytophilic antibodies (113), but such a transfer of delayed hypersensitivity to other antigens has not been achieved (79, 113). In addition, normal guinea-pigs injected with serum containing 7S γ₂-globulin cytophilic antibody do not develop delayed-type hypersensitivity despite the presence of cytophilic antibody in their circulation (75).

In guinea-pigs with delayed-type hypersensitivity, the injection of antigen by any route (but notably intra-peritoneally) is followed promptly by a profound fall in the number of suspended macrophages (114). This macrophage "disappearance" reaction has been studied in some detail and is considered to be a manifestation of delayed-type hypersensitivity (45). Before disappearing from the suspension, it was noted that the macrophages
tended to form clumps, and then adhered to the lining of peritoneal cavity (45). The reaction is immunologically specific, transferable to normal guinea-pigs by cells from hypersensitive guinea-pigs but not by serum alone. The macrophage "disappearance" reaction can also be transferred to normal guinea-pigs by means of normal guinea-pig peritoneal cells treated \textit{in vitro} with immune serum containing cytophilic antibody (115). This finding supports the idea of a role for cytophilic antibody in the macrophage disappearance reaction.

The inhibition of migration of peritoneal macrophages by antigen \textit{in vitro} appears also to be a characteristic manifestation of delayed hypersensitivity (116). The mixture of a small number of peritoneal exudate cells from a hypersensitive donor and macrophages from a normal guinea-pig, plus antigen, resulted in the inhibition of the migration of all the cells in the mixture. However, when normal peritoneal cells were treated with serum from hypersensitive donors and then exposed to varying amounts of antigens, they migrate normally (116,117). This finding tends to suggest that cytophilic antibody is not involved in the migration inhibition reaction.

Although some of the discrepancies between the various results discussed above can probably be explained by differences in methodology, there is as yet no clear-cut evidence that cytophilic antibody plays a role in delayed-type hypersensitivity. Recently, Zembala and
Asherson (119) demonstrated that the 24-hour contact-sensitivity skin reaction in mice to oxazolone could be transferred by both immune serum and by normal peritoneal exudate macrophages incubated with immune serum containing cytophilic antibody. However, they emphasised that their results did not mean that classical delayed hypersensitivity could be transferred by circulating antibodies or by macrophages incubated with immune serum. They suggested, instead, that macrophages coated with cytophilic antibody and circulating antibody played a role in the skin reaction to oxazolone in the mice. This finding would have considerable interest if confirmed.

Antibody-mediated Regulation of Immune Response

One of the most specific means for the regulation of the immune response is with the use of specific antibody. Antibody, introduced into an immunologically responsive system, may also alter the character of the subsequent response. In this section, the effect of passively administered antibody on the immune response is briefly reviewed and the possible part played by cytophilic antibody is discussed. For further information on the regulatory effect of antibody on the immune response, readers are referred to the excellent review of Uhr and Möller (120).

A primary antibody response to an antigen can be readily prevented by mixing the antigen with an excess of
antibody prior to injection (121,122). The inhibition requires the use of specific antibody (23,123-125). Both the ability to prime for a secondary response and to elicit a secondary response can also be inhibited by specific antibody, but to achieve effective inhibition is more difficult (25,126).

Both IgM and IgG antibodies have been shown to be capable of inhibiting the immune response in several systems. However, there is no general agreement towards the relative effectiveness of these two classes of antibodies to suppress antibody induction. Perlman (127), for instance, found IgG and IgM antibodies of equivalent antigen-combining capacity to be equally effective in suppressing the formation of antibody to sheep erythrocytes in rabbit. Rowley and Fitch (126) found a similar situation in rats. In contrast, using more purified preparations, Møller and Wigzell (128) found only IgG antibody to be efficient at suppressing the immune response in mice to sheep erythrocytes. Furthermore, Henry and Jerne (129) found that the primary response in mice to sheep erythrocytes was enhanced by IgM but suppressed by IgG. The discrepancies between these reports could partly be attributed to the difficulties inherent in comparing the two classes of antibody. There may be considerable differences between the ability of IgM and IgG antibodies to cause agglutination, haemolysis, precipitation or neutralisation of infectivity, making it difficult to
compare the quantities of the two classes of antibody. A possible difference in avidity, which for different classes of antibodies may vary during an immune response, also has to be taken into consideration in comparing the effectiveness of IgM and IgG antibodies in influencing the immune responses.

Although the most frequently observed effect of passively administered antibody is inhibition, injected antibody may enhance its own production. Intravenous injection of BSA complexed previously with a small amount of specific antibody, leads to enhanced antibody formation to BSA, compared to control animals, which have received a similar dose of BSA but without the antibody (130,131). The enhancement appears to be specific and only the antigen-antibody combination prepared in antigen excess will elicit an active anti-BSA response (132,133). Similarly, it was found that injection of a mixture of tetanus toxoid with specific horse anti-toxin, stimulated specific antitoxin formation in colostrum-deprived piglets (134,135). The injection of the same dose of toxoid alone did not usually lead to detectable antitoxin formation in such piglets. A similar phenomenon was demonstrated for a cellular antigen, sheep erythrocytes, in mice (128). Walker and Siskind (136), in addition, observed that very low concentrations of high affinity antibody, but not low affinity antibody, increased the expected magnitude of antibody formation to DNP in rabbits.

Thus depending on the regimen of immunisation
(notably the antigen/antibody ratio), passively administered antibody could either suppress or enhance antibody formation.

In general, delayed hypersensitivity to protein antigens appears to be less easily suppressed than is either the primary antibody response or 'priming' for a secondary antibody response. It has been shown that complexes of toxin and antitoxin can induce delayed hypersensitivity indistinguishable from that induced by free toxin (137,138). Indeed, all attempts to demonstrate suppression of delayed hypersensitivity in this system have failed, despite the use of large amounts of heterologous antitoxins (24).

The detailed mechanism of antibody mediated suppression of the antibody response is largely unknown. One interpretation is that passive antibodies compete with immunocompetent cells for antigenic determinants by forming antigen-antibody complexes. It implies that these complexes are less immunogenic than free antigens and are degraded by the animal without the release of immunogenic material (120). This hypothesis has been termed "peripheral" in the sense that the potential of the immunocompetent cells is believed to remain unaffected. On the other hand, data has been reported which suggests that passive antibody, together with antigen, has a "central" effect on the immune system by producing a specific reduction in the number of immunocompetent cells (126,142).
TABLE 4

THE ABILITY OF CYTOPHILIC ANTIBODIES TO SENSITIZE MACROPHAGES OF OTHER SPECIES

<table>
<thead>
<tr>
<th>Cytophilic antibody from</th>
<th>Macrophages from</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>guinea-pig</td>
<td>Guinea-pig</td>
<td>Mouse</td>
<td>Rabbit</td>
<td>Rat</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>±</td>
<td>+b</td>
<td></td>
</tr>
<tr>
<td>mouse</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>rabbit</td>
<td>+</td>
<td>+</td>
<td>+b</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+b</td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>+b</td>
<td>ND</td>
<td>ND</td>
<td>+b</td>
</tr>
</tbody>
</table>

a. data derived from reference 74, 76.
b. candidate's own observation

ND = not determined

+ = positive; ± = trace; - = negative.
In a series of carefully designed experiments, Sinclair and his colleagues (143-145) have shown that removal of the Fc portion reduced the ability of the specific IgG to inhibit the specific antibody response by factors of greater than 10 and sometimes as great as 1,000 fold. Furthermore, these workers were able to show that the more rapid excretion of (Fab')$_2$, compared to IgG, was not the sole reason for its lower effectiveness in suppressing the antibody response. As will be shown in this thesis, the Fc fragment is responsible for the attachment of cytophilic antibody to macrophages, thus, it is conceivable that cytophilic antibody might be involved in the inhibition of antibody response. The following findings support the idea of a role for cytophilic antibody in the antibody-mediated suppression of antibody formation:

1. The exposure of normal spleen cells in vitro or in vivo to specific antibody against SRBC suppressed the PFC response of the spleen cells to this antigen in X-irradiated recipients (126). This observation led Rowley and Fitch (126) to the conclusion that antibody could directly reduce the number of immunocompetent cells reactive to the corresponding antigen. However, this suppression of PFC response could also be interpreted as a consequence of uptake of cytophilic antibody by the splenic macrophages. The mechanism by which macrophages sensitized with
cytophilic antibody may influence the immune response will be discussed later.

2. Chicken anti-HSA hyperimmune serum, which completely suppressed the primary antibody response of chickens to HSA, lost its immunosuppressive activity after repeated adsorption with calf spleen cells (146). Although in this experiment the suppressive antibody, which was presumably cytophilic antibody, was not eluted by heat from the spleen cells used to absorb the immune serum, the result was consistent with the idea that cytophilic antibody may be involved in the antibody-mediated suppression of the antibody response.

3. The work of Pierce (147) also suggested that macrophage cytophilic antibody might be involved in the antibody-mediated regulation of immune response. In this experiment normal mouse spleen cells were separated into a lymphocytes-rich fraction (LR), and a macrophages-rich fraction (MR). Incubation of the LR fraction with mouse anti-SRBC antibody did not impair the ability of these cells, when combined with untreated MR fraction, to develop a PFC response in vitro. However, if the MR fraction was incubated with the antibody either before or after incubation with SRBC, and then combined with the LR fraction, the subsequent PFC response by the lymphoid cells was suppressed.
Although these findings did not prove that cytophilic antibody was the prime factor in the antibody-mediated suppression of the antibody response, they, nevertheless, suggested rather strongly that cytophilic antibody could be one of the factors involved.

III. ANTIBODIES CYTOPHILIC FOR CELLS OTHER THAN MACROPHAGES

1. LYMPHOCYTES

Structure
One of the distinct features of small lymphocytes is the very high ratio of nucleus to cytoplasm. The majority of lymphocytes are small. In so far as their size is concerned, lymphocytes may be roughly graded as large (> 12 μm), medium (10-12 μm) and small (< 10 μm), and the predominant type of lymphocytes in the peripheral blood of a normal human is the small lymphocytes (80-83%) (148).

The nucleus of the small lymphocyte is markedly pachychromatic, i.e. its chromatin is in thick clumps. There is usually a dense accumulation of chromatin immediately adjoining the nuclear membrane, and this membranous condensation is clearly visible in electron micrographs as well as with the light microscope (149). Under the light microscope, the nucleus of the small
lymphocytes usually appear to be round, with occasional small indentations.

**Classification**

One of the points which repeatedly emerges in many of the experiments on the lymphmyeloid complex, is the fact that by all our known criteria, lymphocytes which are morphologically similar, have very different potentialities. Mature lymphocytes are envisaged to be of at least two types, which are referred to as T lymphocytes (thymus-dependant), and B lymphocytes (bursa equivalent or thymus-independant and bone marrow derived). Circulation studies in adult mammals showed that both the T and B cells are derived from the bone marrow. It is thought that T cell precursors from the bone marrow enter the thymus. The mature T cells, possibly several generations removed from their precursors in the thymus, are found in the circulation and in the lymphoid tissues (151,152). Other bone marrow precursor cells differentiate in another direction and develop into the B cells. These are also found in the circulation and in lymphoid tissues. In chicken, it has been clearly shown (153) that the Bursa of Fabricius is the organ where this differentiation occurs, but a similar organ has not yet been identified in mammals. It now seems likely that at least the T cells can further be subdivided into two subclasses which are functionally distinct. The co-operation between the two
classes of lymphocytes is a feature of many immune responses. It is also a subject of intense investigation in current immunological research, the detail of which is beyond the scope of the present review.

Lymphocytes transformation to macrophages

It has generally been accepted that macrophages are derived mainly from blood monocytes. However, there is also evidence that some macrophages may be derived from lymphocytes. The origin of macrophages from lymphocytes appears to be still the subject of controversy.

The view that blood-borne lymphocytes give rise to macrophages was first expressed by Maximow (154), Bloom (155) and later by Rebuck and his colleagues (156,157). Rabinowitz and Schrek (158), on the other hand, showed that when most monocytes were removed by glass beads, very few macrophages formed and when lymphocytes were removed by irradiation, the number of macrophages subsequently found corresponded to the number of monocytes at the start. Fikrig et al (159) also concluded from PHA culture experiments that rabbit macrophages did not arise from leucocytic culture of monocyte-free blood or from lymph. However, Gough et al (160) observed that in the absence of PHA, up to 30 per cent of their blood lymphocytes became macrophages, if living polymorphs were present. The transformation did not occur if the polymorphs were dead. The need for living polymorphs suggested that some kind of symbiotic process was at work. This, in their view,
explains why a number of previous investigators had been unable to demonstrate the lymphocytes to macrophages transformation.

The thoracic duct lymph is the most common source for obtaining pure lymphocytes (free of macrophages). The formation of macrophages from thoracic duct lymphocytes in diffusion chambers (161), on transfusion into another animal (162) or on monolayers (163), seems also to indicate that at least some cells with lymphocyte morphology can become transformed into macrophages.

Coulson et al (164), working with preparations of guinea-pig lymphocytes from which most, but possibly not all, monocytes had been removed, observed the development, probably from lymphocytes, of a number of cells which took up cytophilic antibody and became positively sensitized. In guinea-pigs, cytophilic antibody is thought to be passively adsorbed only by macrophages. However, since lymphocyte cytophilic antibody has been shown to exist, at least in other species, it is doubtful if cytophilic antibody adsorption alone is a good criterion for identifying macrophages.

The question of whether macrophages can be derived from lymphocytes is far from being resolved. Indeed, it becomes rather pointless to indulge in such debate until a marker, capable of distinguishing between lymphocytes and monocytes unequivocally, is found.
Lymphocyte cytophilic antibody

The original experiment on cytophilic antibody was performed on rabbit spleen cells (31). Spleen cells suspension incubated with anti-HSA antisera were found to have the capacity to take up a relatively large amount of 125I-labelled HSA. The cell population in a spleen is highly heterogeneous, and includes lymphocytes, macrophages, plasma cells and others so that it is possible that the cytophilic antibody attached to a number of different cell types. That lymphocyte cytophilic antibody existed was first indicated by Sorkin (discussion in reference 33) who quoted that cells from the thoracic duct of normal rabbits have the ability to adsorb cytophilic antibody, and subsequently, antigens.

Rabbit antibody cytophilic for lymph node lymphocytes has also been described by Rose et al (28), Uhr (165) and Bussard (29) as occurring in antisera to thyroglobulin, bovine serum albumin and bacteriophage \( \Phi X \). Kossard (30) examined rabbit anti-SRBC antiserum containing antibody cytophilic for macrophages and found that it was also capable of sensitizing not only rabbit and guinea-pig macrophages but also guinea-pig small lymphocytes. Koprowski and Fernades (166) incubated normal rat lymph node cells with serum obtained from rats injected with guinea-pig cord tissue. After incubation, these lymph node cells were capable of aggregating on, or around the glial elements in tissue culture monolayers of guinea-pig
brain. In a different situation, Uhr (80) found that complexes of flagellar antigens and guinea-pig antibodies would attach to lymphocytes and plasma cells as well as to macrophages and conferred on all these cells the ability to take up motile bacteria. This form of passive sensitization of lymphocytes was observed only with a small proportion of lymphocytes and seemed to be partly dependent on the presence of complement. Similarly, Lay and Nussenweig (105) also observed that sheep erythrocytes sensitized by rabbit anti-Forssman antibodies adhered to and formed rosettes with mouse lymphocytes. This adherence was also complement dependent and did not occur on thymus lymphocytes.

Basten et al went on to show that a proportion of lymphocytes from primed mice carried receptors for the Fc portion of antibody molecules. The bond between the lymphocytes and the lymphocyte cytophilic antibody was weak, but could be stabilised by addition of the corresponding antigen. For soluble antigens, they found that the attachment of cytophilic antibody to lymphocytes was independent of complement. This finding is in direct contrast to those of Lay and Nussenweig (105) who found that the attachment was complement dependent. The reason for the discrepancy is not apparent but could be due to the difference in antigens used and also the difference in antisera used (e.g., homologous vs. heterologous). Later, the antibody-receptors carrying lymphocytes were identified as B cells (168).
appears that all B lymphocytes, including those from unprimed mice possess receptors which enable them to bind immune complexes. Most, if not all, T cells do not possess receptor for cytophilic antibody. The demonstration of receptors for cytophilic antibody on B cells, but not T cells, led to the development of a technique for depletion of B cells from lymphoid cell populations (174). Lymphocytes from spleen or thoracic duct lymph were incubated with antibody and then passed through a column of beads coated with the corresponding antigen. The cells that emerged from the column were predominantly T cells.

At present, little is known about the properties of lymphocyte cytophilic antibody. It is also not certain if lymphocyte cytophilic antibody is the same as the macrophage cytophilic antibody. However, with the increasing intensity of research on the functional importance of lymphocytes, our knowledge of lymphocyte cytophilic antibody will certainly improve rapidly in the near future.

2. BASOPHILS AND KURLOFF CELLS

Cytophilic antibodies for basophils and Kurloff cells have been detected only recently (169). Both the basophils and Kurloff cells from guinea-pigs can be passively sensitized in vitro by guinea-pig serum containing antibodies to either a particulate antigen (SRBC) or to a soluble antigen (Fab' from rabbit IgG). The passively sensitized basophils and Kurloff cells take up antigen in
the same way as do passively sensitized macrophages. Passive sensitization in vitro of Kurloff cells and basophils was also achieved with fractions of guinea-pig anti-SRBC antiserum. Kurloff-cell cytophilic antibodies, like the cytophilic antibodies for macrophages, were mainly $\gamma_2$-globulins, while antibodies cytophilic for basophils were found predominantly in the $\gamma_1$-globulin fractions.

The demonstration of cytophilic antibodies for basophils will undoubtedly aid studies on anaphylactic reactions. The significance of Kurloff-cell cytophilic antibodies, however, is unknown. The Kurloff cells, which are characterised by the presence of a vacuole with rod-like or spherical inclusion, are apparently unique to guinea-pigs. In these animals, the Kurloff cells are found in many organs of the body, with the notable exception of the lymph nodes where they are extremely scarce (170,171). Morphologically, the Kurloff cells are related to lymphocytes (170,173). No physiological function has as yet been attributed to Kurloff cells, although they are known to increase greatly in number in pregnant guinea-pigs and in guinea-pigs injected with oestrogen (172,173).

3. LYMPHOID FOLLICLES AND DENDRITIC CELLS

The antibodies responsible for the retention of antigens in the lymphoid follicles may also be considered as cytophilic antibodies.
Basically, two types of cells, lymphocytes and dendritic cells are found in lymphoid follicles. In secondary follicles, there is also a small number of tangible body macrophages. The dendritic cells are characterised by extended cytoplasmic processes. The intertwining of these dendritic cell processes creates a weblike structure and fills up the extracellular space in the follicles. Antigens retained in the follicles are found predominantly in association with these processes and are retained extracellularly. The dendritic cells are different from macrophages in that the former are only weakly phagocytic. The exact biological function of the dendritic cells is not known.

The mechanism of antigen retention on the dendritic cells has been studied (for review see reference 22). Evidence obtained to date strongly suggests that antigens become localised in follicles because of the presence of antibodies. In fact, immunoglobulins themselves (either IgG or IgM) are able to localise in this region. Using a radioactivity assay, Herd and Ada (25) found that normal rabbit IgG heavy chain and Fc fragment, but not light chain or Fab fragment, became localised in the follicles of rat popliteal and iliac lymph nodes following injection of these materials into the hind footpads of rats. Since Fc fragment constitutes the C-terminal part of the heavy chains of IgG, this finding indicates that the globulin molecule reacts with the dendritic cell membrane through the Fc portion of the antibody molecule.
The biological significance of the localisation of antigens in lymphoid follicles is not certain. However, it was postulated that antigens in the lymphoid follicles may trigger off primed cells or cause tolerance, and that the feedback inhibition of antibody formation by antibody, might be mediated by antigen caused by antibody to localise in lymphoid follicles (177).

IV. CONCLUSION

Since the discovery of cytophilic antibody eleven years ago, many features still remain a mystery. It is not uncommon, however, to find cytophilic antibody being cited in papers to account for some "hard-to-explain" results. Interest in cytophilic antibody is stimulated, therefore, not only by the inherent fascination of a poorly understood phenomenon but also by the possible association of this antibody with some important aspects of immune responses.

The major unsolved problems in this field are:

1. **The production of cytophilic antibody.** A general agreement on the parameters affecting the production of cytophilic antibody in various laboratory animals is still lacking. The influence of factors such as the adjuvant used and the physical and chemical properties of antigens, have not been well documented.

2. **The mechanism of binding of cytophilic antibody to cells.** So far, there is little information
concerning the nature and structure of the receptor sites on the cells. The means by which cytophilic antibody is attached to cell surface and the nature of the bond between the antibody and the receptor are also obscure. It has been inferred that the Fc portion of the IgG molecule is responsible for the binding of cytophilic antibody to cells, but direct evidence is still lacking.

3. Recent studies have suggested that cytophilic antibody might play an important role in the antibody-mediated regulation of immune response, but direct proof is not yet available.

4. The role of macrophage cytophilic antibody in delayed-type hypersensitivity. Again, no clear-cut conclusion can yet be drawn about the role of cytophilic antibody in delayed-type hypersensitivity. Although it is almost certain that cytophilic antibody does not play an indispensable part as a mediator of the delayed response, the possible role of cytophilic antibody in the induction of delayed hypersensitivity has not been investigated. Recent studies have shown that suppression of antibody formation by a series of chemically modified antigens was accompanied by enhanced delayed-type hypersensitivity to the antigens (178-180). In other words, there appears
to be an inverse relationship between humoral and cell-mediated immunities. If this relation represents a general phenomenon, then the suppression of antibody formation by passively administered antibody would also be expected to enhance cell-mediated immunity. Thus, if cytophilic antibody were one of the main factors in the antibody-mediated suppression of the humoral response, then it is not inconceivable that cytophilic antibody may play an important role in the induction of cell-mediated immunity.

The experiments described in this thesis go some way towards answering some of these problems.
REFERENCES


30. KOSSARD, S., Thesis, Department of Bacteriology, University of Sydney, Australia. 1966. (from reference 45).


34. BOYDEN, S.V., Immunology 7, 474, 1964.


41. RIBBERT, H., Z. Allgem. Physiol. 4, 201, 1904.


46. BALNER, H., Transplantation 1, 217, 1963.


77. NETER, E., Bact. Rev. 20, 166, 1956.


121. BUXTON, J.B., and GLENNY, A.T., Lancet ii, 1109, 1921.


155. BLOOM, W., ibid 24, 567, 1926.


165. UHR, J.W., in discussion of reference 32.


175. WHITE, R.G., Ciba found. study group No. 16, 16, 1963.


"SOME CHEMICAL AND BIOLOGICAL PROPERTIES OF MACROPHAGE CYTOPHILIC ANTIBODIES"
"THE DETECTION AND PRODUCTION OF MACROPHAGE CYTOPHILIC ANTIBODY TO DIFFERENT ANTIGENS IN DIFFERENT LABORATORY ANIMALS"
SUMMARY

An in vitro assay for macrophage cytophilic antibody is described, based on the measurement of the adherence of \(^{125}\)I-labelled antigen to peritoneal exudate cells pretreated with antiserum. Using this assay, the production of cytophilic antibody to SRBC, BSA, polymerised flagellin, flagellin and a CNBr digest of flagellin was investigated in guinea-pigs, rats, rabbits and mice. Antigens injected in either Freund's complete or incomplete adjuvant were almost equally efficient at inducing cytophilic antibody production. In contrast, antigens injected in saline failed to induce detectable cytophilic antibody. The ratio of cytophilic antibody titre/passive haemagglutinating antibody titre was calculated and it was found that the ratio for rabbit > guinea-pig > mouse > rat. Furthermore, in those sera which were examined, cytophilic antibody was only detected in the 7S fractions.
INTRODUCTION

Cytophilic antibody has been of spasmodic interest since its discovery by Boyden and Sorkin (1). This is partly because there is still no clear evidence demonstrating the role it may play in the immune response. It has been suggested (2) that cytophilic antibody might be involved in the delayed-type hypersensitivity phenomenon, but the evidence is inconclusive. Perhaps its main function is simply to aid the carriage of antigen by macrophages, but evidence for such a straightforward role is still lacking. As part of a programme to study the possible role of cytophilic antibody in the immune response, it became necessary to study the parameters affecting the production of cytophilic antibody in different laboratory animals. The influence of such factors as the adjuvant used, and the physical and chemical properties of antigens have not been well documented. To do this adequately, a general method for the estimation of cytophilic antibody to soluble antigens is required. For this purpose, a radioimmunoassay based on the earlier work of Boyden and Sorkin (1) was developed and standardized. This report describes the use of this method in investigating some of the factors which affect the production of cytophilic antibody to different antigens in a range of laboratory animals.
MATERIALS AND METHODS

Animals

Albino guinea-pigs (12-16 weeks old), rabbits (about 20 weeks old) and Walter and Eliza Hall Institute mice (9 weeks old) bred randomly and of both sexes were used. Rats of an inbred strain (Lewis) and about 24 weeks of age were used. All animals were fed on Crajo pelleted diet (Crajo, Sydney) ad libitum.

Antigens

Sheep red blood cells (SRBC) stored in Alsever's solution at 4°C were washed 3 times in saline before use. Bovine serum albumin (BSA, crystallised, Armon, Eastbourne, England) was dissolved in saline to 5 mg/ml and stored at -10°C. Flagellin (FIN) and polymerised flagellin (POL) from Salmonella adelaide 1338 were prepared as described by Ada, Nossal, Pye and Abbot (3). Flagellin has a molecular weight of 40,000 and POL has been estimated to have a mean particle weight corresponding to about 300 flagellin units. The cyanogen bromide (CNBr) digest of flagellin (CNBr.D) was obtained from flagellin by treatment with CNBr as described by Parish and Ada (4). The digest contained four fragments, one of which (fragment A) has a molecular weight of 18,000 and possesses all of the antigenicity of the flagellin molecule.

Immunization

Antigens were injected in one of two ways:
1. As a solution or a suspension (SRBC) in saline or
2. As an emulsion formed by mixing with either Freund's incomplete or complete adjuvant (FIA or FCA, Difco, Detroit). In the latter case, antigens at a concentration of 5 mg/ml or a 30% suspension of SRBC were emulsified with an equal volume of the adjuvant. Rabbits and guinea-pigs had a similar injection scheme. A saline or an adjuvant preparation of the antigen was injected into each of the four footpads at the rate of 0.1 ml/footpad. 10 and 17 days later, the animals were injected intra-dermally with the respective antigens (0.1 ml of 15% SRBC in saline or 0.1 mg in 0.1 ml saline for the other antigens per site) into each of 4 sites.

For rats and mice, the protein antigens, dissolved in 0.15 M NaCl to 2 mg/ml, were mixed with an equal volume of saline or emulsified in an equal volume of FCA, or FIA, and then injected into the hind footpads (0.05 ml/footpad). 10 and 17 days later, the animals were injected intraperitoneally with the respective antigens (0.1 mg in 0.1 ml saline).

Some of the guinea-pigs were bled from an incision in the interdigital web before each of the booster injections. All animals were exsanguinated under either anesthesia 7 days after the last injection. The sera were separated on the same day and stored at -10°.

Fractionation of sera

Sera were fractionated on a sucrose gradient. Serum
(50 µl) was layered on top of a sucrose gradient (20% to 35% w/w) in plastic tubes (2" x 1-1/2") and centrifuged at 60,000 rpm for 15 hours at 4°C. Fractions (0.2 ml), collected by a tube-bottom-puncturing technique, were analysed for passive haemagglutinating antibody. Two well separated peaks of haemagglutinin were always obtained and they were estimated to have sedimentation values of 19S or 7S by analytical ultracentrifugation (performed by my colleague Mr. P.L. Ey on a Beckman Model E centrifuge). Fractions containing 19S and 7S haemagglutinins were analysed for their content of macrophage cytophilic antibody.

Estimation of antibodies

Serum antibody levels were estimated by a passive haemagglutination method (R.E. Langman et al, in preparation) based on that described by Jandl and Simmons (5). Briefly, SRBC stored (0-5 days) in Alsever's solution at 4°C were washed 4 times with saline and resuspended to 5% in saline. To 5 ml of 5% SRBC in saline was added 50 µg of POL. The solution stirred and 65 µl of 0.1% CrCl₃ solution (pH 5.2) added with further mixing. After standing at room temperature for 5 to 10 minutes the cells were washed 3 times with phosphate buffered saline (PBS) and resuspended to 2% in PBS. The POL coated SRBC were used for estimating antibodies to all the flagellar antigens. When BSA was used as the coating antigen the corresponding amounts of reagents used were: BSA, 200 µg; 0.1% CrCl₃
solution, 200 µl. All titrations were done in V-bottom micro-titrating wells. PBS containing 5% FCS or 5% normal serum (for anti-BSA serum) was used as diluent.

Medium

Experiments involving peritoneal exudate cells were carried out in the following medium: 100 ml of medium 199 (10 times concentrated, Commonwealth Serum Laboratories, Melbourne), 10 ml of heparin (10,000 units), 1 ml of NaN₃ (1.5 M) and 10 ml of serum made up to 1 litre with PBS. For anti-POL, anti-FIN and anti-CNBr.D titrations foetal calf serum (FCS) was used. For anti-BSA sera, normal sera were used. Media containing serum at this concentration did not interfere with the adherence of antibodies to macrophages and gave more reproducible results than did medium without serum.

Peritoneal exudate cells

Normal adult animals, which had been injected intraperitoneally with 5 ml of 0.001% (w/v) oyster glycogen solution five days previously, were anaesthetised with ether, exsanguinated and the peritoneal cavity flushed with either 50 ml (guinea-pigs), 10 ml (rats) or 5 ml (mice) of medium. The cells were collected in a pre-chilled beaker, pooled, washed 3 times with cold medium and resuspended at 1.2 x 10⁷ cells per ml in medium. The yield was about 4.0 x 10⁷ cells per guinea-pig, 3.0 x 10⁷ cells per rat and 1.0 x 10⁷ cells per mouse, and 70 to 75%
of the cells were considered to be macrophages as judged by morphology.

**Iodination of Antigens**

Iodination by direct oxidation of carrier free \(^{125}\text{I}\) with Chloramine T was carried out according to Greenwood, Hunter and Glover (6) as modified by Ada, Nossal and Pye (7). The extent of substitution of the labelled proteins were for BSA 1 mole of iodine per 7 to 20 moles of protein and for FIN, 1 mole of iodine per 20 to 25 moles of protein.

**Radioautography**

Radioautography of cell smears was carried out as described by Byrt and Ada (8).

**Radioimmunoassay of cytophilic antibody**

Peritoneal exudate cells (5 x 10^6 cells) in 0.4 ml of medium were exposed at room temperature for 1 hour to 0.1 ml of a series of 2 fold dilutions of immune serum or to 0.1 ml of medium (control). All dilutions were made in the medium. The reactions were carried out in plastic tubes of uniform wall thickness (suitable for radioactivity determination). The cells were then cooled and all subsequent reactions carried out at 0-4°C. Cells were then centrifuged for 5 minutes, washed 4 times in medium, resuspended in 0.1 ml medium and 400 ng of \(^{125}\text{I}\)-labelled antigen (BSA or FIN) in 10 µl PBS containing 1% (w/v)
gelatin added. The contents were mixed and left overnight. At the end of this period, the cells were again washed 4 times and cell associated radioactivity was measured using an auto-gamma scintillation counter (Packard Autogamma Spectrometer). A portion of the cells was then smeared on gelatin coated glass slides in preparation for radioautography. The binding of cytophilic antibody to the cells was expressed as ng of antigen associated with $5 \times 10^6$ cells. The uptake of $^{125}$I-labelled antigen (in ng) was plotted against log$_2$ of serum dilution. The end point was arbitrarily taken as the dilution of serum at which the amount of $^{125}$I-labelled antigen adsorbed was twice the amount of $^{125}$I-labelled antigen bound to control cells, i.e. cells treated with medium only.

RESULTS

THE RADIOIMMUNOASSAY

1. The effect of temperature on the binding of cytophilic antibody to peritoneal exudate cells

There has been some uncertainty in earlier work concerning the optimum temperature for the binding of cytophilic antibody to macrophages. To investigate this parameter, guinea-pig serum containing cytophilic antibody to BSA was incubated with $1 \times 10^7$ normal guinea-pig peritoneal exudate cells at three different temperatures ($0^\circ$, room temperature and $37^\circ$) for 1 hour or for 4 hours. After washing, 1 µg of $^{125}$I-BSA was incubated with the cells for 15 hours at $4^\circ$ and the cell bound radioactivity
measured. Fig. 1 shows that, at each temperature and
time, cells incubated with serum containing cytophilic
antibody adsorbed more $^{125}$I-BSA than did the controls.
However, cells incubated with anti-serum at room
temperature consistently bound more antigen than did
cells treated otherwise. In fact, prolonger incubation
(4 hours) of cells with the anti-serum at $37^\circ$ reduced
the antigen uptake. From these results, the conditions
for routine assay were standardised as incubation of
cells with anti-serum for 1 hour at room temperature.

2. The reproducibility of the assay system

The reproducibility of the radioimmunoassay was
tested using pooled guinea-pig anti-BSA and pooled anti-
FIN hyperimmune sera. Each serum was titrated five times.
The five end-points obtained for each serum agreed to
within ± 0.5 tube. Fig. 2 shows the titration curves of
guinea-pig anti-BSA and anti-FIN anti-sera. In each
case, using the Student's $t$-test, the end-points were
significantly different from control values ($P<0.01$).

3. Radioautography

The degree of uptake of $^{125}$I-BSA by the cells at
different points of the cytophilic antibody titration
curve (Fig. 2) were studied by radioautography. Cells
pretreated with a 1/5 dilution (strongly positive) of
the anti-serum became heavily labelled on subsequent
exposure to $^{125}$I-BSA (Fig. 3a). Cells pretreated with
Fig. 1. Effect of temperature on the binding of cytophilic antibody to peritoneal exudate cells. Normal guinea-pig peritoneal exudate cells incubated with guinea-pig anti-BSA antiserum for either 1 hour (open column), or for 4 hours (solid column). Vertical bars represent range in two experiments.
Fig. 2. The titration curves of guinea-pig anti-BSA (-•-•-) and anti-FIN (---•---) antisera. Each point represents the arithmetic mean of 5 reactions ± the standard error of the mean. The two horizontal lines indicate the end-point levels of the respective antisera. Arrow indicates the end-point.
Fig. 3. Radioautographs of normal guinea-pigs peritoneal exudate cells treated with guinea-pigs anti-BSA antiserum or normal guinea-pig serum and then reacted with $^{125}\text{I-BSA}$. Exposure time was 7 days. Magnification X 500. (a), cells treated with $1/5$ dilution of the antiserum. (b), cells treated with $1/640$ dilution of the antiserum. (c), cells treated with normal guinea-pig serum.
a 1/640 dilution of the anti-serum (i.e. at the end-point) were lightly labelled with $^{125}$I-BSA (Fig. 3b), whereas cells pretreated with medium only (control) were very poorly labelled (Fig. 3c). In order to show the labelling clearly, the photographs were taken by focusing at the level of the grains in the photographic emulsion rather than at the level of the cells. As a result the morphology of some of the cells in Fig. 3 is not clear. However, a general finding has been that cells which, by their morphology are typical lymphocytes, are usually poorly labelled.

4. The quantitation of the assay

Guinea-pig anti-BSA hyperimmune serum (titre 640) was used to estimate the amount of cytophilic antibody present at the end-point of the titration. A series of $5 \times 10^6$ normal guinea-pig peritoneal exudate cells was pretreated with a 1/640 dilution (end-point) of either the hyperimmune serum or normal guinea-pig serum. Increasing amounts of $^{125}$I-BSA were then added to the cells. Fig. 4 shows that the amount of $^{125}$I-BSA bound to the cells increased with increasing antigen concentration. However, cells pretreated with the immune serum adsorbed more radioactivity than did cells pretreated with normal serum. The amount of $^{125}$I-BSA adsorbed to cells via cytophilic antibody appeared to become constant when greater than 250 ng of $^{125}$I-BSA was added. This suggested that at this concentration of $^{125}$I-BSA (250 ng per 0.1 ml), all of the cell-bound cytophilic antibodies may have been
neutralized by antigen. The amount of antigen required for neutralisation was calculated to be approximately 2.7 ng.

If this interpretation is correct, it could be calculated that at the end-point of the titration about 6 ml of the cytotoxic antibody was associated with the cells. This, however, does not tell us the actual sensitivity of the assay. A subsequent attempt to estimate the proportion of cytotoxic antibody in the medium which had been bound to the cells under this condition was unsuccessful.

From the foregoing data, we estimate that about 10,000 molecules of antibody molecules were available for reaction with antigen on the surface of the reticulo-endothelial cells. The peritoneal exudate cells were macrophages, and at the end-point of the titration 10,000 was associated with each cell by the basis of these assumptions a value of 1,600 molecules of antigen per macrophage at the end-point of the titration 10,000.

Fig. 4. The binding of $^{125}\text{I}}$-BSA to normal guinea-pig peritoneal exudate cells with increasing concentration of $^{125}\text{I}}$-BSA. Cells pretreated with guinea-pig anti-BSA antiserum, ■; and cells pretreated with normal guinea-pig serum, □.
neutralized by antigen. The amount of antigen required for neutralisation was calculated to be approximately 2.7 ng.

If this interpretation is correct, it could be calculated that at the end-point of the titration about 6 ng of the cytophilic antibody was associated with the cells. This, however, does not tell us the actual sensitivity of the assay. A subsequent attempt to estimate the proportion of cytophilic antibody in the medium which became bound to the cells under this condition was unsuccessful.

From the foregoing data, an estimate was made of the number of antibody molecules bound to macrophages which were available for reaction with antigen at the end-point of the titration. Two assumptions are required.

1. Approximately 70% of the peritoneal exudate cells were macrophages. 2. The majority of the cytophilic antibody was associated with these cells. On the basis of these assumptions a value of 1,000 molecules of antibody per macrophage at the end-point of the titration (1/640 dilution of antiserum) was obtained. It is obvious from Fig. 2 that even when undiluted antiserum was used, the cells were still not saturated with cytophilic antibody (noting that control values remain constant). This suggests that there are at least $6 \times 10^5$ receptors for cytophilic antibody per macrophage.
THE PRODUCTION OF MACROPHAGE CYTOPHILIC ANTIBODY

1. Guinea-pigs

Table 1 shows the passive haemagglutinating and cytophilic antibody titres of the sera of guinea-pigs at different time points following immunisation. All the sera from the guinea-pigs immunised with the antigens emulsified in FCA or FIA contained cytophilic antibody. Guinea-pigs immunised with the antigens in FCA produced cytophilic antibody more rapidly (see 14 day bleed) than animals immunised with the antigens in FIA. This difference, however, was less pronounced in the later bleed. In contrast, sera from guinea-pigs injected with antigens in saline contained little cytophilic antibody and only moderate titres of passive haemagglutinating antibody.

The possibility existed that cytophilic antibody had been produced following the injection of BSA in saline but became adsorbed onto macrophages in vivo. This was tested by estimating the ability of peritoneal exudate cells from immunised guinea-pigs (BSA) to adsorb $^{125}$I-BSA. The results are shown in Table 2. Peritoneal exudate cells from animals immunised with BSA in FCA or FIA bound respectively 36 and 20 times more $^{125}$I-BSA than did peritoneal exudate cells from unimmunised (control) animals. Peritoneal exudate cells from guinea-pigs injected with BSA in saline did not take up more $^{125}$I-BSA than did peritoneal exudate cells from unimmunised guinea-pigs.
### TABLE 1

**PASSIVE HAEMAGGLUTINATION (HA) AND CYTOPHILIC ANTIBODY (C.Ab)**

**TITRES OF SERA FROM IMMUNE GUINEA-PIGS BEFORE AND AFTER BOOSTER INJECTIONS OF ANTIGENS**

<table>
<thead>
<tr>
<th>Injections</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;&lt;br&gt;HA</th>
<th>C.Ab</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt;&lt;br&gt;HA</th>
<th>C.Ab</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt;&lt;br&gt;HA</th>
<th>C.Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-FCA&lt;sup&gt;†&lt;/sup&gt;</td>
<td>11.25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>11.25</td>
<td>8.00</td>
<td>14.00</td>
<td>9.00</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>7.50</td>
<td>2.00</td>
<td>10.75</td>
<td>6.00</td>
<td>12.25</td>
<td>7.50</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>3.00</td>
<td>&lt;1</td>
<td>6.00</td>
<td>1.00</td>
</tr>
<tr>
<td>SRBC-FCA**</td>
<td>8.00</td>
<td>7.00</td>
<td>9.50</td>
<td>8.00</td>
<td>10.00</td>
<td>9.00</td>
</tr>
<tr>
<td>SRBC-FIA</td>
<td>8.00</td>
<td>4.00</td>
<td>9.00</td>
<td>6.00</td>
<td>9.50</td>
<td>9.00</td>
</tr>
<tr>
<td>SRBC-SAL</td>
<td>5.50</td>
<td>1.00</td>
<td>7.00</td>
<td>1.00</td>
<td>7.75</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<sup>†</sup> BSA = bovine serum albumin; SRBC = sheep red blood cell; FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; SAL = saline.

<sup>+</sup> 1<sup>st</sup>, 14 days after first injection; 2<sup>nd</sup>, 7 days after first booster and 21 days after the first injection; 3<sup>rd</sup>, 7 days after second booster and 28 days after the first injection.

<sup>*</sup> log<sub>2</sub> litre (1 = 1/5 dilution) from pooled sera, 3 animals per group.

**cytophilic antibody estimated by 'Rosette' technique.**
TABLE 2

THE IN VITRO BINDING TO PERITONEAL CELLS FROM HYPERIMMUNE GUINEA-PIGS OF $^{125}$I-BSA

<table>
<thead>
<tr>
<th>Injections</th>
<th>Passive haemagglutination titre*</th>
<th>$^{125}$I-BSA bound to $1 \times 10^7$ cells† (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA-FCA+</td>
<td>14.00</td>
<td>72.64 (65.48 - 78.96) #</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>12.25</td>
<td>41.02 (39.56 - 42.48)</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>6.00</td>
<td>2.90 (1.92 - 3.60)</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>2.01 (1.90 - 2.12)</td>
</tr>
</tbody>
</table>

* Log₂ titre (1 = 1/5 dilution) of pooled sera, 3 animals per group.
† Pooled peritoneal cells (3 animals per group).
+ BSA = bovine serum albumin; FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; SAL = saline.
# Values in brackets show range in three experiments.

Values in brackets show range in three experiments.
Thus, it appears that little or no cytophilic antibody was produced in guinea-pigs after the injection of BSA in saline.

The production of cytophilic antibody in guinea-pigs to flagellar antigens was also investigated. Table 3 shows that, though relatively high titres of passive haemagglutinating antibody were produced when these antigens were injected in saline, negligible amounts of cytophilic antibody were detected. However, antigens injected in FCA or in FIA were equally efficient at inducing cytophilic antibody formation.

2. **Rats**

Cytophilic antibody levels were very low in the sera of Lewis rats immunized with the flagellar antigens (despite relatively high passive haemagglutination titres). This was so whether the cytophilic antibody test was carried out using homologous cells or guinea-pig cells (Table 4). Rats injected with the flagellar antigens in either FCA or FIA produced similar amounts of cytophilic antibody.

3. **Mice**

In contrast to rats, mice produced relatively high cytophilic antibody titres. In mice, slightly but consistently higher levels of cytophilic antibody occurred in animals immunised with antigens in FCA compared with
### TABLE 3
PASSIVE HAEMAGGLUTINATION AND CYTOPHILIC ANTIBODY TITRES
OF SERA FROM GUINEA-PIGS HYPERIMMUNE TO DIFFERENT ANTIGENS

<table>
<thead>
<tr>
<th>Injections</th>
<th>Passive haemagglutination titre*</th>
<th>Cytophilic antibody titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL-FCA+</td>
<td>12.05 ± 0.78</td>
<td>4.10 ± 0.37</td>
</tr>
<tr>
<td>POL-FIA</td>
<td>13.05 ± 0.34</td>
<td>6.45 ± 0.40</td>
</tr>
<tr>
<td>POL-SAL</td>
<td>10.65 ± 0.24</td>
<td>0.20</td>
</tr>
<tr>
<td>FIN-FCA</td>
<td>11.48 ± 0.42</td>
<td>6.30 ± 0.39</td>
</tr>
<tr>
<td>FIN-FIA</td>
<td>12.55 ± 0.59</td>
<td>5.95 ± 0.39</td>
</tr>
<tr>
<td>FIN-SAL</td>
<td>10.05 ± 0.37</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CNBr.D-FCA</td>
<td>7.00 ± 0.31</td>
<td>6.20 ± 0.24</td>
</tr>
<tr>
<td>CNBr.D-FIA</td>
<td>6.50 ± 0.80</td>
<td>7.75 ± 0.83</td>
</tr>
<tr>
<td>CNBr.D-SAL</td>
<td>3.50 ± 0.10</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>BSA-FCA</td>
<td>12.68 ± 0.74</td>
<td>9.20 ± 0.84</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>13.00 ± 0.90</td>
<td>9.00 ± 0.87</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>6.65 ± 0.65</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Log₂ titre, 1 = 1/5 dilution, each figure represents the arithmetic mean of 5 animals ± standard error of the mean.

+ POL = polymerised flagellin; FIN= flagellin; CNBr.D = CNBr digest of flagellin; BSA = bovine serum albumin; FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant; SAL = saline.
TABLE 4
PASSIVE HAEMAGGLUTINATION AND CYTOPHILIC ANTIBODY TITRES
OF SERA FROM RATS HYPERIMMUNE TO DIFFERENT ANTIGENS

<table>
<thead>
<tr>
<th>Injections</th>
<th>Passive haemagglutination titre*</th>
<th>Cytophilic antibody titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL-FCA+</td>
<td>11.35 ± 0.68</td>
<td>2.90 ± 0.43</td>
</tr>
<tr>
<td>POL-FIA</td>
<td>10.15 ± 0.59</td>
<td>3.80 ± 0.58</td>
</tr>
<tr>
<td>POL-SAL</td>
<td>10.10 ± 0.53</td>
<td>0.40 ± 0.24</td>
</tr>
<tr>
<td>FIN-FCA</td>
<td>11.32 ± 0.22</td>
<td>2.30 ± 0.43</td>
</tr>
<tr>
<td>FIN-FIA</td>
<td>11.10 ± 0.79</td>
<td>2.70 ± 0.51</td>
</tr>
<tr>
<td>FIN-SAL</td>
<td>8.93 ± 0.32</td>
<td>0.75 ± 0.47</td>
</tr>
<tr>
<td>CNBr.D-FCA</td>
<td>7.25 ± 0.31</td>
<td>2.50 ± 0.38</td>
</tr>
<tr>
<td>CNBr.D-FIA</td>
<td>8.25 ± 0.31</td>
<td>2.30 ± 0.20</td>
</tr>
<tr>
<td>CNBr.D-SAL</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Log₂ titre, 1 = 1/5 dilution, each figure represents the arithmetic mean of 5 animals ± standard error of mean.

+ For abbreviations see Table 3.

Homologous peritoneal exudate cells were used for cytophilic antibody titration.
animals immunised with antigens in FIA. Antigens injected in saline were again inefficient in inducing cytophilic antibody formation (Table 5).

4. Rabbits

It has been shown by earlier workers (9,10) that rabbit macrophage cytophilic antibody bound equally well to guinea-pig and rabbit macrophages. This was confirmed. Since normal guinea-pig peritoneal exudate cells were more readily available, they were used for the rabbit cytophilic antibody titrations. Although there was only one rabbit used per treatment, each of the eight rabbits produced high and in some cases very high titres of cytophilic antibody after injection of antigens in FCA or in FIA (Table 6). With the three flagellar antigens, injection of the antigens in saline produced titres of passive haemagglutinating antibody as high as those given when adjuvants were used. Despite these high titres, however, injection of antigens in saline failed to produce cytophilic antibody.

FRACTIONATION OF CYTOPHILIC ANTIBODY

Sera were obtained from guinea-pigs after either a single injection of BSA in FCA (1\textsuperscript{O}), after a first booster (2\textsuperscript{O}) or after a second booster (3\textsuperscript{O}) and assayed for the presence of 19S and 7S cytophilic antibody. In all cases, 19S fractions were found to be devoid of
### TABLE 5

PASSIVE HAEMAGGLUTINATION AND CYTOPHILIC ANTIBODY TITRES
OF SERA FROM MICE HYPERIMMUNE TO DIFFERENT ANTIGENS

<table>
<thead>
<tr>
<th>Injections</th>
<th>Passive haemagglutination titre*</th>
<th>Cytophilic antibody titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL-FCA +</td>
<td>10.75 ± 0.28</td>
<td>5.50 ± 0.37</td>
</tr>
<tr>
<td>POL-FIA</td>
<td>9.70 ± 0.22</td>
<td>4.00 ± 0.49</td>
</tr>
<tr>
<td>POL-SAL</td>
<td>10.30 ± 0.60</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>FIN-FCA</td>
<td>9.50 ± 0.45</td>
<td>6.00 ± 0.50</td>
</tr>
<tr>
<td>FIN-FIA</td>
<td>9.57 ± 0.51</td>
<td>5.00 ± 0.35</td>
</tr>
<tr>
<td>FIN-SAL</td>
<td>9.60 ± 0.40</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>CNBr.D-FCA</td>
<td>7.16 ± 0.35</td>
<td>8.00 ± 0.47</td>
</tr>
<tr>
<td>CNBr.D-FIA</td>
<td>7.12 ± 0.51</td>
<td>6.00 ± 0.38</td>
</tr>
<tr>
<td>CNBr.D-SAL</td>
<td>1.80 ± 0.20</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>BSA-FCA</td>
<td>10.80 ± 0.35</td>
<td>5.00 ± 0.36</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>10.25 ± 0.49</td>
<td>3.00 ± 0.51</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>3.00 ± 0.15</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Log₂ titre, 1 = 1/5 dilution, each figure represents the arithmetic mean of 5 animals ± standard error of the mean.

+ For abbreviations see Table 3.
## TABLE 6

**Passive Haemagglutination and Cytophilic Antibody Titres of Sera from Rabbits Hyperimmune to Different Antigens**

<table>
<thead>
<tr>
<th>Injections</th>
<th>Passive haemagglutination titre*</th>
<th>Cytophilic antibody titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL-FCA+</td>
<td>11.75</td>
<td>11.25</td>
</tr>
<tr>
<td>POL-FIA</td>
<td>11.50</td>
<td>11.75</td>
</tr>
<tr>
<td>POL-SAL</td>
<td>11.25</td>
<td>1.00</td>
</tr>
<tr>
<td>FIN-FCA</td>
<td>14.00</td>
<td>12.25</td>
</tr>
<tr>
<td>FIN-FIA</td>
<td>11.25</td>
<td>11.25</td>
</tr>
<tr>
<td>FIN-SAL</td>
<td>12.25</td>
<td>2.00</td>
</tr>
<tr>
<td>CNBr.D-FCA</td>
<td>12.50</td>
<td>10.25</td>
</tr>
<tr>
<td>CNBr.D-FIA</td>
<td>11.25</td>
<td>12.25</td>
</tr>
<tr>
<td>CNBr.D-SAL</td>
<td>11.25</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>BSA-FCA</td>
<td>13.25</td>
<td>9.50</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>14.20</td>
<td>13.50</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>4.00</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

* Log₂ titre, 1 = 1/5 dilution, one rabbit per treatment.  
+ For abbreviations see Table 3.
cytophilic antibody (Table 7). Subsequently, sera from guinea-pigs, rabbits and mice hyperimmunised to FIN were also examined. Again all cytophilic antibody appeared to be in the 7S fractions (Table 8).

DISCUSSION

Cytophilic antibody has mainly been estimated using the 'Rosette' technique (2,11-13). In this paper, a radioimmunoassay is described. This assay system has several advantages compared to the 'Rosette' test. Firstly, it is more readily applicable to soluble antigens as it avoids the difficulties of coating red cells to equal extents with different antigens. Secondly, the technique is convenient, reproducible, objective and quantitative. Thirdly, from preliminary experiments in this laboratory, it appears that the radioimmunoassay to be at least as sensitive as the 'Rosette' method.

A major aspect of the investigation was the study of factors which affect the production of cytophilic antibody to BSA, SRBC and to flagellar antigens in different hosts according to different injection schedules. In guinea-pigs, Boyden (2) found that cytophilic antibody was induced by a single injection of SRBC in FCA but not when the antigen was injected with FIA. In addition, Jonas et al (11) reported that guinea-pigs primed with SRBC in FIA did not produce cytophilic antibody even when boosted with SRBC in saline. In contrast, both past (14) and
TABLE 7

CYTOPHILIC ANTIBODY CONTENTS IN 19S AND 7S IMMUNOGLOBULIN FRACTIONS OF EARLY AND LATE SERA FROM GUINEA-PIGS IMMUNISED WITH BSA IN FCA

<table>
<thead>
<tr>
<th>Immunoglobulin fractions†</th>
<th>1°</th>
<th>2°</th>
<th>3°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
<td>C.Ab</td>
<td>HA</td>
</tr>
<tr>
<td>19S</td>
<td>20†&lt;1</td>
<td>80&lt;1</td>
<td>10&lt;1</td>
</tr>
<tr>
<td>7S</td>
<td>20</td>
<td>8.5</td>
<td>320</td>
</tr>
</tbody>
</table>

* See Table 1.
† Reciprocal of dilution.
+ Fractions prepared from pooled serum,
  3 animals per group.
<table>
<thead>
<tr>
<th>Animal</th>
<th>19S*</th>
<th></th>
<th>7S*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
<td>C.Ab</td>
<td>HA</td>
<td>C.Ab</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>20+</td>
<td>&lt;1</td>
<td>320</td>
<td>120</td>
</tr>
<tr>
<td>Rabbit</td>
<td>120</td>
<td>&lt;1</td>
<td>1,280</td>
<td>120</td>
</tr>
<tr>
<td>Mouse</td>
<td>20</td>
<td>&lt;1</td>
<td>60</td>
<td>10</td>
</tr>
</tbody>
</table>

* Fractions prepared from pooled serum, 5 animals per group.
+ Reciprocal of dilution.
present work from this laboratory showed that SRBC, BSA and different forms of the flagellar antigens all caused the production of cytophilic antibody irrespective of whether FCA or FIA was used. Two possible reasons for the discrepancy between my findings and those of earlier workers are: 1. Different assay systems were used. 2. Animals immunised with antigens in FCA produced cytophilic antibody more rapidly than did animals immunised with antigens in FIA, as seen in the animals after a single injection, which is the schedule used both by Boyden and by Jonas et al. It was only after a series of three injections that animals immunised initially with antigens in FIA produced high levels of cytophilic antibody (Table 1). As has been found by others, injection of antigens in saline in any host failed to induce macrophage cytophilic antibody formation, although particularly in the rabbits, this injection schedule resulted in high to very high passive haemagglutinin titres. The reason why injection in saline fails to induce cytophilic antibody formation is obscure. Perhaps a class of lymphocytes is involved which needs the additional stimulation caused by adjuvants.

Guinea-pigs have in the past been the most commonly used hosts for the production of cytophilic antibody, partly because cytophilic activity has been shown to reside in a particular fraction ($\gamma_2$) of guinea-pig IgG and partly because of the postulated association between cytophilic
antibody and delayed-type hypersensitivity (2). However, in comparing the ratio of cytophilic antibody titre to total haemagglutinating antibody titre (Table 9), it was found that the ratio for rabbit > guinea-pig > mouse > rat. Furthermore, in each species, cytophilic antibody was only detected in the 7S fractions of hyperimmune sera and in the 7S fractions from guinea-pig sera at each stage of the injection schedule. Jonas et al (11) and Berken and Benacerraf (9) also found that the cytophilic antibody of guinea-pigs immunised with SRBC was contained mainly in the $\gamma_2$ IgG fractions. The present results are not necessarily at variance with those of earlier workers. Parish (15) found the cytophilic component of mouse sera in the 7S globulin fraction when he used protein antigens for immunisation, and in the 19S globulin fraction when he determined cytophilic antibodies against *Escherichia coli* polysaccharide. Tizard (16,17) obtained both 19S and 7S cytophilic antibodies in mice immunised with SRBC. It may be that, in mice, the physiochemical characteristics of the cytophilic antibody produced is determined by the type of antigen used.

Finally, a comparison of the passive haemagglutinating antibody and cytophilic antibody titres produced following injection of the different forms of flagellar antigens shows one interesting finding. The CNBr digest of flagellin was as good as POL or FIN at inducing the production of cytophilic antibody but it was generally less effective than
<table>
<thead>
<tr>
<th>Injections</th>
<th>Guineapigs</th>
<th>Rats</th>
<th>Rabbits</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>POL-FCA</td>
<td>0.33</td>
<td>0.25</td>
<td>0.95</td>
<td>0.51</td>
</tr>
<tr>
<td>POL-FIA</td>
<td>0.49</td>
<td>0.37</td>
<td>1.02</td>
<td>0.41</td>
</tr>
<tr>
<td>POL-SAL</td>
<td>0.01</td>
<td>0.03</td>
<td>0.08</td>
<td>0†</td>
</tr>
<tr>
<td>FIN-FCA</td>
<td>0.54</td>
<td>0.20</td>
<td>0.87</td>
<td>0.63</td>
</tr>
<tr>
<td>FIN-FIA</td>
<td>0.47</td>
<td>0.24</td>
<td>1.00</td>
<td>0.52</td>
</tr>
<tr>
<td>FIN-SAL</td>
<td>0</td>
<td>0.08</td>
<td>0.16</td>
<td>0</td>
</tr>
<tr>
<td>CNBr.D-FCA</td>
<td>0.88</td>
<td>0.34</td>
<td>0.82</td>
<td>1.11</td>
</tr>
<tr>
<td>CNBr.D-FIA</td>
<td>1.19</td>
<td>0.27</td>
<td>1.08</td>
<td>0.84</td>
</tr>
<tr>
<td>CNBr.D-SAL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BSA-FCA</td>
<td>0.72</td>
<td>ND</td>
<td>0.71</td>
<td>0.46</td>
</tr>
<tr>
<td>BSA-FIA</td>
<td>0.69</td>
<td>ND</td>
<td>0.95</td>
<td>0.29</td>
</tr>
<tr>
<td>BSA-SAL</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

† Ratio = 0 denotes no detectable cytophilic antibody by the assay method.

* For abbreviation see Table 3.

ND = Not done.
either POL or FIN at inducing passive haemagglutinating antibody (Tables 3-6).

Perhaps the major factor necessary for the production of cytophilic antibody is the use of adjuvant and the form of the antigen used is less important, whereas the latter aspect is still of major importance in determining the total amount of passive haemagglutinating antibody formed. There are experimental approaches available for investigating such a relationship.
REFERENCES


2. BOYDEN, S.V., Immunology 8, 474, 1964.


"THE BINDING OF THE Fc FRAGMENT OF GUINEA-PIG CYTOPHILIC ANTIBODY TO PERITONEAL MACROPHAGES"
SUMMARY

Using radioactivity assays and radioautography, it has been confirmed that the majority of the guinea-pig anti-BSA cytophilic antibodies are found in the $\gamma_2$ IgG fraction. The cytophilic activity was directly demonstrated to be present in the Fc portion of the IgG molecule. Furthermore, the Fc portion quantitatively accounts for the cytophilic activity of the whole IgG molecule.

Earlier workers have demonstrated that both guinea-pig cytophilic antibodies (3) and mouse cytophilic antibodies (7), isolated from hyperimmune sera, lose their ability to adhere to macrophages after pepsin treatment. Since pepsin is known to destroy the Fc portion of immunoglobulin heavy chains, it was inferred that the cytophilic activity of the antibodies was a function of the Fc portion of the IgG molecule.

In this paper, using radioactivity assays and radioautography with guinea-pig anti-BSA antiserum, it is confirmed that the cytophilic activity is present in the $\gamma_2$ IgG fraction. Furthermore, it is demonstrated directly that not only is this activity present in the Fc portion of the molecule but also that the activity of the Fc portion...
INTRODUCTION

Cytophilic antibodies have been defined as globulin components of immune serum which become attached to certain cells in such a way that these cells are subsequently capable of specifically adsorbing antigens (1). Most guinea-pig and mouse cytophilic antibodies to sheep erythrocytes or to soluble proteins have been shown to be present in the slow ($\gamma_2$) 7S-globulin fraction of serum by starch block electrophoresis (2,3) or by DEAE-cellulose column chromatography (4,5). In guinea-pig immune serum, the cytophilic activity, as shown by the uptake of $^{131}$I-HSA by sensitized cells, appears to be present only in the IgG fraction (6).

Earlier workers have demonstrated that both guinea-pig cytophilic antibodies (3) and mouse cytophilic antibodies (7), isolated from hyperimmune sera, lose their ability to adhere to macrophages after pepsin treatment. Since pepsin is known to destroy the Fc portion of immunoglobulin heavy chains, it was inferred that the cytophilic activity of the antibodies was a function of the Fc portion of the IgG molecule.

In this paper, using radioactivity assays and radioautography with guinea-pig anti-BSA antiserum, it is confirmed that the cytophilic activity is present in the $\gamma_2$ IgG fraction. Furthermore, it is demonstrated directly that not only is this activity present in the Fc portion of the molecule but also that the activity of the Fc portion
quantitatively accounts for the activity of the whole IgG molecule.

**MATERIALS AND METHODS**

**Animals**

Albino guinea-pigs bred randomly and of both sexes were used. They were fed on a Crajo, pelleted diet (Crajo, Sydney) ad libitum.

**Immunization**

Guinea-pigs were injected with bovine serum albumin (BSA, crystallised, Armour, Eastbourne, England) emulsified in either Freund's complete or Freund's incomplete adjuvant (FCA or FIA). BSA dissolved in 0.15M NaCl to 5 mg per ml, was emulsified in an equal volume of adjuvant and injected into the 4 footpads at the rate of 0.1 ml per footpad. Ten and seventeen days later, the animals were injected intradermally with BSA (0.1 mg in 0.1 ml saline) into each of 4 sites. The animals were exsanguinated under ether anaesthesia 7 days after the last injection. The serum was separated on the same day and stored at -10°C.

**Isolation of anti-BSA antibodies**

Anti-BSA antibodies were isolated from immune sera by an immune adsorption procedure. Ethylchloroformate aggregated BSA prepared according to the following modification of Avrameas and Ternynck (8) was used as the...
immunoadsorbent. BSA (10 g) was dissolved in 400 ml of acetate buffer (pH 5.0, 0.1 M). The pH was adjusted to 5.0 with \( \text{NH}_3 \text{Cl} \) and kept at that value with \( \text{NaOH} \) during the addition of ethylchloroformate. After standing at room temperature for 1 hour, the solid was dispersed in 500 ml phosphate buffered saline (PBS) using a Virtis homogeniser (Virtis, N.Y.). The insolubilised BSA was washed with PBS until free of soluble protein (O.D. of final wash <0.05 at 280 \( \mu \mu \)). The immunoadsorbent was stored at 4° in PBS containing sodium azide (1.5 mM).

The immunoadsorbent (100 mg) was added to 100 ml of guinea-pig hyperimmune anti-BSA serum. The mixture was stirred at room temperature for 3 hours and then kept at 4° overnight. The suspension was then centrifuged and the pellet washed with PBS until the supernatant had an O.D. of <0.05 at 280 \( \mu \mu \). The specific anti-BSA antibodies were dissociated from the immunoadsorbent with glycine-\( \text{HCl} \) buffer pH 2.2, 0.2 M (4°, 10 minutes). After rapid centrifugation (4°) to deposit the immunoadsorbent, the supernatant was quickly neutralized with 3 \( \text{N} \) NaOH and dialysed against distilled water overnight at 4°. The protein concentrations were estimated from the O.D. measurement at 280 \( \mu \mu \) in neutral solution assuming \( E_1^{1\text{cm}} = 15 \) (9).

**Estimation of antibodies**

Antibody contents of guinea-pig hyperimmune sera and purified antibody preparations were estimated by the
Farr test (10) and the results were expressed as the antigen (BSA) binding capacity (μg) per ml of serum or per ml of preparation.

Preparation of γ₁- and γ₂-globulins

The specific anti-BSA antibodies were separated into γ₁ and γ₂ fractions by chromatography on a DEAE-cellulose column (11).

Preparation and isolation of Fc and Fab fragments

The purified γ₁ and γ₂ guinea-pig anti-BSA antibodies, obtained from the DEAE-cellulose column, were digested with papain as described by Porter (12). The protein was dissolved in phosphate buffer (pH 7.6, 0.1 M) containing NaCl (0.005 M), cysteine (0.01 M) and EDTA (0.002 M) to 10 mg per ml and digested with 2 per cent of its weight of papain (Worthington, New Jersey). The digest mixture was incubated at 37° for 4 hours, centrifuged and dialysed against phosphate buffer (pH 7.6, 0.001 M) at 4°. When γ₁ antibody was digested, Fcγ₁ crystallised out on dialysis. The crystals were isolated by centrifugation and washed several times at 4° with small amounts of the same buffer. Fcγ₂ did not form crystals on dialysis but was separated from Fabγ₂ by repeatedly adsorbing Fabγ₂ from the digest with aggregated BSA. Fabγ₂ was recovered from the immunoadsorbent with glycine-HCl buffer (pH 2.2, 0.2 M, 4°). Fabγ₁ was also recovered from the γ₁ digest by adsorption and elution from the immunoadsorbent.
Experiments involving cells were carried out in the following medium: 100 ml of medium 199 (10x concentrated, Commonwealth Serum Laboratory, Melbourne), 10 ml of Heparin (10,000 units), 50 ml of normal guinea-pig serum, 1 ml of NaN₃ (1.5 M) and made up to 1 litre with PBS. Normal guinea-pig serum used at this concentration did not interfere with the adherence of antibody to macrophages but gave more reproducible results compared to medium without serum.

**Peritoneal cells**

Normal adult guinea-pigs were anaesthetised with ether, exsanguinated and the peritoneal cavity flushed with 50 ml of medium. The cells were collected in a pre-chilled beaker, pooled, washed 3 times with cold medium, and resuspended at 1.0 x 10⁷ cells per ml in medium. The yield was usually 1.5 x 10⁷ cells per guinea-pig and 50 to 60 percent of the cells were macrophages as judged by morphology.

**Indirect cytophilic antibody assay**

Guinea-pig peritoneal cells (5 x 10⁶) in 0.5 ml medium were exposed at 0° for 5 hours to varying dilutions of anti-serum or to varying concentrations of the isolated antibody preparations. All dilutions were made in the medium. The reactions, in duplicates, were carried out in plastic tubes (suitable for radioactivity determination).
The cells were then centrifuged for 5 minutes at 0°, washed 4 times in cold medium, resuspended in 0.5 ml cold medium and 200 ng of ¹²⁵I-labelled BSA in 10 µl PBS containing 1 per cent (w/v) gelatin added. The contents were mixed and left at 4° overnight. At the end of this period, the cells were again washed 4 times and cell-associated radioactivity was measured by an auto-gamma scintillation counter (Packard Autogamma Spectrometer). The binding of cytophilic antibody to the cells was expressed as ng of BSA present in or on 5 x 10⁶ cells.

**Direct cytophilic antibody assay**

The ability of purified γ₁ and γ₂ IgG and of the isolated Fc and Fab fragments to bind to peritoneal cells was tested directly as follows: Each protein preparation was labelled with ¹²⁵I and centrifuged (90,000 g, 2 hours, 4°) to remove aggregated materials. Varying concentrations of each clarified preparation (100 µl) were reacted at 0°, room temperature or 37° for 1 hour or 4 hours with guinea-pig peritoneal cells (5 x 10⁶) in 0.5 ml medium. The cells were then washed 4 times in cold medium, resuspended in 0.5 ml cold medium and cell-associated radioactivity rapidly counted. The cells were then smeared on gelatin coated glass slides preparatory for radioautography study. Experiments were carried out in duplicate and the results were expressed as n mole of protein bound to 5 x 10⁶ cells per µmole of protein added.
Other methods

Radioautography was carried out as described by Byrt and Ada (13). Iodination by direct oxidation of carrier free $^{125}$I with chloramine T was carried out according to Greenwood, Hunter and Glover (14) as modified by Ada, Nossal and Pye (15). The degree of substitution of the $^{125}$I-BSA varied from 1 mole of iodide per 17 to 20 moles of BSA and that of the $^{125}$I-labelled immunoglobulins and their fragments varied from 1 mole of iodide per 30 to 50 moles of protein. Polyacrylamide gel electrophoresis was carried out as described by Parish and Marchalonis (16), and immunoelectrophoresis in agar gel was according to Benacerraf, Ovary, Bloch and Franklin (17).

RESULTS

PREPARATION OF $\gamma_1$- AND $\gamma_2$-IMMUNOGLOBULINS

Anti-BSA specific antibodies were isolated from guinea-pig hyperimmune sera using ethylchloroformate aggregated BSA as immunoadsorbent. The yield of antibody (Farr test) was 30-42 per cent. $\gamma_2$ and $\gamma_1$ immunoglobulins were separated from the specific anti-BSA antibodies by DEAE-cellulose column chromatography. The yield of antibodies (Farr test) from the column was 75-80 per cent, making an overall recovery of 20-35 per cent. Of the final column eluate, 80-90 per cent could be specifically readsobered to the immunoadsorbent. Specific anti-BSA antibodies from sera of guinea-pigs injected with BSA in
FCA or FIA were fractionated and Fig. 1a and 1b represent the protein profile of the specific antibody fractions when subjected to column chromatography. In each case, two well separated protein peaks were obtained. Each was shown to be a single component by polyacrylamide gel electrophoresis (Fig. 2) and was identified as $\gamma_2$ and $\gamma_1$ by immunoelectrophoresis (Fig. 3). In Fig. 3, $\gamma_1$ is the faster migrating band. It is interesting to note that sera prepared in either fashion gave rather similar $\gamma_2/\gamma_1$ ratios, being 0.95 in the case of the BSA-FCA experiment and 0.73 in the case of the BSA-FIA experiment.

BINDING OF $\gamma_1$- AND $\gamma_2$-ANTI-BSA IMMUNOGLOBULINS TO GUINEA-PIG PERITONEAL CELLS

In the following sections the ability of the purified guinea-pig $\gamma_1$ and $\gamma_2$ anti-BSA IgG and of their fragments to bind to macrophages was investigated by two procedures which we have called the indirect and the direct tests. In each case the final measurement is the proportion of radioactivity associated with cells. We realise, however, that the indirect test is the only procedure which measures the antigen binding activity of the intact cytophilic antibodies.

1. Indirect test

Normal guinea-pig peritoneal cells containing 50-60 per cent macrophages were exposed to guinea-pig anti-BSA
Fig. 1a. Separation of $\gamma_1$- and $\gamma_2$-immunoglobulins from purified anti-BSA antibodies, prepared from guinea-pigs injected with BSA in Freund's complete adjuvant, on DEAE-cellulose column with phosphate buffer pH 8.0. Arrow indicates the starting of buffer gradient, 0.01 M to 0.15 M, pH 8.0.
Fig. 1b. Separation of $\gamma_2$- and $\gamma_1$-immunoglobulins from purified anti-BSA antibodies, prepared from guinea-pigs injected with BSA in Freund's incomplete adjuvant, on DEAE-cellulose column with phosphate buffer pH 8.0. Arrow indicates the starting of buffer gradient, 0.01 M to 0.15 M, pH 8.0.
Fig. 2. Polyacrylamide gel electrophoretic patterns of:
1. purified guinea-pig anti-BSA IgG; 2. \( \gamma_1 \) IgG; 3. \( \gamma_2 \) IgG;
4. \( \gamma_1 \) IgG papain digest; 5. Fab\( \gamma_1 \); 6. Fc\( \gamma_1 \); 7. Fc\( \gamma_2 \);
8. Fab\( \gamma_2 \). Both \( \gamma_1 \) and \( \gamma_2 \) IgG (2, 3) show a single band
which migrates to a position expected for a 7S globulin.

IgG papain digest (4) shows no detectable undigested material. Fc\( \gamma_1 \) (6) is from crystallised preparation. The
faint band further away from the origin (0) in Fc\( \gamma_2 \) (7)
is probably the smaller Fc' fragment resulting from
further degradation of the fragment by papain.
Fig. 3. The immunoelectrophoretic pattern of guinea-pig anti-BSA whole serum (WS) of specific anti-BSA antibodies (SAb) isolated from the whole serum, and of $\gamma_1$ and $\gamma_2$ IgG prepared from the specific antibodies. The troughs contain rabbit hyperimmune serum against guinea-pig whole serum. Concentrations of protein preparations: $\gamma_1$, $\gamma_2$, 0.6 mg/ml; specific antibodies, 1.2 mg/ml. The round wells are the points of application of the proteins.
sera or to the $\gamma_1$ or $\gamma_2$ isolates. Each antibody preparation was adjusted to the same BSA binding capacity (Farr test). After washing, the cells were mixed with $^{125}$I-BSA, again washed and the amount of radioactivity bound to the cells estimated. The results are shown in Table 1. The peritoneal cells pretreated with $\gamma_1$ IgG did not adsorb significantly more $^{125}$I-BSA than did the cells pretreated with medium only and was 55 fold less than the amount of $^{125}$I-BSA adsorbed by the cell pre-exposed to $\gamma_2$ IgG. Cells pre-exposed to $\gamma_2$ IgG adsorbed as much $^{125}$I-BSA as did cells pretreated with anti-BSA-FCA serum. Cells treated with anti-BSA-FIA serum adsorbed less antigen than cells treated with anti-BSA-FCA serum and this probably reflects the different $\gamma_2/\gamma_1$ ratios of the two sera (Fig. la and lb).

2. Direct test

Normal guinea-pig peritoneal cells were exposed to $^{125}$I-labelled $\gamma_1$ or $\gamma_2$ preparations or to $^{125}$I-BSA. To minimise pinocytosis, the reactions were carried out at $0^\circ$ in the presence of sodium azide (1.5 mM). The amount of radioactivity which became associated with the cells was estimated and the cells were examined by radioautography. Table 2 shows that when $5 \times 10^6$ cells were exposed either to $^{125}$I-labelled IgG or $^{125}$I-BSA, only the IgG preparations were adsorbed to the cells at an appreciable extent and of these, $\gamma_2$ IgG was adsorbed 10 fold higher than was $\gamma_1$ IgG.
TABLE I

INDIRECT CYTOPHILIC ASSAY OF \( \gamma_1, \gamma_2 \) IMMUNOGLOBULINS FOR CYTOPHILIC ANTIBODY ACTIVITY

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>BSA BINDING CAPACITY (µg/0.5 ml)</th>
<th>125 I-BSA ADDED (µg)</th>
<th>125 I-BSA BOUND TO CELLS* (%)</th>
<th>125 I-BSA BOUND TO CELLS (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal guinea-pig serum</td>
<td>-</td>
<td>1.0</td>
<td>0.02</td>
<td>0.22 (0.01-0.03)</td>
</tr>
<tr>
<td>Anti-BSA-FCA*</td>
<td>3.0</td>
<td>1.0</td>
<td>2.2</td>
<td>22 (1.9-2.4)</td>
</tr>
<tr>
<td>Anti-BSA-FIA*</td>
<td>3.0</td>
<td>1.0</td>
<td>1.1</td>
<td>11 (0.9-1.3)</td>
</tr>
<tr>
<td>( \gamma_1 ) IgG**</td>
<td>3.0</td>
<td>1.0</td>
<td>0.04</td>
<td>0.4 (0.03-0.05)</td>
</tr>
<tr>
<td>( \gamma_2 ) IgG**</td>
<td>3.0</td>
<td>1.0</td>
<td>2.3</td>
<td>23 (2.0-2.5)</td>
</tr>
<tr>
<td>Medium</td>
<td>-</td>
<td>1.0</td>
<td>0.01</td>
<td>0.1 (0.01-0.02)</td>
</tr>
</tbody>
</table>

* % of 125 I-BSA bound to 5 x 10^6 normal guinea-pig peritoneal cells. Figures in brackets refer to range of values of three experiments.
<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE I (cont.)

+ ng = nanogram = $10^{-9}$ g.

# FCA = Freund's complete adjuvant; FIA = Freund's incomplete adjuvant.

** $\gamma_1$ and $\gamma_2$ were samples pooled from both anti-BSA-FCA and anti-BSA-FIA sera as separate tests showed that $\gamma_2$ prepared from either source were equally active in this test.
### TABLE 2

**DIRECT CYTOPHILIC ASSAY OF \( \gamma_1, \gamma_2 \) IMMUNOGLOBULINS FOR CYTOPHILIC ACTIVITY**

<table>
<thead>
<tr>
<th>125 ( \text{I-LABELLED PREPARATION} )</th>
<th>RADIOACTIVITY</th>
<th>AMOUNT OF LABELLED PREPARATION BOUND TO CELLS (%)</th>
<th>AMOUNT OF LABELLED PREPARATION BOUND TO CELLS (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_1 ) IgG 50</td>
<td>0.33</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.28-0.35)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \gamma_1 ) IgG 100</td>
<td>0.22</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.20-0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \gamma_2 ) IgG 50</td>
<td>2.3</td>
<td>1,150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.1-2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA 50</td>
<td>0.06</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.04-0.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values in brackets show range in three experiments.*
3. The effect of temperature on the binding of $\gamma_1$ and $\gamma_2$ to peritoneal cells

There has been some uncertainty in earlier work concerning the optimum temperature for performing the indirect test for cytophilic antibody activity. To investigate this, the direct test for cytophilic antibody activity was carried out at three different temperature ($0^\circ$, room temperature and $37^\circ$) for 1 hour or for 4 hours. Fig. 4 shows that the maximum binding of $\gamma_2$ to peritoneal cells was obtained by exposure for 4 hours at either $0^\circ$ or room temperature. When the reaction was carried out at $37^\circ$ for 4 hours, the amount of $\gamma_2$ recovered with the cells was of the same order as that found after the 1 hour exposure. It is of interest to note that $\gamma_1$ IgG was not adsorbed to the cells at any of these temperatures.

4. Radioautography

Radioautography (Fig. 5) shows that cells treated with $^{125}$I-$\gamma_2$ IgG became heavily labelled whilst cells treated with $^{125}$I-$\gamma_1$ IgG were only lightly labelled. The cells which became heavily labelled upon exposure to $^{125}$I-$\gamma_2$ IgG were, from morphology, judged to be macrophages. Cells which were judged by morphology to be lymphocytes or polymorphonuclear leucocytes were usually not labelled to an appreciable extent.
Fig. 4. Effect of temperature on the binding of guinea-pig $\gamma_1$ and $\gamma_2$ cytophilic antibodies to normal guinea-pig peritoneal cells. 50µg of $^{125}$I-labelled $\gamma_1$ or $\gamma_2$ in 100 µl PBS was reacted at 0°C, room temperature or 37°C for one hour or 4 hours with 5 x $10^6$ cells in 0.5 ml medium. The cells were then washed 4 times and cell associated radioactivity counted. The figure represents the result of a typical experiment. $\gamma_2$, 4 hours, •; $\gamma_2$, 1 hour ▼; $\gamma_1$, 4 hours □; $\gamma_1$, 1 hour □.
Fig. 5. Radioautographs of normal guinea-pig peritoneal cells after reaction with $^{125}$I-labelled proteins. Exposure time was 7 days. Magnification X 500. (a) Cells reacted with $^{125}$I-γ$_2$ IgG. The grains can be seen on macrophages. Insert shows higher magnification (X 1,200) of some of the labelled macrophages. (b) Cells reacted with $^{125}$I-γ$_1$ IgG. (c) Cells reacted with $^{125}$I-BSA.
ISOLATION OF Fc and Fab FRAGMENTS FROM \( \gamma_1 \) AND \( \gamma_2 \) IMMUNOGLOBULINS

No trace of undigested immunoglobulin was detectable by polyacrylamide gel electrophoresis (Fig. 2) in the papain digest of the purified \( \gamma_1 \) and \( \gamma_2 \) anti-BSA immunoglobulin. Whereas the Fc\( \gamma_1 \) was directly isolated by crystallisation, the Fc\( \gamma_2 \) could only be obtained as residual material present in the papain digest after repeated adsorption of the digest with the BSA immunoabsorbent. Fig. 6 shows the immunoelectrophoretic pattern of the purified papain digest fragments. In contrast to the other 3 preparations, the Fc\( \gamma_2 \) preparation shows considerable heterogeneity. To make a more valid comparison of the two Fc preparations, papain digests of \( \gamma_1 \) and \( \gamma_2 \) IgG were both exhaustively adsorbed with the BSA immunoabsorbent. In the case of the \( \gamma_1 \) digest, 75 per cent of the U.V. (280 m\( \mu \)) adsorbing material was removed from the digest in this way. The corresponding figure of the \( \gamma_2 \) digest was 77 per cent. Following this, the Fc\( \gamma_1 \) was crystallised from the residue of the \( \gamma_1 \) papain digest and examined by immunoelectrophoresis. It can be seen that the isolated Fc\( \gamma_1 \) fragment gave the same pattern as did the residue from which it was crystallised (Fig. 6). There is thus reason to believe that the residue after adsorption of the \( \gamma_2 \) papain digest contains predominantly Fc\( \gamma_2 \).

BINDING OF Fc AND Fab FRAGMENTS TO PERITONEAL CELLS

The Fc and Fab fragments of \( \gamma_1 \) and \( \gamma_2 \) IgG were
Fig. 6. Immunoelectrophoretic analysis of the fragments isolated after papain digestion of purified guinea-pig anti-BSA antibodies (γ1 and γ2). Fcγ1c: Fcγ1 crystallised. Fcγ1r: Fcγ1 residue from γ1 papain digest after adsorbing out Fabγ1 with immunoadsorbent. A rabbit anti-serum against guinea-pig whole serum was placed in the troughs. Concentrations of proteins: 0.6 mg/ml.
labelled with $^{125}\text{I}$ and their ability to adsorb to peritoneal cells were tested by the direct method with the results shown in Table 3. Fab fragments from both $\gamma_1$ and $\gamma_2$ bound poorly to peritoneal cells and to no greater extent than did BSA. Fc$\gamma_1$ bound rather better (8 times) to peritoneal cells than did either of the Fab fragments, but only about one third as well as did the Fc$\gamma_2$ fragment. When expressed on a molar basis, Fc$\gamma_2$ bound to the cells as well as $\gamma_2$ IgG.

Radioautography confirmed these results. Heavily labelled cells were found in cell populations treated with $^{125}\text{I-FCY}_2$ (Fig. 7a) whereas cells treated with $^{125}\text{I-FCY}_1$ were more lightly labelled (Fig. 7b). Cells treated with $^{125}\text{I-FABY}_1$ and $^{125}\text{I-FABY}_2$ were very poorly labelled (Fig. 7c).

**DISCUSSION**

The results reported in this paper confirm the following findings of earlier workers. 1. Most cytophilic activity in hyperimmune guinea-pig serum is recovered in the $\gamma_2$ IgG fraction. 2. This activity is directed towards macrophages and not towards lymphocyte like cells or polymorphonuclear leucocytes (18,19). 3. Digestion of the $\gamma_2$ IgG with papain yields Fab fragments which are unable to bind to macrophages (3,7).

The principle findings of this paper, as shown by radioactivity counting and radioautography, are two fold. 1. The Fc fragment isolated from $\gamma_2$ guinea-pig IgG has
TABLE 3

THE BINDING TO GUINEA-PIG PERITONEAL CELLS OF $^{125}$ I-LABELLED $\gamma_1$, $\gamma_2$ IgG AND OF THE Fab, Fc FRAGMENTS ISOLATED FROM THEM BY PAPAIN DIGESTION.

<table>
<thead>
<tr>
<th>125 I-LABELLED PREPARATION</th>
<th>AMOUNT ADDED TO $5 \times 10^6$ CELLS (µg)</th>
<th>RADIOACTIVITY BOUND TO CELLS (%)</th>
<th>AMOUNT OF LABELLED PREPARATION BOUND TO CELLS (ng)</th>
<th>n MOLE OF PREPARATION BOUND µ MOLE OF PREPARATION ADDED</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma_1$ IgG</td>
<td>32.0</td>
<td>0.14 (0.13, 0.15)</td>
<td>47</td>
<td>1.48</td>
</tr>
<tr>
<td>$\gamma_2$ IgG</td>
<td>34.5</td>
<td>2.88 (2.75, 3.01)</td>
<td>992</td>
<td>28.7</td>
</tr>
<tr>
<td>Fc$\gamma_1$</td>
<td>21.9</td>
<td>0.87 (0.85, 0.89)</td>
<td>190</td>
<td>8.66</td>
</tr>
<tr>
<td>Fc$\gamma_2$</td>
<td>20.8</td>
<td>2.83 (2.77, 2.89)</td>
<td>582</td>
<td>28.0</td>
</tr>
<tr>
<td>Fab$\gamma_1$</td>
<td>36.9</td>
<td>0.01 (0.01, 0.01)</td>
<td>55</td>
<td>1.48</td>
</tr>
<tr>
<td>Fab$\gamma_2$</td>
<td>15.3</td>
<td>0.01 (0.005, 0.02)</td>
<td>14</td>
<td>0.90</td>
</tr>
<tr>
<td>BSA</td>
<td>50.0</td>
<td>0.06 (0.03, 0.09)</td>
<td>30</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Figures in brackets are individual values.
Fig. 7. Radioautographs of normal guinea-pig peritoneal cells after reaction with $^{125}$I-labelled fragments isolated from papain digests of purified anti-BSA antibodies ($\gamma_1$ and $\gamma_2$). Exposure time 7 days. Magnification X 500. (a) Cells reacted with $^{125}$I-Fc$\gamma_2$. (b) Cells reacted with $^{125}$I-Fc$\gamma_1$. (c) Cells reacted with $^{125}$I-Fab$\gamma_2$. 
cytophilic activity. 2. This Fc fragment is as active, on a molar basis, as the intact $\gamma_2$ IgG molecule. These findings, taken together with the demonstration that the Fab fragment of the molecule has no detectable cytophilic activity, show that the cytophilic activity of the intact molecule is a function solely of the Fc portion of the molecule. The isolated Fc$\gamma_1$ fragment also appears to have, in contrast to the intact $\gamma_1$ IgG molecule, an appreciable amount of cytophilic activity (see Table 4). However, in this case, radioautography of the smeared cell pellet showed only very light labelling of macrophages compared to macrophages exposed to the Fc$\gamma_2$. It is possible that the high count of the cell pellet exposed to the Fc$\gamma_1$ preparation may have been due, in part to some nonspecific trapping of the labelled protein. Whether association with antigens affects the cytophilic activity of an IgG molecule is not clear at present because it is difficult to devise a satisfactory method for clearly differentiating opsonizing and cytophilic activity. Parish (18) was able to distinguish between the two activities to some extent but found a considerable overlap between them.

Cytophilic activity can now be added to those other biological activities which have been shown to be a direct function of the Fc portion of IgG. They are: ability to localise in lymph node follicles (20), to fix complements (21), to transfer antibodies from mother to foetus (22) and to fix antibody to skin (23). It is now possible to
TABLE 4
A COMPARISON OF THE CYTOPHILIC ACTIVITY OF $\gamma_1$, $\gamma_2$, IgG AND THEIR Fc FRAGMENTS

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>RELATIVE CYTOPHILIC ACTIVITY*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma_2$ IgG</td>
<td>47.8</td>
</tr>
<tr>
<td>$\gamma_1$ IgG</td>
<td>2.5</td>
</tr>
<tr>
<td>Fc $\gamma_2$</td>
<td>46.6</td>
</tr>
<tr>
<td>Fc $\gamma_1$</td>
<td>1.4</td>
</tr>
<tr>
<td>BSA</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Relative cytophilic activities are derived from the last column of table 3, taking the value for BSA as 1.0.
look into the mechanism of the binding of the Fc fragment to cell membranes using approaches such as varying the charge of the molecule and by controlled degradation studies.
REFERENCES


The ability of passively administered antibody to regulate the humor al and cell-mediated immune response to flagellin, polymerized flagellin and sheep red blood cell was investigated in rats. It was found that for all three antigens, suppression of antibody formation by passive antibody was accompanied by enhanced delayed-type hypersensitivity. This phenomenon was observed with both homologous and heterologous passive antibody. However, with flagellin and sheep red blood cell, high concentrations of passive antibody led to a decrease in delayed-type hypersensitivity. Immune antibody was not transferable by serum, but specific passive antibody persisted for at least 4 weeks after immunization and was transferable by immune cells but not by serum.
SUMMARY

The ability of passively administered antibody to regulate the humoral and cell-mediated immune responses to flagellin, polymerised flagellin and sheep red blood cell was investigated in rats. It was found that for all three antigens, suppression of antibody formation by passive antibody was accompanied by enhanced delayed-type hypersensitivity. This phenomenon was observed with both homologous and heterologous passive antibody. However, with flagellin and sheep red blood cell, high concentrations of passive antibody led to a decline of delayed-type hypersensitivity.

This regulation of immune responses by passive antibody was antigen specific. The delayed-type hypersensitivity induced by antigen in the presence of specific passive antibody persisted for at least 4 weeks after immunisation and was transferable by immune cells but not by serum.
INTRODUCTION

A large amount of data has now accumulated (1-11) indicating that passively administered antibody has a depressive effect on the immune response both in vivo and in vitro (12-14). Such suppression is specific and may be most readily achieved by antibody administered just prior to the antigenic stimulus. The finding that immune suppression can also be obtained by passive antibody given subsequent to the antigen has led to the suggestion that actively formed serum antibody may have a regulatory role (1,4,15).

Many workers have studied the effect of passive antibody on the subsequent humoral antibody response to soluble antigens. In contrast, the effect of passive antibody on cell-mediated immunity to soluble antigens has not been studied in such detail. However, studies have demonstrated that delayed-type hypersensitivity to protein antigens appears to be less easily suppressed than is either the primary antibody response or "priming" for a secondary antibody response (16). In fact, Uhr et al (17) reported that the induction of delayed hypersensitivity to soluble protein antigens was facilitated by antigen-antibody complexes. On the other hand, the effect of passive antibody on the induction of transplantation immunity has been examined in great detail both at the humoral and cell-mediated level (16,18-20).

In this paper we investigated the effect of passively
administered antibody on humoral and cell-mediated immunity in rats. Evidence will be presented that the suppression of humoral antibody production by passive antibody led to the concomitant enhancement of cell-mediated immunity. Both the suppression and the enhancement were specific and were applicable to soluble as well as particulate antigens.

MATERIALS AND METHODS

Animals

Female out-bred Wistar rats of 9-12 weeks of age and albino guinea-pigs of both sexes between 10-16 weeks of age were used. All animals were fed on Crago pelleted diet (Crago, Sydney) ad libitum.

Antigens

Sheep red blood cells (SRBC) stored in Alsever's solution at 4°C were washed 4 times in saline before use. Flagellin and polymerised flagellin (POL) from Salmonella adelaide (strain SW1338; H antigen, fg; O antigen, 35) were prepared as described by Ada, Nossal, Pye and Abbot (21). Flagellin has a molecular weight of 40,000 and POL has been estimated to have a mean particle weight corresponding to 300-1,600 flagellin units.

Preparation of antisera

Guinea-pig anti-POL and guinea-pig anti-SRBC
antisera were prepared as follows: Polymerised flagellin at a concentration of 5 mg/ml in saline or SRBC as a 30% suspension in saline were emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit). The adjuvant preparation of each antigen was injected into each of the 4 footpads of guinea-pigs at the rate of 0.1 ml/footpad. Ten and 17 days later, the animals were injected intradermally with the respective antigens (0.1 ml of 15% SRBC in saline or 0.1 mg of POL in 0.1 ml saline) into each of 4 sites.

Rat anti-POL antiserum was prepared as follows: POL (2 mg/ml in saline) was emulsified with an equal volume of Freund's complete adjuvant and injected into the hind footpads of rats (50 µl/footpad). Ten and 17 days later, the rats were injected intradermally with POL. (0.1 mg in 0.1 ml saline).

All animals were exsanguinated under ether anesthesia 7 days after the last injection. The sera were separated on the same day and stored at -10°C.

**Immunization procedures**

Anti-sera of known antibody titre were diluted with phosphate buffered saline (PBS) to form a series of antibody dilutions. Groups of rats (8 rats per group) were injected intraperitoneally with 1.0 ml of the various antibody dilutions. Control rats were injected with normal serum of appropriate dilution. One hour later, each animal
received intraperitoneally 500 ng of POL or 500 ng of flagellin or $5 \times 10^7$ SRBC in 0.5 ml saline. The levels of serum antibody due to passive immunization were determined in groups of rats which received antibody but no antigen. Rats were bled weekly from the tail for 4 weeks following immunization. On day 28 the animals were given a second dose of the appropriate antigen and bled weekly for 2 more weeks. The second injection also served as the eliciting injection for both immediate and delayed-type hypersensitivity. Antigen (500 ng of flagellin in 50 µl saline or $5 \times 10^7$ SRBC in 50 µl saline) was injected into the right hind footpad and saline alone into the left hind footpad. Footpad thickness was measured at 3, 6, 9, 12 and 24 hr after the injections and specific footpad swelling was determined by subtracting the thickness of the left hind footpad from the right. Footpad thickness was measured by a dial calliper gauge AO2T (Schnelltaster, System Kröplin). Flagellin and POL sensitization induced no significant immediate hypersensitivity compared with control animals. Thus, in subsequent experiments footpad measurements were made only at 24 hr post challenge.

In an experiment to determine the effect of antigen-antibody complexes on the immune response, different concentrations of guinea-pig anti-POL anti-serum (10 ml volumes) were incubated with 5 µg of POL at 37° for 1 hr. After incubation, 1 ml of the mixture was injected intraperitoneally into each rat. Humoral and cell-mediated
immune responses were determined as above.

**Antibody estimations**

Antibodies to SRBC were estimated by haemagglutination. Antibodies to flagellin and POL were estimated by a passive haemagglutination method based on that described by Jandl and Simmons (22). Briefly, SRBC stored (0-5 days) in Alsever's solution at $4^\circ$C were washed 4 times with saline and resuspended to 5% in saline. To 5 ml of 5% SRBC in saline was added 50 $\mu$g of POL. The solution was stirred and 0.65 ml of 0.01% CrCl$_3$ solution (pH 5.2) added with further mixing. After standing at room temperature for 5 to 10 min the cells were washed 3 times with phosphate buffered saline (PBS) and resuspended to 2% in PBS. All titrations were done in V-bottomed micro-titrating wells. PBS containing 5% foetal calf serum (FCS) was used as diluent.

**Collection of serum and peritoneal cells**

Serum samples were routinely obtained by bleeding rats from the tail. To obtain larger quantities of serum for transfer experiments rats were exsanguinated and their serum pooled. Peritoneal cells were obtained by washing out the peritoneal cavities of rats with 5 ml of a solution of 5% FCS in PBS containing 10 units/ml of heparin. The peritoneal cells were pooled and washed 4 times with the same medium but lacking heparin. (Most rats yielded approximately 2.5-3.0 ml of serum and $2.0-2.5 \times 10^7$ peritoneal cells). Finally, peritoneal cells ($2 \times 10^7$
cells/rat in 0.05 ml) were injected intravenously into a lateral tail vein of recipient rats.

Statistical Methods

Standard errors of the means and P values according to the Student's t Test were calculated on an IBM computer.

RESULTS

The effect of guinea-pig anti-POL antibody on the immune response to POL in rats

Adult rats (7 - 8 per group) were injected intraperitoneally with different concentrations of guinea-pig anti-POL antiserum (titres ranging from 2 to 1,250). Control animals received a 1/20 dilution of normal guinea-pig serum which corresponded to the lowest dilution of anti-serum used. One hour later, each rat was injected intraperitoneally with 500 ng of polymerized flagellin (POL) in saline. Animals were injected into the hind footpads 28 days later with 500 ng of flagellin in saline and delayed-type hypersensitivity measured by footpad swelling (see Materials and Methods). Antibody titres were estimated at weekly intervals during the experiment.

It was found that all concentrations of passively administered anti-POL antibody significantly suppressed both the primary and second antibody responses. In fact, POL injected with the two highest concentrations of passive antibody induced only a transitory secondary response (Fig. 1).
Fig. 1. The effect of passive guinea-pig anti-POL antibody on the subsequent antibody response to POL in rats. Groups of rats were injected intraperitoneally with 1.0 ml of the various antiserum dilutions. One hour later, each rat received intraperitoneally 500 ng of POL in 0.5 ml saline. On day 28, each animal was injected into the hind footpad with 500 ng of flagellin in 50 µl of saline. The concentrations (antibody titres) of passive antibody injected were: 1,250 (○), 250 (●), 50 (△), 10 (▲), 2 (□), and normal guinea-pig serum (■). The broken line represents the antibody response of control rats which were not treated on day 0 and challenged with flagellin (500 ng) on day 28. Vertical bars represent standard errors of the means, 7 rats per group. The levels of serum antibody due to passive immunization were subtracted.
Fig. 2. Comparison of the effect of different concentrations of passively administered guinea-pig anti-POL antibody on the humoral and cell-mediated immune responses to POL. For experimental details see Materials and Methods. Antibody responses represent the mean primary antibody titres (data derived from Fig. 1). Cell-mediated immunity is expressed as 24 hour footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group.
Figure 2 compares the effect of different concentrations of guinea-pig anti-POL antibody on the subsequent humoral (primary antibody) and cell-mediated immune responses to POL. Control animals which received POL with normal serum expressed very weak delayed-type hypersensitivity (1.0 unit). However, when POL was injected with anti-POL antibody, delayed-type hypersensitivity was enhanced, and this enhancement steadily increased with increasing concentrations of passive antibody. In contrast, the production of anti-POL antibody was suppressed as the concentration of passive antibody was increased (data in Fig. 2 taken from Fig. 1).

The effect of guinea-pig anti-POL antibody on the immune response to flagellin in rats

The experimental protocol was the same as described in the preceding section except that in this case 500 ng of flagellin, the monomeric unit of polymerized flagellin (POL), was used as antigen. Again all concentrations of passively administered anti-POL antibody significantly suppressed both the primary and secondary antibody responses to flagellin. In fact, there was a suggestion that the highest concentration of passive antibody (titre: 1,250) induced antibody tolerance, although this suppression could be due to persisting passive antibody. The degree of suppression increased with increasing concentrations of passive antibody (Fig. 3). The delayed-type hypersensitivity
Fig. 3. The effect of passive guinea-pig anti-POL antibody on the subsequent antibody response to flagellin in rats. For experimental protocol see legend to Fig. 1. The concentrations (antibody titres) of passive antibody injected were: 1,250 (○), 250 (●), 50 (△), 10 (▲), 2 (□), and normal guinea-pig serum (■). The broken line represents the antibody response of control rats which were not treated on day 0 and challenged with flagellin (500 ng) on day 28. Vertical bars represent standard errors of the means, 7 rats per group. The levels of serum antibody due to passive immunization were subtracted.
Fig. 4. Comparison of the effect of different concentrations of passively administered guinea-pig anti-POL antibody on the humoral and cell-mediated immune responses to flagellin. For experimental details see Materials and Methods. Cell-mediated immunity was expressed as 24 hr footpad swelling and the antibody responses represent the mean primary antibody titres (data derived from Fig. 2.). Vertical bars represent standard errors of the means, 7 rats per group.
responses to flagellin were more complex than those to POL (Fig. 4). Flagellin, unlike POL, provoked significant delayed-type hypersensitivity when injected in the absence of passive antibody. The delayed response to flagellin was steadily enhanced as the antigen was injected with increasing concentrations of passive antibody. However, when the highest concentration of passive antibody was administered (titre: 1,250) the delayed response was no greater than that given by flagellin itself.

The effect of guinea-pig anti-SRBC antibody on the immune response to SRBC

The experimental protocol was similar to that described for POL and flagellin. Rats were injected intraperitoneally with different concentrations of guinea-pig anti-SRBC antiserum (titre ranging from 1 to 500), and one hour later injected in the same site with $5 \times 10^7$ SRBC in saline. Secondary antibody responses and hypersensitivity reactions were elicited in the hind footpads 28 days later with $5 \times 10^7$ SRBC in saline.

Figure 5 depicts the effect of different concentrations of passively administered anti-SRBC antibody on the subsequent antibody responses to SRBC. As was the case with POL and flagellin increasing concentrations of passive antibody steadily suppressed the primary antibody response to SRBC. In contrast to POL and flagellin, pre-treatment with antibody had little or
Fig. 5. The effect of passive guinea-pig anti-SRBC antibody on the subsequent antibody response to SRBC in rats. The concentrations (antibody titres) of passive antibody injected were: 500 (○), 125 (●), 25 (▲), 5 (▼), 1 (■), and normal guinea-pig serum (■). The broken line represents the antibody response of control rats which were not treated on day 0 and challenged with SRBC (5 x 10^7 cells) on day 28. Vertical bars represent standard errors of the means, 7 rats per group. The levels of serum antibody due to passive immunization were subtracted.
no effect on the antibody response to a second dose of SRBC. In all cases there was an obvious priming effect compared with the control rats. It is worth noting that SRBC injected into the hind footpad of rats induced a much lower antibody response than a similar dose of SRBC injected intraperitoneally.

Figure 6 compares the effect of different concentrations of passive antibody on the primary antibody and delayed-type hypersensitivity responses to SRBC. Similar to POL and flagellin, suppression of primary antibody formation was accompanied by enhanced delayed hypersensitivity. However, there was not a linear relationship between concentration of passive antibody and the level of delayed hypersensitivity, the three highest concentrations of passive antibody inducing similar delayed responses.

The effect of antibody preincubated with antigen on the subsequent humoral and cell-mediated immune responses to flagellin.

In the preceding sections antigen and antibody were injected separately into rats. In this section, an experiment was carried out to determine the effect of in vitro incubation of antigen with antibody prior to injection. The rationale of this experimental procedure was to allow passive antibody to first react with antigen, rather than competing with receptors on immunocompetent cells. Different concentrations of guinea-pig anti-POL
Fig. 6. Comparison of the effect of different concentrations of passively administered guinea-pig anti-SRBC antibody on the humoral and cell-mediated immune responses to SRBC. For experimental details see Materials and Methods. Cell-mediated immunity is expressed as 24 hour footpad swelling and the antibody responses represent the mean primary antibody titres (data derived from Fig. 5). Vertical bars represent standard errors of the means, 7 rats per group.
antiserum were incubated with 500 ng of POL at 37° for 1 hr. The mixture was then injected intraperitoneally into adult rats and 28 days later animals were elicited with 500 ng of flagellin injected into the hind footpads as described previously.

It was found that anti-POL antibody when preincubated with POL was less effective at suppressing both the primary and secondary antibody responses. In fact, to achieve the same degree of suppression as separate injection of antibody and antigen, 25 times more passive anti-POL antibody was required (compare Fig. 1 and Fig. 7). Again, there was a suggestion that high concentrations of passive antibody might be inducing antibody tolerance. Similarly, the preincubation procedure tended to reduce the ability of passive antibody to enhance delayed-type hypersensitivity (compare Fig. 2 and Fig. 8), although a strict comparison of the two figures is difficult as control animals (NGPS) gave different footpad swellings. In contrast with POL, in a subsequent experiment it was found that when anti-POL antibody was either preincubated with flagellin or injected separately, the passive antibody was equally effective at suppressing both the primary and secondly antibody responses to flagellin. Furthermore, either preincubation or separate injection of passive antibody and flagellin resulted in comparable delayed hypersensitivity reactions.
Fig. 7. The ability of antigen-antibody "complexes" of POL to induce antibody production to POL in rats. Different concentrations of guinea-pig anti-POL antibody (10 ml volumes) were incubated with 5 μg of POL at 37° for 1 hour. After incubation, 1 ml of the mixture was injected intraperitoneally into each rat. Each animal was challenged with 500 ng of flagellin 28 days later. The concentrations (antibody titres) of passive antibody used were: 1,250 (○), 250 (●), 50 (△), 10 (▲), 2 (□), and normal guinea-pig serum (■). The broken line represents the antibody response of control rats which were not treated on day 0 and challenged with flagellin (500 ng) on day 28. Vertical bars represent standard errors of the means, 7 rats per group. The levels of serum antibody due to passive immunization were subtracted.
Fig. 8. The ability of antigen—antibody "complexes" of POL to induce humoral and cell-mediated immune responses to flagellin in rats. For experimental details see Fig. 7. Cell-mediated immunity is expressed as 24 hour footpad swelling. Antibody responses represent the mean primary antibody titres (data derived from Fig. 7). Vertical bars represent standard errors of the means, 7 rats per group.
The effect of rat anti-POL antibody on the immune response to POL and flagellin in rats

In the experiments described so far heterologous passive antibody was used, i.e. guinea-pig antibody. To show that homologous antibody had the same effects as heterologous antibody, experiments were performed with rat anti-POL antiserum. The same experimental protocol was used as for heterologous antibody. The effect of different concentrations of passively administered rat anti-POL antibody on the subsequent antibody response to POL and to flagellin is presented in Table 1. As was the case with heterologous antibody, increasing concentrations of homologous antibody steadily suppressed the primary antibody response to both POL and flagellin. There was also a suggestion that the secondary antibody response to POL was suppressed by the highest concentration of passive antibody. Fig. 9 compares the antibody and cell-mediated immune responses to both POL and flagellin in the presence of different concentrations of rat anti-POL antibody. With both antigens, suppression of antibody production was accompanied by a concomitant enhancement of cell-mediated immunity.

The specificity of the immunological effects induced by passive antibody

The experimental procedure was similar to that described in the preceding sections. Adult rats were injected intraperitoneally with 1 ml of a 1/20 dilution
<table>
<thead>
<tr>
<th>Concentration of antibody injected (500 ng)</th>
<th>Antibody titer (days)</th>
<th>Second antibody titer after second injection (500 ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat serum</td>
<td>POL (^a)</td>
<td>4.3 ± 0.6 5.4 ± 0.7 5.1 ± 0.6 5.8 ± 0.6 flagellin 9.7 ± 0.6 10.3 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>POL</td>
<td>1.0 ± 0.4 4.9 ± 0.3 5.0 ± 0.4 5.8 ± 0.4 flagellin 10.0 ± 0.3 10.2 ± 0.3</td>
</tr>
<tr>
<td>50</td>
<td>POL</td>
<td>&lt;0.5 1.6 ± 0.8 3.6 ± 0.7 4.4 ± 0.8 flagellin 10.0 ± 0.5 10.4 ± 0.3</td>
</tr>
<tr>
<td>250</td>
<td>POL</td>
<td>&lt;0.5 &lt;0.5 2.0 ± 0.7 2.0 ± 0.7 flagellin 8.0 ± 0.9 7.3 ± 0.7</td>
</tr>
<tr>
<td>Normal rat serum</td>
<td>flagellin</td>
<td>&lt;0.5 4.0 ± 0.9 4.2 ± 0.5 4.8 ± 0.5 flagellin 11.3 ± 0.4 9.6 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>flagellin</td>
<td>&lt;0.5 3.3 ± 0.8 3.9 ± 0.3 5.0 ± 0.3 flagellin 10.6 ± 0.4 9.5 ± 0.5</td>
</tr>
<tr>
<td>50</td>
<td>flagellin</td>
<td>&lt;0.5 1.7 ± 0.4 3.8 ± 0.5 4.8 ± 0.5 flagellin 9.8 ± 0.3 9.0 ± 0.3</td>
</tr>
<tr>
<td>250</td>
<td>flagellin</td>
<td>&lt;0.5 1.0 ± 0.1 1.3 ± 0.5 3.7 ± 0.3 flagellin 8.7 ± 0.6 9.1 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) POL = polymerised flagellin

\(^b\) Log\(_2\) antibody titer, tube 1 = 1/10 dilution. Each value represents the arithmetic mean of 7-8 animals ± standard error of the mean. Antibody due to passive immunisation was subtracted.
Fig. 9. Comparison of the effect of different concentrations of passively administered rat anti-POL antibody on the humoral and cell-mediated immune responses to POL (□) and flagellin (■). Cell-mediated immunity is expressed as 24 hour footpad swelling. Antibody responses represent the mean primary antibody titres (data derived from Table 1). Vertical bars represent standard errors of the means, 7 rats per group.
of either guinea-pig anti-POL antiserum (undiluted titre 25,000), or guinea-pig anti-SRBC antiserum (undiluted titre 2,000). One hour later each rat received intraperitoneally either 500 ng of POL in 0.5 ml saline or $5 \times 10^7$ SRBC in 0.5 ml saline. Animals were bled 5, 14 and 28 days after injection. Each rat was tested for cell-mediated immunity to SRBC or to flagellin on day 28 as described in the Materials and Methods.

It was found that the immunological effects of passive antibody were highly specific. Passively administered anti-POL antibody suppressed the antibody response to POL but not SRBC. Conversely, the suppressive effect of anti-SRBC antiserum was SRBC specific (Table 2). Similarly, the enhancement of delayed-type hypersensitivity by passive antibody was antiserum specific (Table 3).

The time course of induction of delayed-type hypersensitivity to flagellin by passive antibody

Groups of adult rats (8 per group) were injected intraperitoneally (1 ml/rat) with a 1/20 dilution of guinea-pig anti-POL antiserum (undiluted titre 25,000) and 1 hr later injected in the same site with 500 ng POL. These rats were tested for delayed-type hypersensitivity 4 weeks, 3 weeks, 2 weeks, 1 week or 1 hr following this injection schedule. Delayed-type hypersensitivity was elicited by the injection of 500 ng of flagellin in saline into the right hind footpads and specific footpad swelling
**TABLE 2**

SPECIFICITY OF THE SUPPRESSION OF ANTIBODY FORMATION BY PASSIVE ANTIBODY

<table>
<thead>
<tr>
<th>Antibody injected</th>
<th>Antigen injected</th>
<th>Antibody produced</th>
<th>Anti-POL titer (days)</th>
<th>Anti-SRBC titer (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Guinea-pig anti-POL</td>
<td>POL</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Guinea-pig anti-SRBC</td>
<td>POL</td>
<td>6.5 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Normal Guinea-pig serum</td>
<td>POL</td>
<td>6.0 ± 0.4</td>
<td>6.4 ± 0.4</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Guinea-pig anti-POL</td>
<td>SRBC</td>
<td>3.9 ± 0.1</td>
<td>4.0 ± 0.4</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>Guinea-pig anti-SRBC</td>
<td>SRBC</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Normal guinea-pig serum</td>
<td>SRBC</td>
<td>3.9 ± 0.1</td>
<td>3.4 ± 0.2</td>
<td>3.1 ± 0.3</td>
</tr>
</tbody>
</table>

a Each rat was injected intraperitoneally with 1 ml of a 1/20 dilution of either guinea-pig anti-POL antiserum (titer 25,000) or guinea-pig anti-SRBC antiserum (titer 2,000). One hour later each rat received intraperitoneally either 500 ng of POL in 0.5 ml saline or 5 x 10⁷ SRBC in 0.5 ml saline.

b Log₂ antibody titer, tube 1 = 1/10 dilution. Each value represents the arithmetic mean of 7-8 animals ± standard error of the mean. Antibody due to passive immunisation was subtracted.

c POL = polymerized flagellin, SRBC = sheep red blood cells.
TABLE 3

SPECIFICITY OF THE ENHANCEMENT OF CELL-MEDIATED IMMUNITY BY PASSIVE ANTIBODY

<table>
<thead>
<tr>
<th>Antibody injected</th>
<th>Antigen injected</th>
<th>Eliciting antigen</th>
<th>Footpad swelling (1/10th mm) 24 hr after eliciting injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea-pig anti-SRBC</td>
<td>SRBCb</td>
<td>SRBC</td>
<td>3.0 ± 0.3 C</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>flagellin</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>POLb</td>
<td>flagellin</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>SRBC</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Normal guinea-pig serum</td>
<td>SRBC</td>
<td>SRBC</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Guinea-pig anti-POL</td>
<td>POL</td>
<td>flagellin</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>SRBC</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>flagellin</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Normal guinea-pig serum</td>
<td>POL</td>
<td>flagellin</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>flagellin</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

---

a Each rat was injected intraperitoneally with 1 ml of a 1/20 dilution of either guinea-pig anti-POL antiserum (titer 25,000) or guinea-pig anti-SRBC antiserum (titer 2,000). One hour later each rat received intraperitoneally either 500 ng of POL in 0.5 ml saline or 5 x 10⁷ SRBC in 0.5 ml saline. Each rat was tested for cell-mediated immunity to SRBC or to flagellin on day 28 as described in Materials and Methods.

b POL = Polymerised flagellin; SRBC = sheep red blood cells.

c Each figure represents the arithmetic mean of 7-8 rats ± standard error of the mean.
determined 24 hr after the eliciting injection. Normal rats elicited in the same way served as experimental controls. It was found that the delayed response had reached its peak 7 days after POL priming and this level of reactivity persisted for at least four weeks (Fig. 10). Thus, delayed responses in this system can be elicited between 7 and 28 days following immunization.

The ability of cells and serum to transfer delayed-type hypersensitivity to flagellin induced by passive antibody

Adult rats (24 per group) were injected intraperitoneally (1 ml/rat) with either a 1/20 dilution of guinea-pig anti-POL antiserum (undiluted titre 25,000) or a 1/20 dilution of normal guinea-pig serum (control). One hour later, each rat received intraperitoneally 500 ng of POL in 0.5 ml saline. Two weeks later peritoneal cells and serum were harvested from 16 rats in each group and the remaining 8 rats tested for delayed-type hypersensitivity. Normal rats (9 per group) were then injected with either peritoneal cells (5 x 10⁷ cells/rat, i.v., approximately two donors to one recipient) or serum (2.5 ml/rat, i.p.) and 30 minutes later hypersensitivity reactions elicited by the injection of 500 ng of flagellin in saline into the right hind footpads. Specific footpad swelling was determined at 24 hr after the eliciting injection. It was observed that delayed-type hypersensitivity was very efficiently transferred from sensitized rats by peritoneal cells. In
Fig. 10. The time course of induction of delayed-type hypersensitivity to flagellin by passive antibody. For experimental details see text. delayed-type hypersensitivity is expressed as 24 hour footpad swelling. Vertical bars represent standard errors of the means, 8 rats per group.
contrast, peritoneal cells and serum from control rats, or serum from sensitized rats failed to transfer delayed responsiveness (see Table 4). Furthermore, histological analysis of footpads at the peak of the delayed response (24 hr footpad swelling) showed a large infiltration of mononuclear cells, a histology which is characteristic of delayed-type hypersensitivity (Fig. 11).

DISCUSSION

The data presented in this paper demonstrates that suppression of antibody formation by passively administered antibody can be accompanied by a concomitant enhancement of cell-mediated immunity. This phenomenon was observed with soluble (flagellin), particulate (polymerized flagellin) and cellular (SRBC) antigens and was also obtained with both homologous and heterologous passive antibody. These findings are consistent with recent studies in this laboratory which demonstrated that suppression of antibody formation by a series of acetoacetyl derivatives of flagellin was also accompanied by enhanced cell-mediated immunity (23,24). Thus, results from two separate experimental systems suggest that antibody formation and cell-mediated immunity may well be opposing immunological processes in adult animals.

The inverse relationship between humoral and cell-mediated immunity was observed for all antigens studied, although the exact nature of this relationship was highly dependent upon the antigen used. The most striking
TABLE 4

ABILITY OF SERUM AND PERITONEAL CELLS FROM HYPERSENSITIVE RATS TO TRANSFER DELAYED-TYPE HYPERSENSITIVITY TO FLAGELLIN

<table>
<thead>
<tr>
<th>Transfer</th>
<th>Amount</th>
<th>Footpad swelling (1/10th mm) 24 hr after eliciting injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Cellular immune&quot; (^c)</td>
<td>5 x 10^7 cells</td>
<td>2.4 ± 0.2(^a)</td>
</tr>
<tr>
<td>Peritoneal cells</td>
<td></td>
<td>(2.7)(^b)</td>
</tr>
<tr>
<td>Control peritoneal (^d)</td>
<td>5 x 10^7 cells</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>&quot;Cellular immune&quot; (^c)</td>
<td>2.5 ml</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control serum (^d)</td>
<td>2.5 ml</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Each figure represents the arithmetic mean of 8 rats ± standard error of mean.

\(^b\) Figures in brackets represent delayed-type hypersensitivity in actively sensitized rats. Similarly sensitized rats were used as cell and serum donors.

\(^c\) Peritoneal cells and serum from rats which had been injected two weeks previously with 1 ml of guinea-pig anti-POL antiserum (1/20 dilution) and 500 ng of POL. The hypersensitivity responses in recipient rats were elicited with 0.5 \(\mu\)g of flagellin.

\(^d\) Peritoneal cells and serum from rats which received normal guinea-pig serum (1/20 dilution) with POL instead of guinea-pig anti-POL antiserum. These donors showed no delayed-type hypersensitivity.
Fig. 11. Histology of delayed-type hypersensitivity to flagellin in rats, 24 hour after eliciting injection. Rats were injected intraperitoneally with guinea-pig anti-POL antibody (titre, 1,250; 1.0 ml/rat), and one hour later with POL (500 ng/rat) at the same site. Ten days later, delayed hypersensitivity was elicited by injecting flagellin (500 ng in 50 µl saline) into the right hind footpad and saline (50 µl) into the left hind footpad. (a) Transverse section of the hypodermis of left hind footpad. (b) Transverse section of the hypodermis of right hind footpad. Note the marked infiltration of mononuclear cells in (b). Magnification x 310. Sections stained with haematoxylin and eosin.
comparison was between flagellin and polymerized flagellin (POL), two antigens which are almost identical antigenically but differ in physical state (21). In the absence of passive antibody, flagellin induced significant delayed-type hypersensitivity whereas POL induced little or no delayed reactivity. For both antigens passive antibody enhanced cell-mediated immunity and suppressed antibody formation. However, the highest concentration of passive antibody administered did not enhance the delayed hypersensitivity induced by flagellin but continued to enhance the delayed response induced by POL. In the case of sheep red blood cells (SRBC) the kinetics of enhancement of delayed hypersensitivity by passive antibody differed from flagellin and POL. Thus, it appears that the immunological effects of passive antibody depend profoundly upon the physical nature of the antigen.

One of the interesting findings of this study was that passive antibody, when preincubated with POL rather than injected prior to antigen, was 25 times less efficient at suppressing antibody formation and there was also a suggestion that it was less effective at enhancing delayed-type hypersensitivity. A similar result was obtained by Ryder and Schwartz (10) who found that anti-SRBC antibody when preincubated with SRBC was less effective at suppressing antibody formation than when antigen and antibody were injected separately. Contrary to these findings, it was found that passive antibody, either preincubated with
flagellin or injected separate to flagellin, had the same effect on the subsequent humoral and cell-mediated immune responses. The explanation of these findings is unknown. However, it could be argued that preformed antigen-antibody complexes localised differently in the lymphoid system and this altered localisation could have a profound effect on immunogenicity.

The 24 hr footpad swellings reported in this paper represented genuine delayed-type hypersensitivity. The footpad swelling response was transferred by cells and not by serum (Table 4) and histological studies have revealed an infiltration of mononuclear cells. Furthermore, the delayed-type hypersensitivity observed was antigen specific (Table 3) and persisted for at least four weeks after sensitization (Fig. 10.).

It could be argued that the inverse relationship between humoral and cell-mediated immunity represents a masking of delayed-type hypersensitivity by serum antibody. If this were the case, passive administration of antibody to hypersensitive animals should suppress the delayed response. In contrast, peritoneal cells from animals with high antibody titres but little or no delayed hypersensitivity should readily transfer delayed responsiveness to normal animals. However, it has already been reported that passive administration of specific hyperimmune serum into hypersensitive rats failed to mask delayed-type hypersensitivity (24). In addition, peritoneal cells from POL
primed rats were unable to transfer the delayed response even though these animals had high antibody titres (Table 4). On the other hand, rats primed with POL together with anti-POL antibody failed to produce serum antibody but expressed delayed-type hypersensitivity, and this delayed responsiveness could be readily transferred by cells (Table 4).

Other workers have investigated the effect of passive antibody on the induction of delayed-type hypersensitivity to soluble antigens. Crowle and Hu (25,26) observed that passively administered antibody could suppress the induction of delayed hypersensitivity to a range of soluble protein antigens. However, these findings are not necessarily in conflict with our results, as in these earlier studies, multiple doses of high titre antiserum were used. On the other hand, it has been shown that complexes of antigen and antibody can induce delayed hypersensitivity indistinguishable from that induced by free antigen (16,28). Indeed, all attempts to demonstrate suppression of delayed hypersensitivity in this system have failed, despite the use of large amounts of heterologous antibody (1).

In contrast to these findings, Axelrad (27) reported that passive antibody can actively suppress the delayed-type hypersensitivity induced by sheep erythrocytes. Furthermore, numerous studies have demonstrated that passive antibody can readily suppress the induction of
cell-mediated immunity against tissue grafts (18-20). The discrepancy between these findings and the results presented in this paper could be explained in two ways. Firstly, in these other studies comparatively large doses of undiluted, hyperimmune serum were injected to induce the suppressive effects. Secondly, in the case of tissue grafts, the antigen source was usually implanted as a tissue mass in a site which was peripheral to the lymphoid system.

The mechanism of regulation of the immune response by passive antibody is uncertain. However, several workers have suggested that passive antibody has a "peripheral" effect on the immune response by complexing with antigenic determinants and thereby preventing these determinants from reacting with immunocompetent cells (16). On the other hand, data has been reported which suggests that passive antibody together with antigen has a "central" effect on the immune system by producing a specific reduction in the number of immunocompetent cells capable of producing antibody against an antigen (2,14). The data presented in this paper is consistent with passive antibody playing both a central and peripheral role. The lower concentrations of passive antibody had a central effect on the immune system as suppression of antibody formation was accompanied by enhanced delayed-type hypersensitivity. Thus, rather than passive antibody simply producing a reduction in the number of immunocompetent
cells capable of producing antibody (14) it appears that the immune response is deviated into cell-mediated immunity rather than antibody formation. On the other hand, high concentrations of passive antibody probably had a peripheral effect on the immune response as antibody formation was suppressed and a decline in cell-mediated immunity was observed.

In subsequent papers a more comprehensive study of this phenomenon will be presented and a hypothesis proposed to explain these findings.
REFERENCES


18. KALISS, N., Cancer Res. 18, 992, 1958.


PAPER IV

"THE EFFECTS OF DIFFERENT CLASSES OF PASSIVE ANTIBODY ON HUMORAL AND CELL-MEDIATED IMMUNITY"

The ability of different classes of passively administered antibody (γ, κ, and μ) to regulate humoral and cell-mediated immunity to flagellin, polymerized flagellin (POL) and sheep red blood cells (SRBC) was investigated in rats. It was found that at high concentrations, all classes of antibody suppressed the primary antibody response and usually enhanced the delayed-type hypersensitivity induced by the three antigens. With flagellin and SRBC, the different classes of passive antibody used at lower concentrations, γ and μ enhanced the primary antibody response and suppressed the delayed hypersensitivity induced by flagellin. Such an effect was not observed with either POL or SRBC. Priming for a secondary antibody response was less readily suppressed by all classes of passive antibody. The removal of macrophage cytotoxic antibody from γ converted this antibody to a preparation (γ absorbed) which had effects on humoral and cell-mediated immunity approaching that of μ antibody.

These results indicated that the immunological effects of passive antibody varied according to the physical nature of the antigen injected. Furthermore, it appears that the differences in the regulatory properties of various classes of passive antibody are dependent upon...
The ability of different classes of passively administered antibody ($\gamma_1$, $\gamma_2$ and IgM) to regulate humoral and cell-mediated immunity to flagellin, polymerized flagellin (POL) and sheep red blood cells (SRBC) was investigated in rats. It was found that at high concentrations, all classes of antibody suppressed the primary antibody responses and usually enhanced the delayed-type hypersensitivity induced by the three antigens. With flagellin and SRBC, the different classes of passive antibody varied in their suppressing and enhancing properties, being in the order: $\gamma_2 > \gamma_1 = \text{IgM}$. At low concentrations, $\gamma_1$ and IgM enhanced the primary antibody response and suppressed the delayed hypersensitivity induced by flagellin. Such an effect was not observed with either POL or SRBC. Priming for a secondary antibody response was less readily suppressed by all classes of passive antibody. The removal of macrophage cytophilic antibody from $\gamma_2$ converted this antibody to a preparation ($\gamma_2$ absorbed) which had effects on humoral and cell-mediated immunity approaching that of $\gamma_1$ antibody.

These results indicated that the immunological effects of passive antibody varied according to the physical nature of the antigen injected. Furthermore, it appears that the differences in the regulatory properties of various classes of passive antibody are dependent upon...
their content of macrophage cytophilic antibody. In addition, the results obtained support the notion that an inverse relationship exists between humoral and cell-mediated immunity.

INTRODUCTION

Evidence presented in an earlier paper (1) suggested that suppression of antibody formation by passively administered antibody could be accompanied by enhanced cell-mediated immunity. This phenomenon was observed with soluble (flagellin) particulate (polymerized flagellin, POL) and cellular (sheep red blood cells, SRBC) antigens. This phenomenon was further investigated in the present paper by determining the ability of different classes of antibody to both suppress and enhance humoral and cell-mediated immunity. An attempt was also made to demonstrate that macrophage cytophilic antibody plays an important regulatory role.

MATERIALS AND METHODS

Animals

Female outbred Wistar rats (9-12 weeks old) and albino guinea-pigs (10-16 weeks old) of both sexes were used. All animals were fed on Crajo pelleted diet (Crajo, Sydney) ad libitum.

Antigens

Sheep red blood cells (SRBC) stored in Alsever's
solution at 4° were washed 4 times in saline before use. Flagellin and polymerized flagellin (POL) from *Salmonella adelaide* (strain SW1338, H antigen, fg; o antigen, 35) were prepared according to Ada, Nossal, Pye and Abbot (2). Flagellin has a molecular weight of 40,000 and POL has been estimated to have a mean particle size equivalent to between 300 and 1600 flagellin units.

**Preparation of antisera**

The preparation of guinea-pig anti-POL and guinea-pig anti-SRBC hyperimmune sera was described in the preceding paper of this series (1). Briefly, POL (5 mg/ml in saline) or SRBC (30% suspension in saline) was emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit). Guinea-pigs were injected in each of the 4 footpads with the antigen preparations at the rate of 0.1 ml per footpad. Ten and 17 days later, the animals were injected intradermally with the respective antigens (0.1 ml of 15% SRBC in saline or 0.1 mg of POL in 0.1 ml saline) into each of 4 sites. The animals were exsanguinated under ether anaesthesia 7 days after the last injection.

For the preparation of IgM rich antisera, the following procedure was followed. For anti-POL anti-sera, POL (2.5 mg/ml in saline) was injected into each of the 4 footpads of 10 guinea-pigs at the rate of 0.1 ml per footpad (1 mg/guinea-pig). The animals were exsanguinated 7 days after the injection and the sera from all 10 guinea-pigs pooled.
For anti-SRBC antiserum, each of a group of 10 guinea-pigs was injected intraperitoneally with 5 ml of a 5% suspension of SRBC in saline (i.e., $2.5 \times 10^9$ SRBC per guinea-pig). The guinea-pigs were bled out 6 days after the injection and the sera pooled. All sera were stored at $-10^\circ \text{C}$.

**Fractionation of antibodies**

For the preparation of $\gamma_1$ and $\gamma_2$ antibodies, the hyperimmune sera were first dialysed against distilled water, overnight at $4^\circ \text{C}$, and then precipitated with 37% saturated ammonium sulphate at $4^\circ \text{C}$. $\gamma_1$ and $\gamma_2$ immunoglobulins were then isolated from the ammonium sulphate precipitated protein by chromatography on a DEAE-cellulose column (3). The yield of haemagglutinating activity was 75-80% and $\gamma_1$ and $\gamma_2$ contained almost equal activity. The yield of cytophilic activity was about 80%, the majority of which was found in the $\gamma_2$ fraction. IgM antibodies were prepared from the early sera on a sucrose gradient as described elsewhere (4). The 7 day serum from guinea-pigs injected with POL in saline contained no detectable 7S anti-POL antibody. The 6 day guinea-pig anti-SRBC anti-serum, on the other hand, contained both 19S and 7S antibody and was fractionated twice on sucrose gradients.

All the $\gamma_2$ preparations were found to contain macrophage cytophilic antibody. In some experiments, the $\gamma_2$
preparations were depleted of macrophage cytophilic antibody by absorbing 3 times with normal guinea-pig peritoneal exudate cells which had been previously washed 3 times with excess Hanks' solution. At each absorption, 10 ml of a \( \gamma_2 \) preparation in phosphate buffered saline (PBS), containing heparin (10 units/ml) and sodium azide (1.5 mM) was mixed with 1.0 ml of packed peritoneal exudate cells and incubated at room temperature for 1 hr. At the end of the incubation the cells were centrifuged at 1,500 rpm for 10 min. The \( \gamma_2 \) preparations thus absorbed were dialysed against PBS and assayed for haemagglutinating and cytophilic activity. Attempts to eluate cytophilic activity from the PEC used to absorb cytophilic antibody from \( \gamma_2 \) were not successful, despite the fact that these cells could be shown to have taken up cytophilic antibody.

**Immunisation procedures**

Anti-sera or antibody preparations of known antibody titres were diluted with PBS to form a series of antibody dilutions. Groups of rats (7 per group) were injected intraperitoneally with 1.0 ml of one of the various antibody dilutions. Control rats were injected with either normal serum, a guinea-pig anti-BSA antibody preparation of the appropriate dilution, or received no injection. Rats injected with normal guinea-pig serum or guinea-pig anti-BSA antibody responded to the different antigens in exactly the same way as rats which received no injection. Thus,
in subsequent experiments untreated rats were used as controls. One hour after the injection of passive antibody each animal was injected intraperitoneally with the appropriate antigen, i.e., 500 ng of POL, or 500 ng of flagellin or \(5 \times 10^7\) SRBC in 0.5 ml saline. The levels of serum antibody due to passive immunisation were determined in groups of rats which received antibody but no antigen. Antibody titres were estimated at weekly intervals following injection from serum samples which had been collected from the tail. Rats which received flagellin or POL on day 0 were given a second dose of flagellin on day 28, whereas rats which were injected with SRBC on day 0 received a second dose of SRBC on day 14. All rats were bled for one more week after the second injection. The second injection also served as the eliciting injection for delayed-type hypersensitivity. Antigen (500 ng of flagellin in 50 µl saline or \(5 \times 10^7\) SRBC in 50 µl saline) was injected into the right hind footpads and saline alone into the left hind footpads. Specific footpad swelling was measured at 24 hr by subtracting the thickness of the left hind footpad from the right. Footpad thickness was measured by a dial calliper gauge A02T (Schnelltaster, System Kröplin).

**Antibody estimation**

Antibodies to SRBC were estimated by haemagglutination. Antibodies to flagellin and POL were estimated by a passive haemagglutination method based on that of Jandl and Simmons (5) and was described previously (1).
Macrophage cytophilic antibodies to flagellar proteins were estimated by a radioimmunoassay system (4). Macrophage cytophilic antibodies to SRBC were estimated by the rosette method of Boyden (6) as modified by Jonas et al (7).

**Peritoneal exudate cells**

Peritoneal exudate cells were obtained from normal adult guinea-pigs which had been injected intraperitoneally with 10 ml of a 0.1% (w/v) oyster glycogen solution 2 days previously.

**Statistical methods**

Statistical calculations were performed on an IBM computer and standard errors of the means and P values were calculated according to the Student's t Test.

**RESULTS**

**Effect of anti-POL antibody on the immune response to flagellin**

(a) **Unfractionated anti-POL anti-serum**

In an earlier paper (1), it was reported that unfractionated anti-POL antiserum at high concentrations (titres 2 to 1250) could suppress the antibody response to flagellin. It also appeared that at certain concentrations of passive antibody (titres 50 and 250) delayed-type hypersensitivity to flagellin was enhanced. In this paper
these studies were extended to much lower concentrations of passive antibody (titres $4 \times 10^{-4}$ to $4 \times 10^2$).

Groups of rats (7 per group) were injected intraperitoneally with different concentrations of guinea-pig anti-POL antiserum (titres ranging in ten fold dilution steps from $4 \times 10^2$ to $4 \times 10^{-4}$). Control rats received no antibody. One hour later, each rat was injected intraperitoneally with 500 ng of flagellin in saline. Twenty-eight days later, delayed hypersensitivity reactions and secondary antibody responses were elicited by the injection of 500 ng of flagellin in saline into the hind footpads. Antibody titres were estimated 21, 28 and 35 days following primary immunisation. The primary anti-flagellin antibody titres peaked at 21 and 28 days following injection. Usually the antibody levels in these two bleeds were similar. For convenience, only the 28 day titres are presented in the Figures.

The results of this experiment are presented in Fig. 1. From these results the following observations were made.

1. The highest concentrations (titre $4 \times 10^2$) of passive anti-POL antiserum suppressed the primary antibody response to flagellin, whilst lower concentrations (titres, $4$ to $4 \times 10^{-2}$) of antiserum significantly enhanced ($P < 0.05$) the primary antibody response to flagellin. However, very low concentrations (titres, $4 \times 10^{-3}$ to $4 \times 10^{-4}$) of passive antiserum had no effect on the production of antibody to flagellin.
Fig. 1. The effect of unfractionated passive anti-POL antiserum on the humoral and cell-mediated immune responses to flagellin. Groups of rats were injected intraperitoneally with 1.0 ml of the various antiserum dilutions, antibody concentrations (antibody titers) ranging from $4 \times 10^{-2}$ to $4 \times 10^{-4}$. Control rats received no injection. One hour later, each animal was injected intraperitoneally with 500 ng of flagellin in 0.5 ml saline. On day 28, each animal was elicited for delayed hypersensitivity by injecting into the hind footpads 500 ng of flagellin in 50 μl saline. Cell-mediated immunity is expressed as 24 hr footpad swelling. Antibody responses represent the 28 day primary antibody titers (reciprocal of dilution). Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
It should be noted that in a previously reported study (1) high concentrations of passive anti-POL antiserum (titres: 2 to 1,250) also suppressed the antibody response to flagellin. However, in this earlier study a different batch of antiserum was used which appeared to be more potent at suppressing antibody formation (Fig. 1).

2. The delayed-type hypersensitivity induced by flagellin, in the presence of high (titres: $4 \times 10^2$ to $4 \times 10^{-1}$) and low (titres: $4 \times 10^{-3}$ to $4 \times 10^{-4}$) concentrations of passive anti-POL antiserum was no different from that given by flagellin itself. One fact that should be noted is that 500 ng of flagellin induced a substantial delayed response when injected alone in saline (footpad swelling 2.4). In contrast, the intermediate doses of the passive antiserum (titres: $4 \times 10^{-1}$ to $4 \times 10^{-2}$) significantly suppressed ($P < 0.05$) the delayed response to flagellin.

3. At the intermediate doses of passive antiserum, there was an apparent inverse relationship between humoral and cell-mediated immunity. Enhancement of antibody formation was accompanied by a suppression of delayed-type hypersensitivity.

Table 1 presents the secondary antibody responses to flagellin in rats given different concentrations of passive antiserum. It can be seen that only the highest concentration of passive antiserum (titre $4 \times 10^2$)
significantly suppressed the ability of flagellin to prime animals for a secondary antibody response whereas all other concentrations of passive antibody did not significantly affect the induction of immunological memory.

(b) **Fractionated anti-POL antiserum**

The ability of $\gamma_1$, $\gamma_2$ and IgM antibodies to regulate the immune response to flagellin was investigated. Adult rats (7 per group) were injected intraperitoneally with different concentrations of one of the three anti-POL antibody preparations ($\gamma_1$, $\gamma_2$ or IgM) and one hour later injected in the same site with 500 ng of flagellin in saline. Secondary antibody responses and delayed-type hypersensitivity were elicited in the hind footpads 28 days later with 500 ng of flagellin in saline.

Fig. 2 depicts the effects of different concentrations of passively administered $\gamma_1$ and $\gamma_2$ anti-POL antibodies on the primary antibody and cell-mediated immune responses to flagellin.

At high concentrations, both $\gamma_1$ and $\gamma_2$ passive antibodies suppressed the antibody response to flagellin. However, $\gamma_2$ was about 150 times more efficient than $\gamma_1$ at suppressing antibody production. At lower concentrations (titres: $4 \times 10^{-1}$ to $4 \times 10^{-2}$) $\gamma_1$ antibody enhanced the antibody response to flagellin. In contrast, this enhancement was not achieved with $\gamma_2$ antibody at all the concentrations tested. At very low concentrations (titres
Fig. 2. The effect of passive $\gamma_1$ (□) and $\gamma_2$ (■) anti-POL antibody on the humoral and cell-mediated immunity to flagellin. For experimental detail see legend to Fig. 1. Antibody responses represent the 28 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
\[ \leq 4 \times 10^{-2} \], both the \( \gamma_1 \) and the \( \gamma_2 \) antibodies had no effect on the production of antibody to flagellin.

In contrast to the humoral responses, at high concentrations (titres: \( 4 \times 10^1 \) to \( 4 \times 10^2 \)) passive \( \gamma_2 \) anti-POL antibody enhanced the delayed-type hypersensitivity induced by flagellin. However, with low concentrations of \( \gamma_2 \) (titres: \( 4 \) to \( 4 \times 10^{-4} \)), the delayed response was no greater than that given by flagellin itself. In contrast to \( \gamma_2 \), certain concentrations of \( \gamma_1 \) antibody (titres: \( 4 \times 10^1 \) to \( 4 \)) significantly \( (P < 0.05) \) suppressed the delayed reaction to flagellin. The \( \gamma_1 \) passive antibody, on the other hand, did not enhance delayed hypersensitivity at all the concentrations of antibody tested.

Passively administered anti-POL IgM antibody had remarkably similar effects on the immune response to flagellin as \( \gamma_1 \) antibody (compare Fig. 2 and 3). However, for reasons to be mentioned in the discussion, it is difficult to compare the immunological effectiveness of passive IgG and IgM antibodies.

The effect of passively administered \( \gamma_1 \), \( \gamma_2 \) and IgM anti-POL antibodies on the secondary antibody response to flagellin are summarised in Table 2. It was found that \( \gamma_2 \) was 10 to 100 times more effective than \( \gamma_1 \) in suppressing the secondary antibody response. The effect of IgM on the secondary antibody response was, again, similar to that of \( \gamma_1 \). It should be noted that the three classes of antibody did not significantly enhance secondary antibody responsiveness.
Fig. 3. The effect of passive IgM anti-POL antibody on the humoral and cell-mediated immunity to flagellin. For experimental detail see legend to Fig. 1. Antibody responses represent the 28 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
TABLE 2

THE EFFECT OF DIFFERENT CLASSES OF ANTI-POL ANTIBODY ON THE SECONDARY ANTIBODY RESPONSE TO FLAGELLIN<sup>a</sup>

<table>
<thead>
<tr>
<th>CONCENTRATION OF ANTIBODY INJECTED (ANTIBODY TITRE&lt;sup&gt;c&lt;/sup&gt;)</th>
<th>SECONDARY ANTIBODY TITRE&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Y&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IgM</td>
<td></td>
</tr>
<tr>
<td>960</td>
<td>3.9 ± 1.3</td>
<td>-</td>
<td>7.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>8.0 ± 0.6</td>
<td>0.5 ± 0.2</td>
<td>9.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10.5 ± 0.2</td>
<td>2.9 ± 0.6</td>
<td>11.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11.7 ± 0.4</td>
<td>10.3 ± 0.7</td>
<td>10.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>4 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>11.6 ± 0.5</td>
<td>11.3 ± 0.5</td>
<td>10.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>4 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>11.6 ± 0.5</td>
<td>10.9 ± 0.3</td>
<td>11.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>11.3 ± 0.1</td>
<td>11.1 ± 0.3</td>
<td>11.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>10.7 ± 0.3</td>
<td>11.6 ± 0.2</td>
<td>11.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10.3 ± 0.4</td>
<td>10.3 ± 0.4</td>
<td>10.3 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

For details see text and legend to Table 1.

Log<sub>2</sub> antibody titre, \( l = 1/10 \) dilution. Each value represents the arithmetic mean of 7 rats ± standard error of the mean.

Reciprocal of dilution.
Fig. 4. The effect of unfractionated passive anti-POL antiserum on the humoral and cell-mediated immunity to POL. Groups of rats were injected intraperitoneally with 1.0 ml of the various antiserum dilutions, antibody concentrations (antibody titers) ranging from $4 \times 10^2$ to $4 \times 10^{-4}$. Control rats received no injection. One hour later, each rat was injected intraperitoneally with 500 ng of POL in 0.5 ml saline. On day 28, each animal was elicited for delayed-type hypersensitivity by injecting into the hind footpads 500 ng of flagellin in 50 µl saline. Cell-mediated immunity is expressed as 24 hr footpad swelling. Antibody responses represent the 28 day primary antibody titers. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
TABLE 3

THE EFFECT OF UNFRACTIONATED ANTI-POL ANTISERUM ON
THE SECONDARY ANTIBODY RESPONSE TO POL\textsuperscript{a}

<table>
<thead>
<tr>
<th>CONCENTRATION OF ANTIBODY INJECTED (ANTIBODY TITRE\textsuperscript{b})</th>
<th>SECONDARY ANTIBODY TITRE\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 x 10\textsuperscript{2}</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>4 x 10\textsuperscript{1}</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>11.9 ± 0.3</td>
</tr>
<tr>
<td>4 x 10\textsuperscript{-1}</td>
<td>12.6 ± 0.4</td>
</tr>
<tr>
<td>4 x 10\textsuperscript{-2}</td>
<td>12.3 ± 0.5</td>
</tr>
<tr>
<td>4 x 10\textsuperscript{-3}</td>
<td>11.1 ± 0.3</td>
</tr>
<tr>
<td>4 x 10\textsuperscript{-4}</td>
<td>11.4 ± 0.2</td>
</tr>
<tr>
<td>0</td>
<td>11.6 ± 0.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Groups of rats were injected intraperitoneally with 1.0 ml of different concentrations of passive anti-POL antiserum followed 1 hr later by an injection (intraperitoneally) of 500 ng POL. 28 days later, all animals were injected into the footpad with 500 ng of flagellin. Secondary antibody responses to flagellin were estimated 7 days after the last injection.

\textsuperscript{b} reciprocal of dilution.

\textsuperscript{c} log\textsubscript{2} antibody titre, 1 = 1/10 dilution. Each value represents the arithmetic mean of 7 rats ± standard error of the mean.
different batch of passive antibody also suppressed antibody formation and enhanced delayed hypersensitivity to POL.

(b) Fractionated anti-POL antiserum

In this section, the ability of $\gamma_1$, $\gamma_2$ and IgM anti-POL antibody to influence the immune response to POL was determined. The experimental design was the same as described in earlier sections.

Fig. 5 shows that the effects of passive $\gamma_1$ and $\gamma_2$ anti-POL antibodies on the immune response to POL were remarkably similar. At high concentrations, both $\gamma_1$ and $\gamma_2$ antibodies suppressed the primary antibody response and enhanced the delayed-type hypersensitivity induced by POL. There was no significant difference between the ability of the two classes of antibody to both suppress antibody formation and enhance delayed reactivity. In addition, both $\gamma_1$ and $\gamma_2$ antibodies did not enhance antibody production to POL. Passive IgM anti-POL antibody had a similar effect on the immune response to POL as $\gamma_1$ and $\gamma_2$ (compare Figs. 5 and 6). At all concentrations tested, none of the three classes of passive antibody significantly affected the induction of immunological memory by POL. A similar result was obtained with the unfractionated antiserum (see Table 3).

It should be emphasized that with all three classes of passive antibody, there was an apparent inverse
Fig. 5. The effect of passive $\gamma_1$ (□) and $\gamma_2$ (■) anti-POL antibody on the humoral and cell-mediated immunity to POL. For experimental detail see legend to Fig. 4. Antibody responses represent the 28 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
relationship between the humoral and cell-mediated immune responses, although an exact correlation between these two responses was not obtained.

Effect of anti-SRBC antibody on the immune response to SRBC

In the preceding section, the regulatory effect of passive antibody on the immune response to a soluble antigen (flagellin) and to a particulate antigen (POL) was investigated. In this section the effect of passive antibody on the immune response to a cellular antigen (SRBC) was studied.

(a) Unfractionated anti-SRBC antiserum

Adult rats were injected intraperitoneally with different concentrations of anti-SRBC antiserum and one hour later injected in the same site with $5 \times 10^7$ SRBC in saline. Secondary antibody responses and delayed hypersensitivity were elicited in the hind footpad 14 days later with $5 \times 10^7$ SRBC in saline. Antibody against SRBC was measured 7, 14 and 21 days following primary immunisation. The primary anti-SRBC antibody responses peaked between 7 and 14 days, and for convenience, only the 14 day titres are reported.

Fig. 7 shows the effect of different concentrations of anti-SRBC antibody on the humoral and cell-mediated immune responses to SRBC. At high concentrations (titres:
Fig. 7. The effect of unfraccionated passive anti-SRBC antiserum on the humoral and cell-mediated immunity to SRBC. Groups of rats were injected intraperitoneally with 1.0 ml of the various antiserum dilutions, antibody concentrations (antibody titers) ranging from 50 to $5 \times 10^{-5}$. Control rats received no injection. One hour later, each animal was injected intraperitoneally with $5 \times 10^7$ SRBC in 0.5 ml saline. On day 14, each rat was elicited for delayed-type hypersensitivity by injecting into the hind footpads $5 \times 10^7$ SRBC in saline. Cell-mediated immunity is expressed as 24 hr footpad swelling. Antibody responses represent the 14 day primary antibody titers. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
5 x 10^2 to 5 x 10^{-1}), the passive antibody suppressed the antibody response but enhanced the delayed hypersensitivity induced by SRBC. However, there was not a linear relationship between the concentrations of passive antibody and the level of delayed hypersensitivity, the three highest concentrations of passive antibody inducing similar delayed responses. At lower concentrations (titres: ≤ 5 x 10^{-2}) the passive antibody had no effect on both the humoral and cell-mediated immune responses induced by SRBC. Following the second injection of 5 x 10^7 SRBC in saline, all groups of rats expressed 21 day (7 day secondary) antibody titres which did not significantly differ. Thus, similar levels of immunological memory were induced by SRBC both in the presence and absence of passive antibodies. In subsequent experiments the secondary antibody responses to SRBC were not measured.

(b) Fractionated anti-SRBC antiserum

The experimental design was the same as described in the preceding section except that in this case different classes of passive anti-SRBC antibody (Y_1, Y_2 and IgM) isolated from the anti-SRBC antiserum, were used.

The result of this experiment is presented in Fig. 8 and 9. At the highest concentrations (titres: 5 x 10^1 and 5), both Y_1 and Y_2 passive anti-SRBC antibodies suppressed the antibody response and enhanced the delayed hypersensitivity induced by SRBC (Fig. 8). However, the
Fig. 8. The effect of passive $\gamma_1$ (□) and $\gamma_2$ (■) anti-SRBC antibody on the humoral and cell-mediated immune responses to SRBC. For details see Fig. 7. Cell-mediated immunity is expressed as 24 hr footpad swelling. Antibody responses represent the 14 day primary antibody titers. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
Fig. 9. The effect of passive IgM anti-SRBC antibody on the humoral and cell-mediated immune responses to SRBC. For detail see legend to Fig. 7. Antibody responses represent the 14 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
two classes of passive antibody significantly differed in their ability to modify the immune response to SRBC. Compared with $\gamma_1$, on the basis of titre, $\gamma_2$ passive antibody was about 100 times more effective at enhancing the delayed response, and about 10 times more effective in suppressing the antibody response to SRBC. At lower concentrations (titres: $5 \times 10^{-2}$ to $5 \times 10^{-3}$), $\gamma_1$ antibody may have enhanced the anti-SRBC antibody response, but the results were only marginally significant ($0.02 < P < 0.05$).

Fig. 9 depicts the effect of passive IgM anti-SRBC antibody on the immune response induced by SRBC. At the highest concentration (titre, 5) IgM suppressed anti-SRBC antibody formation. In contrast, except in one group of rats (titre, $5 \times 10^{-1}$), IgM did not significantly enhance the delayed hypersensitivity provoked by SRBC.

Influence of macrophage cytophilic antibody on humoral and cell-mediated immune responses

Results from the preceding sections revealed that $\gamma_1$ and $\gamma_2$ antibodies markedly differed in their regulatory effects on the immune response to flagellin and SRBC. In guinea-pigs, the majority of macrophage cytophilic antibody is present in the $\gamma_2$ fraction, whereas little or no macrophage cytophilic activity is found in the $\gamma_1$ fraction. It is therefore, possible that $\gamma_2$ and $\gamma_1$ antibodies differ in their immuno-regulatory properties due to the presence or absence of macrophage cytophilic antibody in these two fractions.
Table 4 compares the ratios of macrophage cytophilic antibody titre to total haemagglutinating titre in the various antibody preparations. In both the anti-POL and anti-SRBC antibody preparations $\gamma_2$ had a high cytophilic antibody content. In contrast, both the $\gamma_1$ and IgM preparations contained little or no cytophilic antibody. To investigate the possible regulatory role of macrophage cytophilic antibody on the immune response, the following experiments were carried out.

The $\gamma_2$ preparations were absorbed repeatedly with normal guinea-pig peritoneal exudate cells to remove the macrophage cytophilic antibody. The absorption procedure reduced by $>99\%$ the cytophilic-antibody content of the $\gamma_2$ preparations, but left the haemagglutinating titres undiminished (Table 5). Groups of rats (7 per group) were injected intraperitoneally with different concentrations of either the $\gamma_2$ preparation or the absorbed $\gamma_2$ preparation ($\gamma_2$ absorbed). One hour later, each rat was injected intraperitoneally with the appropriate antigen. Secondary antibody responses and delayed hypersensitivity reactions were elicited on day 14 in the SRBC experiment and on day 28 in the POL and flagellin experiment. Antibody titres were measured just before elicitation and one week after the eliciting injection. The results obtained with the three antigens, flagellin, POL and SRBC, are presented in the following sections.
TABLE 4

THE RELATIVE RATIOS OF MACROPHAGE CYTOPHILIC ANTIBODY TITRE TO TOTAL HAEMAGGLUTINATING ANTIBODY TITRE IN VARIOUS ANTIBODY PREPARATIONS

<table>
<thead>
<tr>
<th>ANTIBODY PREPARATIONS</th>
<th>C.Ab(^a) TITRE</th>
<th>H.A.(^b) TITRE</th>
<th>C.Ab/H.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-POL whole serum</td>
<td>960(^c)</td>
<td>24,000(^c)</td>
<td>0.04</td>
</tr>
<tr>
<td>&quot; γ₁</td>
<td>&lt;5</td>
<td>960</td>
<td>0(^d)</td>
</tr>
<tr>
<td>&quot; γ₂</td>
<td>320</td>
<td>4,000</td>
<td>0.08</td>
</tr>
<tr>
<td>&quot; IgM</td>
<td>&lt;5</td>
<td>960</td>
<td>0</td>
</tr>
<tr>
<td>Anti-SRBC whole serum</td>
<td>100</td>
<td>1,000</td>
<td>0.10</td>
</tr>
<tr>
<td>&quot; γ₁</td>
<td>&lt;5</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>&quot; γ₂</td>
<td>100</td>
<td>500</td>
<td>0.20</td>
</tr>
<tr>
<td>&quot; IgM</td>
<td>&lt;5</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) C.Ab = cytophilic antibody.

\(^b\) H.A. = haemagglutinating antibody.

\(^c\) Reciprocal of dilution

\(^d\) Ratio = 0 denotes no detectable cytophilic antibody by the assay method.
TABLE 5
THE RELATIVE CYTOPHILIC ANTIBODY CONCENTS IN $\gamma_2$ IgG PREPARATION BEFORE AND AFTER ABSORPTION WITH PERITONEAL EXUDATE CELLS

<table>
<thead>
<tr>
<th>ANTIBODY PREPARATION</th>
<th>C.Ab TITRE$^a$</th>
<th>H.A.$^b$ TITRE</th>
<th>C.Ab H.A.$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-POL $\gamma_2$</td>
<td>1,600$^c$</td>
<td>4,000$^c$</td>
<td>0.40</td>
</tr>
<tr>
<td>Anti-POL $\gamma_2$ Absorbed$^e$</td>
<td>5</td>
<td>4,000</td>
<td>0.001</td>
</tr>
<tr>
<td>Anti-SRBC $\gamma_2$</td>
<td>100</td>
<td>500</td>
<td>0.20</td>
</tr>
<tr>
<td>Anti-SRBC $\gamma_2$ Absorbed$^e$</td>
<td>&lt;5</td>
<td>500</td>
<td>0$^d$</td>
</tr>
</tbody>
</table>

For experimental details, see Materials and Methods

$^a$ C.Ab = cytophilic antibody

$^b$ H.A. = haemagglutinating antibody

$^c$ Reciprocal of dilution

$^d$ Ratio = 0 denotes no detectable cytophilic antibody by the assay method.

$^e$ $\gamma_2$ antibody preparation absorbed three times with peritoneal exudate cells
(a) Flagellin

Fig. 10 shows the effect of depleting anti-POL $\gamma_2$ of cytophilic antibody on the ability of the antibody to influence the immune response to flagellin. The $\gamma_2$ absorbed preparation, in term of titre, was 10 times less effective in suppressing the antibody response to flagellin than the undepleted preparation of $\gamma_2$. At the same time, the ability of $\gamma_2$ absorbed preparation to enhance delayed hypersensitivity also decreased about 10 fold. Furthermore, at lower concentrations, the $\gamma_2$ absorbed preparation was able to enhance antibody production and to suppress delayed reactivity to flagellin. It appears that after the removal of cytophilic antibody, $\gamma_2$ antibody had a similar effect on the immune response to flagellin as $\gamma_1$ antibody. It should be noted that the $\gamma_2$ and $\gamma_2$ absorbed preparations had a similar effect on the immunological memory induced by flagellin (Table 6).

(b) Polymerised flagellin

In contrast with flagellin, the immune response to POL was equally affected by the $\gamma_2$ preparation either depleted or undepleted of cytophilic antibody (Fig. 11). In addition, the $\gamma_2$ and $\gamma_2$ absorbed preparations had no effect on the immunological memory induced by POL (Table 6).

(c) Sheep red blood cells

Fig. 12 compares the effect of $\gamma_2$, $\gamma_2$ absorbed and
The effect of passive $\gamma_2$ (■) and $\gamma_2$ absorbed (□) anti-POL antibody on the humoral and cell-mediated immune responses to flagellin. For detail see legend to Fig. 1. Antibody responses represent the 28 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
TABLE 6

THE EFFECT OF PASSIVE ANTI-POL $\gamma_2$ ANTIBODY AND $\gamma_2$ ABSORBED ANTIBODY ON THE SECONDARY ANTIBODY RESPONSE TO FLAGELLIN$^a$

<table>
<thead>
<tr>
<th>CONCENTRATION OF ANTIBODY INJECTED (ANTIBODY TITRE$^d$)</th>
<th>SECONDARY ANTIBODY TITRE$^b$</th>
<th>$\gamma_2$</th>
<th>$\gamma_2$ Absorbed$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4 \times 10^2$</td>
<td></td>
<td>4.5 ± 0.5</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>$4 \times 10^1$</td>
<td></td>
<td>9.6 ± 0.5</td>
<td>9.9 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>11.2 ± 0.1</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>$4 \times 10^{-1}$</td>
<td></td>
<td>10.6 ± 0.3</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>$4 \times 10^{-2}$</td>
<td></td>
<td>10.7 ± 0.3</td>
<td>11.0 ± 0.3</td>
</tr>
<tr>
<td>$4 \times 10^{-3}$</td>
<td></td>
<td>10.4 ± 0.7</td>
<td>10.7 ± 0.2</td>
</tr>
<tr>
<td>$4 \times 10^{-4}$</td>
<td></td>
<td>10.6 ± 0.2</td>
<td>10.4 ± 0.3</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>10.5 ± 0.3</td>
<td>10.5 ± 0.3</td>
</tr>
</tbody>
</table>

$^a$ For experimental details see text

$^b$ Log$_2$ antibody titre, 1 = 1/10 dilution. Each value represents the arithmetic mean of 7 rats ± standard error of the mean

$^c$ $\gamma_2$ antibody preparation absorbed three times with peritoneal exudate cells

$^d$ Reciprocal of dilution
Fig. 11. The effect of passive $\gamma_2$ (■) and $\gamma_2$ absorbed (□) anti-POL antibody on the humoral and cell-mediated immune responses to POL. For detail see legend to Fig. 4. Antibody responses represent the 28 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
Fig. 12. The effect of passive $\gamma_1$ ( ), $\gamma_2$ ( ■ ) and $\gamma_2$ absorbed ( ○ ) anti-SRBC antibody on the humoral and cell-mediated immune responses to SRBC. For detail see legend to Fig. 7. Antibody responses represent the 14 day primary antibody titers. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group. The broken lines represent the immune responses of control rats.
\( \gamma_1 \) anti-SRBC antibody preparations on the immune response to SRBC. The three preparations were derived from the same serum and the experiments were carried out at the same time. It can be seen that the ability of the \( \gamma_2 \) absorbed preparation to suppress antibody formation and enhance delayed hypersensitivity to SRBC was in between that of the \( \gamma_2 \) and \( \gamma_1 \) preparations.

**DISCUSSION**

The results presented in this paper confirm the earlier finding (1) that suppression of antibody formation by high concentrations of passive antibody may be accompanied by enhanced cell-mediated immunity. This phenomenon was observed with flagellin, POL and SRBC. However, upon closer examination it was found that at both high and low concentrations of passive antibody the immunological effects observed were highly dependent upon the class of antibody and the type of antigen used. These results are summarised in Table 7 from which the following observations can be made and conclusions drawn.

1. At high concentrations all classes of passive antibody significantly suppressed the primary antibody responses and usually enhanced the delayed-type hypersensitivity induced by flagellin, POL or SRBC.

2. With flagellin and SRBC, the different classes of passive antibody varied in their suppressing (antibody formation) and enhancing (cell-mediated immunity) properties, being in the order: \( \gamma_2 > \gamma_1 = \text{IgM} \). In contrast, all three
### Table 7

The effect of different classes of passive antibody on the humoral and cell-mediated immunity induced by different antigens.

<table>
<thead>
<tr>
<th>Passive Antibody</th>
<th>Antibody Concentration</th>
<th>Delayed Hypersensitivity</th>
<th>Primary Antibody Response</th>
<th>Secondary Antibody Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Flagellin</td>
<td>POL</td>
<td>SRBC</td>
</tr>
<tr>
<td>Whole Serum</td>
<td>High</td>
<td>±&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>γ&lt;sub&gt;2&lt;/sub&gt;</td>
<td>High</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>γ&lt;sub&gt;1&lt;/sub&gt;</td>
<td>High</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>γ&lt;sub&gt;2&lt;/sub&gt; absorbed</td>
<td>High</td>
<td>±&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgM</td>
<td>High</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enhancement of delayed hypersensitivity reported previously (1).

**Symbols:**
- ++ = Strong enhancement
- + = Enhancement
- ± = Weak enhancement
- 0 = No effect
- - = Suppression
- -- = Strong suppression
classes of antibody had similar immunological effects on POL.

3. At low concentrations, \( \gamma_1 \) and IgM enhanced the primary antibody response and suppressed the delayed hypersensitivity induced by flagellin. Such an effect was not observed with POL or SRBC.

4. Priming for a secondary antibody response was less readily suppressed by all classes of passive antibody.

5. The depletion of macrophage cytophilic antibody from \( \gamma_2 \) converted this antibody to a preparation (\( \gamma_2 \) absorbed) which had effects on humoral and cell-mediated immunity more like that of \( \gamma_1 \) antibody.

One of the most striking features of this study was the apparent inverse relationship between humoral and cell-mediated immunity. A similar inverse relationship was observed in earlier studies with chemically modified antigens (8 - 10). At high concentrations of passive antibody, suppression of primary antibody formation was usually accompanied by enhanced delayed-type hypersensitivity. On the other hand, low concentrations of whole serum, \( \gamma_1 \), \( \gamma_2 \) absorbed and IgM enhanced the primary antibody response to flagellin but suppressed the delayed hypersensitivity induced by this antigen. It should be noted, however, that the suppression of delayed-type hypersensitivity was only observed with flagellin, as POL and SRBC failed to induce a detectable delayed response when injected in the absence of passive antibody. However, there was not always a perfect inverse relationship between humoral and cell-mediated
immunity. This was particularly evident in the case of POL where it was observed that substantially lower concentrations of passive antibody were required to enhance delayed hypersensitivity than to suppress primary antibody formation. This result could be partly explained by the fact that antibody titre may not be a true reflection of the number of antibody forming cells. The recent observation by Siskind et al (11) that in the presence of passive antibody, antigens induce higher affinity antibody is consistent with this interpretation.

Earlier works have shown that the presence of small amounts of passive antibody can enhance antibody production (12 - 19). This enhancement was more frequently observed with soluble rather than particulate antigens. However, it has been reported that IgM can enhance the primary antibody response to SRBC (20 - 21). In the present study IgM did not enhance the antibody response to SRBC, although there was an indication that \( \gamma_1 \) could enhance antibody production. This discrepancy is difficult to explain, although earlier works measured the number of antibody forming cells rather than antibody titre, and also used homologous passive antibody in mice rather than heterologous antibody in rats. A further complication is the fact that IgM and IgG antibodies considerably differ in their agglutinating and haemolytic activity, a factor which makes it difficult to compare the relative quantity of the two types of antibody.

A notable feature of this study was the observation
that remarkably low concentrations of passive antibody had an immunological effect. If one assumes that an antibody titre of 1 represents an antibody concentration of approximately 20 ng/ml of IgG (22), then in the case of POL and flagellin (500 ng) a titre of 100 is required to obtain an equimolar ratio between antigen and antibody. However, since flagellar antigens are much more rapidly eliminated from recipient animals than passive antibody (23, 24) the actual antigen to antibody ratio in vivo may be lower than that calculated in vitro. Bearing this factor in mind, it was calculated that the antibody response to flagellin can be enhanced by antigen/antibody ratios ranging from 2.5 - 2500/1. Similar antigen/antibody ratios enhanced the delayed hypersensitivity induced by POL. However, such a calculation could not be obtained with SRBC due to their complex antigenic nature.

The absorption experiments imply that macrophage cytophilic antibody may play an important role in regulating the immune responses to flagellin and SRBC but not to POL. This result suggests that the physical form of an antigen determines the regulatory effect of cytophilic antibody. In addition, these results indicate that the differences in the regulatory properties of various classes of passive antibody are dependent upon their content of macrophage cytophilic antibody. Results from other laboratories also indicate that macrophage cytophilic antibody may play an important immunoregulatory role (25 - 26). The mechanism of action of macrophage cytophilic antibody will be discussed
in detail in the next paper (27) where it will be demonstrated that antigens bound to macrophages via cytophilic antibody have quantitatively altered immunological properties.

5. ZOYBER, E.V., Immunology 4, 474, 1964.
12. NICHOLS, S., Antibiotics and Chemotherapy 18, 92, 1963
REFERENCES


26. PAPER V, THIS THESIS.
"THE EFFECT OF MACROPHAGE CYTOPHILIC ANTIBODY ON HUMORAL AND CELL-MEDIATED IMMUNITY"

The role of macrophage cytophilic antibody in regulating humoral and cell-mediated immunity was investigated by cell-transfer experiments in rats. It was found that flagellin, polymerized flagellin (POL) and sheep red blood cells (SRBC) differed from one another in their immunological behaviour when they were bound to macrophages. Flagellin and SRBC bound to macrophages either nonspecifically or via cytophilic antibody induced higher levels of primary antibody than did free antigens. However, this cell-bound antigen was less effective than free antigen at inducing a primary antibody response. In contrast, POL-bound to macrophages via cytophilic antibody failed to induce delayed-type hypersensitivity, but produced higher levels of primary antibody than did free antigen. Antigens bound to macrophages either nonspecifically or via cytophilic antibody differed somewhat in their immunological properties. However, for any particular antigen, similar immunological effects were induced when the antigen was either complexed to macrophages via cytophilic antibody or injected in the presence of macrophages sensitized with cytophilic antibody. These results are interpreted as indicating that the physical form of an antigen and its presentation in vivo are important factors in determining the type of immune response which occurs.
SUMMARY

The role of macrophage cytophilic antibody in regulating humoral and cell-mediated immunity was investigated by cell-transfer experiments in rats. It was found that flagellin, polymerised flagellin (POL) and sheep red blood cells (SRBC) differed from one another in their immunological behaviour when they were bound to macrophages. Flagellin and SRBC bound to macrophages either in the presence or absence of cytophilic antibody induced higher levels of delayed-type hypersensitivity than did free antigen. However, this cell-bound antigen was less effective than free antigen at inducing a primary antibody response. In contrast, POL bound to macrophages via cytophilic antibody failed to induce delayed-type hypersensitivity, but produced higher levels of primary antibody than did free antigen. Antigens bound to macrophages either nonspecifically or via cytophilic antibody differed somewhat in their immunological properties. However, for any particular antigen, similar immunological effects were induced when the antigen was either complexed to macrophages via cytophilic antibody or injected in the presence of macrophages sensitized with cytophilic antibody. These results are interpreted as indicating that the physical form of an antigen and its presentation in vivo are important factors in determining the type of immune response which occurs.
INTRODUCTION

In the preceding paper of this series (1) evidence was presented which indicated that macrophage cytophilic antibody was involved in regulating the immune response to certain antigens. It was shown that the depletion of macrophage cytophilic antibody from guinea-pigs $\gamma_2$ antibody preparations markedly altered the ability of this class of antibody to influence the immune response to flagellin and sheep red blood cell (SRBC) but not to polymerised flagellin (POL).

In this paper the role of macrophage cytophilic antibody in regulating the immune response was further investigated by cell transfer experiments. It was found that flagellin and SRBC, when bound to macrophages via cytophilic antibody were more effective in inducing delayed hypersensitivity responses, but tended to be less effective at inducing primary humoral antibodies. In contrast, macrophage bound POL failed to induce delayed hypersensitivity but provoked enhanced antibody formation.

These results are interpreted as indicating that the physical form of an antigen and its presentation in vivo are important factors in determining the type of immune response which occurs.
MATERIALS AND METHODS

Animals

Inbred Lewis rats (8-12 weeks of age) and albino guinea-pigs of both sexes (10-16 weeks of age) were used.

Antigens

Sheep red blood cells (SRBC), polymerised flagellin (POL) and flagellin were prepared as described in an earlier paper (2).

Immune sera

Guinea-pig anti-POL and anti-SRBC antisera were prepared as described previously (1).

Iodination

Iodination by direct oxidation of carrier free 125I with chloramine T was carried out according to the method of Greenwood, Hunter and Glover (3) as modified by Ada, Nossal and Pye (4).

Medium

Hanks' balanced salt solution containing 10 units/ml of heparin and 1.5 mM NaN₃ was used.

Peritoneal exudate cells (PEC)

Normal adult Lewis rats were injected intraperitoneally with 5.0 ml of a 0.1% (w/v) oyster glycogen solution. Two days later the animals were anaesthetised with ether,
exsanguinated and the peritoneal cavity flushed with 10 ml of medium. The cells were collected in a prechilled beaker, pooled and washed 3 times with cold medium. The yield was about $3.0 \times 10^7$ cells per rat, and 70 to 75% of the cells were considered to be macrophages as judged by morphology.

Preparation of macrophage-antigen complexes

In a typical experiment, PEC ($8 \times 10^7$) were exposed at room temperature for 1 hr to 4.0 ml of either a guinea-pig antiserum or normal guinea-pig serum. The cells were then cooled to 0-4°C, washed four times with medium and resuspended in 1.0 ml of medium. PEC so treated with antiserum were sensitized with macrophage cytophilic antibody (M-CAb). The extent of sensitization of these cells was determined by their ability to take up $^{125}$I-labelled antigen. Under these reaction conditions most of the cells in the peritoneal exudate that absorbed cytophilic antibody were macrophages as judged by morphology (5).

To obtain macrophage-cytophilic antibody-antigen complexes (M-CAb-antigen), the M-CAb preparations were exposed at 4°C for 4 hours to a predetermined amount of the appropriate $^{125}$I-labelled antigen. Macrophage-antigen complexes (M-antigen) were obtained by reacting normal serum treated macrophages with a predetermined amount of a $^{125}$I-labelled antigen. This reaction was also carried out at 4°C for 4 hours. At the end of the reaction period both the
M-CAb-antigen and the M-antigen complexes were washed 4 times with cold phosphate buffered saline (PBS) and resuspended in 8.0 ml PBS. The amount of cell-bound antigen was determined by measuring the radioactivity of 0.5 ml aliquots with an auto-gamma scintillation counter (Packard Autogamma Spectrometer). The viability of the PEC at the end of the reaction procedure was 85-90%.

**Immunization**

Groups of adult rats (7-8 per group) were injected intraperitoneally with 1.0 ml of the various PEC-antigen preparations. In the cases where antigen not bound to cells was injected, the antigen was mixed with the cell preparations immediately before injection. Rats were bled weekly from the tail following immunisation. Secondary antibody responses and delayed-type hypersensitivity reactions were elicited on day 14 in the SRBC experiment and on day 28 in the POL and flagellin experiments. The eliciting dose of antigen (500 ng of flagellin in 50 µl saline or 5 x 10^7 SRBC in 50 µl saline) was injected into the right hind footpads, and saline alone into the left hind footpads. Footpad thickness was measured at 3 hour (immediate hypersensitivity) and 24 hour (delayed hypersensitivity) after elicitation and specific footpad swelling was determined.

**Assay for hypersensitivity**

Immediate and delayed-type hypersensitivity
reactions were determined by measuring specific footpad swelling as described previously (2). At the dose levels used, flagellin and POL sensitisation induced no significant immediate hypersensitivity compared with control animals. Thus, in experiments with flagellin and POL, footpad measurements were made only 24 hr post challenge.

Estimation of antibody

Anti-SRBC antibody was estimated by haemagglutination. Antibody to flagellar antigens was estimated by a passive haemagglutination method as described in a previous paper (2).

Statistical methods

Standard errors of the means and P values according to the Student's t-test were calculated on an IBM computer.

RESULTS

The binding of antigens to peritoneal exudate cells (PEC) in vitro.

Experiments were carried out to investigate the uptake of antigens by normal and cytophilic antibody sensitized PEC. From these experiments, standard curves were constructed from which the amounts of antigen bound to PEC could be predetermined.
(a) **Flagellin**

Normal rat PEC (1 x 10⁷ cells/reaction mixture) were treated at room temperature for 1 hr with 0.5 ml of either guinea-pig anti-POL antiserum or normal guinea-pig serum. The cells were then cooled and washed four times. Increasing amounts of ¹²⁵I-flagellin were then added to the cells and the contents mixed and left to react at 4°C for 4 hr. At the end of this period the cells were again washed four times and cell associated radioactivity measured.

Fig. 1 shows that the amount of ¹²⁵I-flagellin bound to the cells increased with increasing antigen concentration. However, at any-one antigen concentration cell pretreated with the immune serum absorbed more antigen than did cells pretreated with normal serum. On the other hand, cells treated with normal serum could absorb comparable amounts of antigen as cytophilic antibody sensitized cells, if the cells were reacted with larger amounts of antigen. By referring to the standard curves presented in Fig. 1, the amounts of flagellin bound to PEC in the presence or absence of cytophilic antibody could be predetermined.

(b) **Polymerised flagellin**

The experimental procedure was the same as described in the preceding section for flagellin except that ¹²⁵I-POL was used as antigen. Fig. 2 depicts the binding of ¹²⁵I-POL to normal serum treated PEC (M) and to PEC sensitized with
Fig. 1. The binding of $^{125}$I-flagellin to peritoneal exudate cells pretreated with either normal serum (□) or anti-POL antiserum (■). For experimental details see text. Each point represents the mean of duplicate reactions.
The binding of $^{125}$I-POL to peritoneal exudate cells pretreated with either normal serum (□) or anti-POL antiserum (■). For details see text. Each point represents the mean of duplicate reactions.
cytophilic antibody (M-CAb). It should be noted that at the same antigen concentration, cytophilic antibody sensitized PEC more efficiently absorbed POL than did flagellin (compare Fig. 1 and 2). In contrast, similar amounts of POL and flagellin were nonspecifically bound to normal PEC.

(c) Sheep red blood cells

Due to the particulate nature of SRBC and macrophages, it was found difficult to separate macrophage bound SRBC (rosettes) from unattached SRBC. Although techniques have been developed for separating rosettes from leucocytes and SRBC by virtue of their differences in density and sedimentation velocity, in this paper no attempt was made to utilize these separation techniques to purify rosettes.

The induction of humoral and cell-mediated immunity by macrophage bound antigens

(a) Flagellin

Normal rat PEC were treated with either guinea-pig antiserum or normal guinea-pig serum. A portion of the macrophages sensitized with cytophilic antibody (M-CAb) was exposed to $^{125}$I-flagellin (FIN) to form M-CAb-FIN complexes. Groups of rats (7-8 per group) were injected intraperitoneally with 1.0 ml of one of the following preparations:

Each rat received $1 \times 10^7$ PEC and, with the exception of the control groups (groups 3 and 6), received 42 ng of flagellin, either bound to the cells or unattached to the cells. In the cases where free flagellin was injected (groups 2 and 5) the antigen was mixed with the cell preparations immediately before injection. Secondary antibody responses and delayed hypersensitivity reactions were elicited 28 days later with 500 ng of flagellin in saline. Antibody titres were measured at weekly intervals following primary injection and on days 4, 7 and 14 after secondary challenge.

The antibody responses to flagellin are shown in Fig. 3. At a dose of 42 ng both macrophage bound flagellin and free flagellin provoked low primary antibody responses. Control groups of rats (M-CAb and M) contained no detectable antibody prior to challenge. It should be noted that there was no significant difference ($p > 0.05$) in the primary antibody titres induced by macrophage bound or free flagellin. In contrast, rats primed with the M-CAb-FIN or the M-CAb + FIN preparations gave significantly higher ($p < 0.01$) secondary antibody responses than rats primed with the M-FIN or the M+ FIN preparations.

Fig. 4 presents the delayed hypersensitivity responses in rats injected with the various PEC preparations. Rats which received flagellin with normal PEC (M + FIN) expressed little or no delayed reactivity. However, when flagellin was injected with cytophilic antibody
Fig. 3. The antibody response to flagellin in rats injected intraperitoneally with 1.0 ml of one of the following preparations: M-CAb-FIN (■), M-CAb + FIN (□), M-FIN (▼), M + FIN (▼), M-CAb (●), and M (○). FIN = flagellin, M = peritoneal exudate cells, CAb = cytophilic antibody. Each rat received $1 \times 10^7$ peritoneal exudate cells and, with the exception of the control groups (●, ○), received 42 ng of flagellin, either bound to the cells or unattached to the cells. Secondary antibody responses and delayed-type hypersensitivity reactions were elicited on day 28 with 500 ng of flagellin in saline. Vertical bars represent standard errors of the means, 7 rats per group.
Fig. 4. The induction of cell-mediated immunity by flagellin bound to peritoneal exudate cells. Data obtained from the same experiment as Fig. 3. Cell-mediated immunity is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group.
sensitized PEC (M-CAb + FIN) or in complexes with PEC (M-CAb-FIN and M-FIN), delayed hypersensitivity was induced. It should be noted that the delayed responses induced by the three preparations (M-FIN, M-CAb-FIN and M-CAb + FIN) did not significantly differ.

In a further experiment, the effect of antigen dose on the immune response induced by macrophage bound flagellin was investigated. Three doses of flagellin were employed, namely 15, 42 and 85 ng. For each antigen dose, three different PEC preparations were injected (i.e., M-CAb-FIN, M-FIN and M+FIN). The experimental protocol was identical to that described earlier in this section.

Table 1 summarizes the immune responses induced by the different flagellin preparations. It can be seen that 15 ng of flagellin either free or bound to macrophages did not provoke a detectable primary antibody response but was still capable of priming animals for secondary antibody response. Rats injected with 42 ng of flagellin expressed a weak primary antibody response (titre = 1.6), and similar antibody levels were induced by antigen injected either free or bound to macrophages. A dose of 85 ng of flagellin induced moderate primary antibody titres (titre = 4.5). However, in contrast to the 42 ng dose, the primary antibody titres induced by 85 ng of free flagellin were significantly higher than those induced by 85 ng of macrophage bound flagellin. At all three doses of flagellin, flagellin bound to macrophages via cytophilic antibody induced the strongest immunological memory.
<table>
<thead>
<tr>
<th>PREPARATION OF ANTIGEN</th>
<th>ANTIGEN DOSE (ng)</th>
<th>DELAYED HYPERSENSITIVITY b</th>
<th>PRIMARY ANTIBODY c</th>
<th>SECONDARY ANTIBODY d</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-C.Ab-FIN a</td>
<td>15</td>
<td>2.6 ± 0.2</td>
<td>&lt;0.5</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>M - FIN</td>
<td>15</td>
<td>2.0 ± 0.2</td>
<td>&lt;0.5</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td>M + FIN</td>
<td>15</td>
<td>0.5 ± 0.2</td>
<td>&lt;0.5</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>M-C.Ab-FIN</td>
<td>42 e</td>
<td>2.0 ± 0.2</td>
<td>2.1 ± 0.5</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>M - FIN</td>
<td>42</td>
<td>1.9 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>M + FIN</td>
<td>42</td>
<td>0.4 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>M-C.Ab-FIN</td>
<td>85</td>
<td>2.7 ± 0.2</td>
<td>2.2 ± 0.5</td>
<td>11.7 ± 0.6</td>
</tr>
<tr>
<td>M - FIN</td>
<td>85</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.6</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>M + FIN</td>
<td>85</td>
<td>0.3 ± 0.2</td>
<td>4.5 ± 0.5</td>
<td>8.5 ± 0.8</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
<td>0.5 ± 0.1</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

a. M = peritoneal exudate cells, C.Ab = cytophilic antibody, FIN = flagellin.
b. 24 hr footpad swelling in 1/10th mm.
c. 28 day primary antibody titres, log₂ scale, 1 = 1/10 dilution. Each figure represents the arithmatic mean of 7-8 rats ± standard error of the mean.
d. 7 day post challenge titres.
e. Data for 42 ng antigen dose derived from Fig. 3.
From Table 1 it also appeared that macrophage bound flagellin, either in the presence or absence of cytophilic antibody, was more effective than free flagellin at inducing delayed-type hypersensitivity. As little as 15 ng of macrophage bound flagellin was able to induce delayed responsiveness, whereas the unattached flagellin failed to induce detectable delayed hypersensitivity even at a dose of 85 ng. It should be noted that all three doses of macrophage bound flagellin induced comparable levels of delayed hypersensitivity.

At a dose of 85 ng, it was observed that macrophage bound flagellin induced a significantly lower primary antibody response than free flagellin. This result suggested that cytophilic antibody may suppress the primary antibody response to flagellin. To further investigate this possibility, rats were injected with $2 \times 10^7$ PEC sensitized with cytophilic antibody and at the same time were injected with 120 ng of flagellin.

The antibody responses in this experiment are presented in Fig. 5. It can be seen that animals treated with normal PEC gave a significant primary antibody response to flagellin. In contrast, animals which received PEC sensitized with cytophilic antibody expressed little or no primary antibodies. This result was consistent with the suggestion that cytophilic antibody can actively suppress the primary antibody response to flagellin (1). However, flagellin induced comparable immunological memory when injected into animals which had received either normal PEC
Fig. 5. The induction of antibody production by flagellin injected with peritoneal exudate cells (PEC) pretreated with either normal serum (■) or anti-POL antiserum (□). Each rat was injected intraperitoneally with \(2 \times 10^7\) PEC and 120 ng of flagellin in saline. All animals were challenged on day 28 with 500 ng of flagellin in saline. Vertical bars represent standard errors of the means, 8 rats per group.
or cytophilic antibody treated PEC (Fig. 5). It was also found that animals injected with flagellin and normal PEC failed to exhibit significant delayed-type hypersensitivity (footpad swelling = 0.2 ± 0.1), whereas animals injected with flagellin and cytophilic antibody treated PEC expressed significant delayed responsiveness (footpad swelling = 1.9 ± 0.2).

(b) Polymerised flagellin

The experimental design was the same as described in the preceding section except that in this case 120 ng of POL was used. Fig. 6 presents the antibody responses induced by the different PEC-POL preparations. The ability of the different preparations to induce a primary antibody response was in the following order: M - CAb - POL > M - CAb + POL > M + POL > M - POL. Thus, in contrast to flagellin, cytophilic antibody for macrophages enhances the primary antibody response to POL (compare Fig. 5 and 6). However, there was no significant difference in the ability of the different preparations of POL to induce immunological memory. Unlike the case with flagellin, none of the POL preparations induced significant delayed-type hypersensitivity (Table 2).

(c) Sheep red blood cell

Due to the difficulty in obtaining macrophage-SRBC rosettes, the experimental design with SRBC was different from that of the preceding sections. Normal rat PEC
Fig. 6. The induction of antibody formation by polymerised flagellin (POL) bound to peritoneal exudate cells (M). Groups of rats were injected intraperitoneally with 1.0 ml of one of following preparations: M-CAb-POL ( ■ ), M-CAb + POL ( □ ), M-POL ( ▽ ), M + POL ( ▼ ), M-CAb ( ● ) and M ( ○ ). M-CAb = peritoneal exudate cells sensitized with cytophilic antibody. Each rat received 1 x10⁷ cells and, with the exception of control groups ( ● , ○ ), received 120 ng of POL, either bound to the cells or unattached to the cells. Secondary antibody responses were elicited on day 28 with 500 ng of flagellin in saline. Vertical bars represent standard errors of the means, 7 rats per group.
TABLE 2

THE INDUCTION OF CELL-MEDIATED IMMUNITY BY POLYMERIZED FLAGELLIN BOUND TO PERITONEAL EXUDATE CELLS

<table>
<thead>
<tr>
<th>PREPARATION OF ANTIGEN</th>
<th>ANTIGEN DOSE (ng)</th>
<th>24 HR FOOTPAD SWELLING (1/10th MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-C.Ab-POL b</td>
<td>120</td>
<td>1.1 ± 0.3c</td>
</tr>
<tr>
<td>M-C.Ab+POL</td>
<td>120</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>M-C.Ab</td>
<td>-</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>M-POL</td>
<td>120</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>M+POL</td>
<td>120</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

Rats were injected intraperitoneally with 1.0 ml of the various cell and antigen preparations. Each animal was tested for cell-mediated immunity to flagellin on day 28 as described in the Materials and Methods.

b M = peritoneal exudate cells (1 x 10^7 cells/ml).
C.Ab = Cytophilic antibody
POL = Polymerized flagellin

c Each figure represents the arithmetic mean of 7-8 rats ± standard error of the mean.
were treated with either guinea-pig anti-SRBC antiserum or normal guinea-pig serum to form M - CAb and M respectively. Groups of rats (6 per group) were injected intraperitoneally with 1.0 ml of a solution containing 5 x 10^7 SRBC and numbers of M - CAb ranging from 1 x 10^7 to 5 x 10^7. Two groups of control rats were used. One group received 5 x 10^7 normal serum treated PEC (M) and 5 x 10^7 SRBC, whereas the other control group received no injection. Fourteen days later, hypersensitivity and secondary antibody responses were elicited by injecting 5 x 10^7 SRBC in saline. Serum antibody levels were measured at weekly intervals following primary immunisation.

The antibody responses are presented in Fig. 7. It was found that the primary antibody response to SRBC was suppressed by M - CAb and the suppression increased with increasing amounts of M - CAb. In contrast to the primary antibody response, however, the antibody response to a second dose of SRBC was not affected by the M - CAb treatment.

Fig. 8 depicts the effects of M - CAb on the immediate and delayed hypersensitivity reactions induced by SRBC. As was the situation with the primary antibody response, increasing amounts of M - CAb steadily decreased the immediate response to SRBC. In contrast, the delayed-type hypersensitivity to SRBC was enhanced by M - CAb. Furthermore, the three different doses of M - CAb injected induced similar levels of delayed responsiveness.
Fig. 7. The induction of antibody formation by $5 \times 10^7$ SRBC injected intraperitoneally with one of the following preparations of peritoneal exudate cells (M): $5 \times 10^7$ M (■), $1 \times 10^7$ M-CAb (□), $2.5 \times 10^7$ M-CAb (●), $5 \times 10^7$ M-CAb (○). M-CAb = peritoneal exudate cells sensitized with cytophilic antibody. Control rats (▼) received no injection on day 0. All animals were challenged in the hind footpads with $5 \times 10^7$ SRBC on day 14. Vertical bars represent standard errors of the means, 6 rats per group.
Fig. 8. The induction of immediate and delayed hypersensitivity responses by SRBC injected with peritoneal exudate cells pretreated with either normal serum (M) or anti-SRBC antiserum (M-Cab). Data obtained from the same experiment as Fig. 7. Immediate hypersensitivity (open column) is expressed as 3 hr footpad swelling, and delayed hypersensitivity (hatched column) is expressed as 24 hr footpad swelling. Vertical bars represent standard errors of the means, 7 rats per group.
DISCUSSION

In this paper the role of macrophage cytophilic antibody in regulating humoral and cell-mediated immunity was investigated. The experimental results are summarised in Table 3 from which the following observations were made and conclusions drawn:

1. Antigens differed from one another in their immunological behaviour when they were injected bound to macrophages.

2. For any particular antigen similar immunological effects were induced when the antigen was either complexed to macrophages via cytophilic antibody or injected in the presence of macrophages sensitized with cytophilic antibody.

3. Flagellin and SRBC bound to macrophages either in the presence or absence of cytophilic antibody induced higher levels of delayed hypersensitivity than free antigen. However, this cell-bound antigen was less effective than free antigen at inducing a primary antibody response.

4. In contrast to flagellin and SRBC, POL bound to macrophages via cytophilic antibody failed to induce delayed hypersensitivity, but caused higher levels of primary antibody than did free antigen.

5. Antigens bound to macrophages either non-specifically or via cytophilic antibody differed somewhat in their immunological properties.

6. Remarkably low doses (15 ng) of flagellin bound to macrophages could induce significant levels of
TABLE 3

COMPARISON OF THE EFFECTS OF MACROPHAGE CYTOPHILIC ANTIBODY ON THE IMMUNE RESPONSES TO DIFFERENT ANTIGENS

<table>
<thead>
<tr>
<th>PREPARATION OF ANTIGEN</th>
<th>ANTIGEN</th>
<th>DELAYED HYPERSENSITIVITY</th>
<th>PRIMARY ANTIBODY</th>
<th>SECONDARY ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-C.Ab-Ag</td>
<td>flagellin</td>
<td>++</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>t</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M-C.Ab+Ag</td>
<td>flagellin</td>
<td>++</td>
<td>±</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>t</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M-Ag</td>
<td>flagellin</td>
<td>++</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>t</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M+Ag</td>
<td>flagellin</td>
<td>t</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>t</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>SRBC</td>
<td>t</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

M = peritoneal exudate cell, C.Ab = cytophilic antibody, Ag = antigen, POL = polymerized flagellin, SRBC = sheep red blood cells.

± = trace, + = moderate, ++ = high, +++ = very high.

ND = not determined.
delayed hypersensitivity.

Many workers have studied the ability of antigens associated with macrophages to induce immune responses (6 - 11). These studies revealed that usually macrophage bound antigen induced variable quantities of primary antibody, but primed animals for a secondary antibody response more efficiently than did equivalent amounts of free antigen. However, the results obtained were highly dependent upon the type of antigen used (6,7,11); in fact, Mitchison (7) has placed antigens in a hierarchy according to the immunogenicity of macrophage bound antigen relative to the free form. In contrast, our results suggest that a difference in physical size rather than a difference in antigenic specificity (and immunogenicity) is an important factor in determining the type of immune response which occurs. For example, monomeric flagellin and polymerised flagellin (POL) when injected bound to macrophages differ markedly from one another in their immunological properties, even though they are antigenically almost identical.

The immunogenicity of antigen carried by macrophages could be attributed either to surface-bound antigen or to internally held antigen which is gradually released over a considerable period of time. Evidence has been reported supporting both possibilities (10,11). It should be noted, however, that in all these studies antigens have been reacted with macrophages in the absence of cytophilic antibody.
Results presented in this paper indicate that antigen bound to macrophages via cytophilic antibody differs somewhat in its immunogenicity from antigen nonspecifically bound to macrophages. This phenomenon was apparent under two different experimental conditions. Firstly, flagellin bound to macrophages via cytophilic antibody (M-CAb-FIN) more effectively primed animals for a secondary antibody response than the same dose of nonspecifically bound antigen (M-FIN) (Fig. 3). Secondly, POL bound to macrophages via cytophilic antibody (M-CAb-POL) was more efficient at inducing a primary antibody response than the same dose of nonspecifically bound POL (M-POL) (Fig. 6). It has already been reported that cytophilic antibody differs from opsonins (12,13). In fact, Boyden and his colleagues (Dr. S.V. Boyden, personal communication) observed that SRBC attached to macrophages via cytophilic antibody were not phagocytosed by the macrophages even when the rosettes were incubated at 37° in the presence of foetal calf serum. Preliminary electron microscopic studies in this laboratory have confirmed this observation, and have also demonstrated that, in contrast to cytophilic antibody, SRBC treated with complete anti-SRBC antiserum were readily phagocytosed. It is therefore conceivable that antigen bound by cytophilic antibody is presented on the surface of macrophages more efficiently than nonspecifically bound antigen.

The effect of macrophage-bound antigen on the induction of delayed-type hypersensitivity has not been fully explored. Recently, Unanue and Feldman (14)
demonstrated that when heat aggregated human serum albumin (HSA) was injected bound to macrophages, it induced a state of delayed hypersensitivity equivalent to that provoked by the same dose of free antigen. Furthermore, it was observed that primary antibody formation was more efficiently induced by macrophage-bound antigen. This immunological activity is similar to POL, an antigen which provokes enhanced antibody formation but little or no delayed hypersensitivity when bound to macrophages. It is not surprising that this similarity occurred as both polymerised flagellin and heat aggregated HSA are particulate antigens.

There are several reports in the literature suggesting that macrophage cytophilic antibody may play an important role in the passive antibody-mediated suppression of antibody formation. The most direct evidence that macrophage cytophilic antibody plays a regulatory role was presented by Ivanyi (15), who reported that a passive antiserum lost its immuno-suppressive activity after repeated absorption with spleen cells. In addition, the removal of the Fc fragment from antibody drastically reduced its inhibitory effect (16). More indirect evidence is the observation by several workers (17,18) that pre-exposure of spleen cells to antibody can markedly suppress the subsequent antibody response to sheep erythrocytes.

In conclusion, a hypothesis will be proposed which attempts to explain antibody-mediated regulation of the immune response. The hypothesis proposes that: (a) Antigen mediated interaction of thymus-derived T cells and bursa-
equivalent B cells is essential for the induction of antibody formation, but suppresses the expression of cell-mediated immunity. (b) Separate interaction of B and T cells with antigen fails to provoke antibody formation, but results in the stimulation of T cells to proliferate and express cell-mediated immunity (19). Based on this proposal, it would be predicted that presentation of antigen in a form which promotes cell-cell interaction would enhance antibody formation. By contrast, antigen presented in a state favouring separate interaction with immunocompetent cells would augment cell-mediated immunity.

The following observations presented in this paper and in two earlier papers (1,2) can be accommodated by these predictions as follows:

1. In the presence of very high concentrations of passive antibody, where masking of most antigenic determinants would be anticipated, antigen induced low levels of both humoral and cell-mediated immunity.

2. With high concentrations of all classes of passive antibody, suppression of antibody formation was accompanied by enhanced delayed hypersensitivity, a phenomenon which was found to be antigen independent. Such a phenomenon is consistent with the notion that high concentrations of passive antibody reduce the number of exposed antigenic determinants on antigens, thereby lowering the probability of antigen-mediated cell-cell interaction (i.e., antibody production) and thus favouring the separate interaction of antigen with immunocompetent cells (i.e.,
induction of cell-mediated immunity).

3. The immunological effect of very low concentrations of passive antibody appeared to be highly dependent upon the physical state of the antigen. Thus, in the case of soluble antigens such as flagellin, low concentrations of passive antibody enhanced antibody formation but suppressed delayed hypersensitivity. This result is not surprising as it would be anticipated that low concentrations of antibody would aggregate flagellin and present it in a multi-determinant form which more efficiently induced cell-cell interaction (i.e., antibody formation). On the other hand, low concentrations of passive antibody did not modify the immune response to POL or SRBC. Since both these antigens already exist in a particulate, repeating determinant form, further aggregation would be expected to have little or no effect on their immunogenicity.

4. In contrast to free antibody, cell-bound antibody (i.e., cytophilic antibody) produced markedly different immunological effects. These effects were again highly dependent upon the physical state of the antigen. Fig. 9 schematically depicts the manner by which flagellin, POL and SRBC may be presented to immunocompetent cells when bound to the surface of macrophages. It can be seen from this model that flagellin bound to macrophages would be incapable of mediating cell-cell interaction (Fig. 9a). Conversely, polymerised flagellin (POL) due to its filamentous nature, would be presented in a form which
Fig. 9. Schematic models for the presentation of macrophage bound antigens to immunocompetent cells. For explanation see the text.
could still promote cell-cell interaction (Fig. 9b). The fact that POL bound to macrophages induces an enhanced primary antibody response is probably not due to its presentation in a form which more efficiently favours cell-cell interaction, but because it is retained more effectively in the lymphoid system. In fact, it would be anticipated that a portion of each POL filament would be bound to the surface of macrophages in a form which would not favour cell-cell interaction. However, since POL exists as long filaments (0.5 - 3.5 μ in length) (20) a substantial portion of each filament would probably protrude from the surface of cells.

If intimate contact between immunocompetent cells is essential for the induction of antibody formation, then it would be anticipated that intact SRBC, due to their size, may be incapable of mediating such contact. This interpretation implies that intact SRBC bound to macrophages would promote cell-mediated immunity rather than antibody formation (see Fig. 9c). The fact that SRBC must be degraded by macrophages before they are capable of inducing antibody formation is consistent with this interpretation (21).

An alternative interpretation of the data presented in this paper is that macrophage associated antigen is released in a degraded form which favours cell-mediated immunity (22,23). This explanation is unlikely in view of the fact that macrophage-bound POL induces heightened levels of primary antibody. The same argument can also be directed
against the proposal by Fishman and others (24,25) that macrophages "process" antigen.

Finally, it should be emphasized that minute amounts of macrophage bound antigen would be expected to be immunologically active due to the highly efficient localisation of this antigen in lymphoid tissue (26).
REFERENCES

1. Paper IV, this thesis.


PART C

CONCLUDING DISCUSSION
I. INTRODUCTION

The experimental work reported in this thesis can be conveniently divided into three sections. The first section (paper I) dealt with the production of macrophage cytophilic antibody. In this section, a systematic study of the parameters affecting the production of macrophage cytophilic antibody to different antigens in different laboratory animals was attempted. The second section (paper II) investigated the binding of cytophilic antibody to macrophages. In the last section (papers III-V), the possible role of macrophage cytophilic antibody in the immune response was explored. The possible significance of the findings has been discussed at the end of each paper. In this concluding section, the results are summarised and some further interpretations discussed.

II. THE PRODUCTION OF MACROPHAGE CYTOPHILIC ANTIBODY

The experiments described in paper I showed that antigens injected in either Freund's complete or incomplete adjuvant were almost equally efficient in inducing cytophilic antibody production. In contrast, antigens injected in saline failed to induce detectable cytophilic antibody. These conclusions were drawn from experiments with four different laboratory animals (guinea-pig, rabbit, mouse, rat) and with five different antigens (SRBC, BSA, polymerized flagellin, flagellin and CNBr digested flagellin). In each species, cytophilic antibody was only detected in the 7S fraction of hyperimmune sera.
The reason why injection in saline fails to induce cytophilic antibody is obscure. Perhaps a class of lymphocytes is involved which needs the additional stimulation caused by adjuvants. The recent finding by Alison et al (1) that T cells are preferentially stimulated by antigen injected in adjuvant, is consistent with this interpretation.

A comparison of the passive haemagglutinating antibody and cytophilic antibody titres produced following injection of the different forms of flagellar antigens, shows one interesting finding. The CNBr digest of flagellin was as good as polymerised flagellin (POL) or flagellin in inducing the production of cytophilic antibody, but it was generally less effective than either POL or flagellin in inducing haemagglutinating antibody. It may be that the major factor necessary for the production of cytophilic antibody is the use of adjuvant and the form of the antigen is less important, whereas the latter aspect is still of major importance in determining the total amount of passive haemagglutinating antibody formed.

Experiments reported in this thesis were mainly concerned with macrophage cytophilic antibody. No attempt was made to study the nature of cytophilic antibodies for other cell types. In mice, it was found that the majority of the lymphocyte cytophilic antibodies belonged to the $\gamma_1$ IgG class (2). It would be interesting to know if the factors affecting the production of lymphocyte cytophilic antibody are in fact different from those affecting
the production of macrophage cytophilic antibody.

III. THE BINDING OF CYTOPHILIC ANTIBODY TO MACROPHAGES

Earlier workers have demonstrated that both guinea-pig cytophilic antibody (3) and mouse cytophilic antibody (4), isolated from hyperimmune sera, lose their ability to adhere to macrophages after pepsin treatment. Since pepsin is known to destroy the Fc portion of immunoglobulin heavy chains, it was inferred that the cytophilic activity was a function of the Fc portion of the IgG molecule. In paper II, using a radioactivity assay and radioautography, it was demonstrated directly that the cytophilic activity is present in the Fc portion of the molecule. Furthermore, this Fc fragment is as active, on a molar basis, as the intact $\gamma_2$ IgG molecule. These findings, taken together with the demonstration that the Fab fragment of the molecule has no detectable cytophilic activity, show that the cytophilic activity of the intact molecule is a function solely of the Fc portion of the molecule.

Whether association with antigens affects the cytophilic activity of an IgG molecule is not clear at present, because it is difficult to devise a satisfactory method for clearly differentiating opsonising and cytophilic activity. Using the indirect test, it was found that the specific antibody preparation (purified by the immunoadsorption method) had a 10 times higher cytophilic antibody to haemagglutinating antibody titres ratio compared
with the whole serum, from which the specific antibody was isolated (5). This is consistent with the notion that cytophilic antibody, which has had a previous association with antigen, has an augmented affinity for macrophages, a situation not unlike that of opsonising antibody. However, it could also be argued that the apparent lower cytophilic antibody to haemagglutinating antibody titres ratio in the whole serum was due to the presence of cytophilic antibody directed against other antigens. These cytophilic antibodies with other specificities would be expected to compete with the specific cytophilic antibody for the receptors on macrophages.

There are certain similarities between opsonic adherence and cytophilic antibody mediated absorption of antigen to macrophages. For example, both reactions result in the adherence of an antigen-antibody complex to macrophage membrane. In addition, both cytophilic and opsonic adherence reactions can be mediated by IgG globulins which attach to macrophage receptors by binding sites on the Fc portion of the immunoglobulin molecules (3,6,7).

However, previous investigators (4,8) have demonstrated that the adherence of antigens to mouse macrophages, mediated by macrophage cytophilic antibody is not identical to the adherence mediated by opsonising antibody. The differences which have been demonstrated included the immunoglobulin classes involved, the cells on to which
absorption takes place, and the factors affecting this absorption. Macrophage cytophilic antibody absorbs on to macrophages by its Fc piece in the absence of antigen, whereas opsonising antibody does not attach to macrophages in the absence of antigen.

The important question, however, is whether there is a functional difference between opsonising antibody and macrophage cytophilic antibody. Berken and Benacerraf (3) reported that SRBC attached to macrophages via cytophilic antibody were phagocytosed by the macrophages, if the rosettes were incubated at 37°. By contrast, Boyden and his colleagues (9) were unsuccessful in their attempts to induce phagocytosis of SRBC attached to macrophages sensitized with cytophilic antibody. The discrepancy between these two observations can partly be due to the uncertainty of the phagocytosis score, as, under the light microscope, it often proves rather difficult to determine whether a sheep erythrocyte is intracellular or extracellular. Preliminary electron-microscopic studies carried out in this laboratory have confirmed Boyden's observation, and have also demonstrated that, in contrast to cytophilic antibody, SRBC treated with complete anti-SRBC antiserum were readily phagocytosed. Electron microscopic radioautography studies are in progress to investigate the localising pattern of soluble antigens associated with macrophages sensitized with cytophilic antibody. These studies would also determine whether the receptors on the macrophages are 'patchy' or randomly distributed.
IV. THE ROLE OF MACROPHAGE CYTOPHILIC ANTIBODY IN THE IMMUNE RESPONSE

A major aspect of the work reported in this thesis has been concerned with the possible role of macrophage cytophilic antibody in the immune response. The approach has been to investigate the part played by macrophage cytophilic antibody in the regulation of the immune response by passive antibody. This investigation was carried out in three stages.

1. In paper III, the general effects of passive antibody on the immune response were reported. It was found that, for a certain range of concentrations of passive antibody, the suppression of antibody production was accompanied by enhanced delayed-type hypersensitivity. This phenomenon was observed with soluble (flagellin), particulate (polymerised flagellin) and cellular (SRBC) antigens and was also obtained with both homologous and heterologous passive antibodies.

2. In paper IV, the ability of different classes of passive antibody (\(\gamma_1, \gamma_2, \text{IgM}\)) to regulate humoral and cell-mediated immunity to flagellin, POL and SRBC was investigated. It was found that different classes of passive antibody varied in their ability to influence the immunological response to flagellin and SRBC. This variation was especially marked between \(\gamma_1\) and \(\gamma_2\) antibodies. However, removal from \(\gamma_2\) preparation of macrophage cytophilic activity converted this antibody to a preparation (\(\gamma_2\) absorbed) which demonstrated that an inverse relationship between humoral
had an effect on humoral and cell-mediated immunity approaching that of $\gamma_1$. This is the first strong indication that macrophage cytophilic antibody is involved in the regulation of the immune response.

3. In paper V, the role of macrophage cytophilic antibody in regulating the immune response was further investigated by cell transfer experiments. It was found that flagellin and SRBC, when bound to macrophages via cytophilic antibody were more effective than unattached SRBC in inducing delayed-type hypersensitivity, but tended to be less effective in inducing primary antibody responses. In contrast, macrophage-bound POL failed to induce delayed hypersensitivity but provoked enhanced antibody formation.

These studies (papers III-V) shed some light on the mechanism of the regulation of the immune response by passive antibody. The studies also emphasised that the physical form of an antigen and its presentation in vivo are important factors in determining the type of immune response which occurs. The role of macrophage cytophilic antibody in the immune response appears to be primarily concerned with the presentation of antigens to immunocompetent cells.

The mechanism of the regulation of the immune response by passive antibody and the possible role of macrophage cytophilic antibody in this aspect has been discussed in detail in paper V.

In a number of experimental systems it has now been demonstrated that an inverse relationship between humoral
and cell-mediated immunity exists (10-13). It is well known that humoral and cell-mediated immunity play different roles in protecting animals against diseases (14-20). Thus, it becomes highly desirable that for adequate disease resistance, the levels of both types of immunity be carefully regulated. By virtue of their capacity to suppress and enhance humoral and cell-mediated immunity, antibodies and, in some cases, macrophage cytophilic antibodies represent an important regulatory mechanism.

V. CONCLUSIONS

In the introductory review (part A) to this thesis, four major problems in the study of cytophilic antibody were cited. Partial answers to these problems are now available. The answered and the unanswered problems are listed as follows:

1. The production of macrophage cytophilic antibody

The incorporation of antigen in Freund's adjuvants (complete or incomplete) has been found to be necessary for the effective production of macrophage cytophilic antibody in all four species of animals tested. A second injection of antigen in saline is usually followed by a sharp rise in the level of macrophage cytophilic antibody in the serum. However, the factors affecting the production of cytophilic antibodies for other cell types are yet to be determined.
2. The mechanism of binding of cytophilic antibody to cells

Experiments described in paper II demonstrated that the Fc portion of the IgG molecule is responsible for the binding of cytophilic antibody to macrophages, and that the Fab fragment does not have affinity for macrophages. However, the experiments described in paper II offer no clue to the detailed mechanism by which the Fc fragment attaches to macrophages. The problems such as the chemical grouping involved in the binding and the distribution of the receptors for cytophilic antibody on macrophages (and other cell types) require further investigation.

3. The role of macrophage cytophilic antibody in delayed-type hypersensitivity

At the dose levels studied flagellin and SRBC bound to macrophages are capable of inducing higher levels of delayed hypersensitivity than free antigen. Since macrophage cytophilic antibody promotes the attachment of antigens to macrophages, thus macrophage cytophilic antibody indirectly contributes to the induction of delayed-type hypersensitivity. This phenomenon, however, was not observed for particulate antigens such as polymerised flagellin (POL).

4. The role of macrophage cytophilic antibody in the antibody mediated regulation of the immune response

The experiments described in paper IV strongly suggested that macrophage cytophilic antibody played an important part in the antibody mediated regulation of the
immune response to soluble and cellular antigens. The role of macrophage cytophilic antibody in the regulation of the immune response appears to be the presentation of antigens to immunocompetent cells (paper V).
REFERENCES

2. BASTON, A., and WARNER, N.L., Personal communication.
5. LIEW, F.Y., Unpublished results.
9. BOYDEN, S.V., Personal communication.
11. Appendix, this thesis.


PART D
PAPER VI

APPENDIX

"ENHANCED CELL-MEDIATED IMMUNITY DURING HIGH AND
LOW DOSE ANTIBODY TOLERANCE TO FLAGELIN"
PAPER VI

"ENHANCED CELL-MEDIATED IMMUNITY DURING HIGH AND LOW ZONE ANTIBODY TOLERANCE TO FLAGELLIN"

High zone and low zone antibody tolerance to bacterial flagellin can be induced in adult strain W Wistar rats by multiple injections of CMBr digest of flagellin at regularly spaced dose levels. Intermediate doses of the CMBr digest produced enhanced antibody titres to flagellin rather than antibody tolerance. Studies reported in this paper revealed that both high and low zone antibody tolerance to flagellin were present and active at the cellular level and that an inverse relationship between tolerance and antibody responses was observed. This inverse relationship between humoral and cell-mediated immunity was very striking in strain W Wistar rats but was not quite as clear cut in another strain of Wistar rats (strain J). Strain J rats were resistant to the induction of antibody tolerance and gave higher immunological responses to flagellin than strain W animals. In addition, it was observed that, in contrast to adult tolerance, administration of the CMBr digest to neonatal rats induced complete tolerance at the level of both humoral and cell-mediated immunity.
High zone and low zone antibody tolerance to bacterial flagellin can be induced in adult strain W Wistar rats by multiple injections of a cyanogen bromide (CNBr) digest of flagellin at two widely spaced dose levels. Intermediate doses of the CNBr digest produce enhanced antibody titres to flagellin rather than antibody tolerance. Studies reported in this paper revealed that both high and low zone antibody tolerance to flagellin were accompanied by heightened levels of delayed-type hypersensitivity. Conversely, when enhancement of the antibody response occurred suppression of delayed hypersensitivity was observed.

This inverse relationship between humoral and cell-mediated immunity was very striking in strain W Wistar rats but was not quite so clear cut in another strain of Wistar rats (strain J). Strain J rats were resistant to the induction of antibody tolerance and gave higher immunological responses to flagellin than strain W animals. In addition, it was observed that, in contrast to adult tolerance, administration of the CNBr digest to neonatal rats induced complete tolerance at the level of both humoral and cell-mediated immunity.
INTRODUCTION

Recent studies have indicated that humoral and cell-mediated immunity may be intimately related (1-3). Using a series of acetoacetylated derivatives of flagellin it was observed that increasing acetoacetylation steadily destroyed the ability of flagellin to initiate antibody formation but enhanced the capacity of the molecule to induce flagellin-specific delayed-type hypersensitivity and antibody tolerance. Thus, in this system antibody tolerance in adult rats was accompanied by enhanced cell-mediated immunity. If this relationship represents a general phenomenon then it would be predicted that all states of antibody tolerance in adult animals should be accompanied by enhanced cell-mediated immunity. Using bacterial flagellin as the antigen, studies were initiated to test this prediction.

It has been previously reported that antibody tolerance to flagellin can be induced in adult rats by multiple injections of a cyanogen bromide (CNBr) digest of flagellin at two widely spaced dose levels (4). This phenomenon is called high and low zone antibody tolerance (5). Intermediate doses of the CNBr digest resulted in very high antibody titres rather than in antibody tolerance. Data presented in this paper reveals that, as predicted, both high and low zone antibody tolerance to flagellin were associated with heightened levels of delayed-type hypersensitivity. Furthermore, when enhancement of the antibody response occurred, suppression of delayed-type
hypersensitivity was observed. Thus, an inverse relationship appears to exist between humoral and cell-mediated immunity. However, this relationship is not invariably true as rats from another strain exhibited only a partial inverse relationship. In addition, it was confirmed (3) that administration of antigen to neonatal rats induced tolerance at the level of both humoral and cell-mediated immunity.

MATERIALS AND METHODS

Animals

Outbred Wistar rats were obtained from two separate colonies and according to their source were termed strain W or strain J Wistar rats. Strain W Wistar rats were either obtained directly from the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, or bred in Canberra from Wistar breeders supplied by the Walter and Eliza Hall Institute. Strain W rats bred in either Parkville or Canberra expressed similar immune responsiveness. Strain J Wistar rats were obtained from the Wistar rat colony present at the John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. In studies with both strains, adult (6-10 weeks of age) Wistar rats of either sex were used. In the case of strain J Wistar rats both adult and neonatal rats were used.
Antigens

Flagellin from Salmonella adelaide (strain SW 1338; H antigen - fg) was prepared as described previously (6). A cyanogen bromide (CNBr) digest of flagellin was prepared as described elsewhere (7). A stock solution of 1 mg/ml was prepared and to avoid antigen losses by adsorption ten fold dilution steps were made in saline containing 0.1% gelatin. Acetoacetylated flagellin (16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) was obtained by a previously described method (1, 2).

Antigens were injected in saline either i.p. (0.1 ml), intradermally into the flanks (50 µl at two separate sites), i.v. into a lateral tail vein (0.5 ml) or subcutaneously into the hind footpads (50 µl per footpad). In some experiments, flagellin was emulsified in Freund's complete adjuvant (FCA), in the ratio of 3 volumes of antigen to one volume of adjuvant, and injected intradermally (50 µl per site).

Antibody Estimations

Antibody to flagellin was estimated by haemagglutination using sheep erythrocytes sensitized with S. adelaide polymerized flagellin by a chromic chloride procedure (8).

Assay for Delayed-Type Hypersensitivity

Hypersensitivity reactions were determined by measuring the increase in footpad thickness following the injection of antigen in saline into the hind footpads.
Flagellin specific reactions were elicited by injecting 0.5-100 µg (50 µl) of flagellin in saline into the right hind footpads and saline (50 µl) alone into the left hind footpads. Footpad thickness was measured at 3, 6, 24 and 48 hr following challenge and specific footpad swelling was determined by subtracting the thickness of the left hind footpad from the right. Footpad thickness was measured by a dial calliper gauge A02T (Schnelltaster, System Kroplin) which had 0.1 mm graduations. Compared with control animals no significant immediate-type hypersensitivity (3 hr footpad swelling) was observed in any of the experiments reported in this paper. It was also found that all the delayed responses detected, although still being prominent at 48 hr, peaked at the 24 hr time point. Thus, only 24 hr footpad swellings are reported in the figures and tables.

It was found that the hind feet of adult rats ranged in thickness from 4 to 5 mm. Footpad thicknesses were measured to the nearest 0.05 of a mm. Repeated measurements (6-10 times) of the same footpad gave standard errors ranging from ± 0.01 to ± 0.03 mm. The left and right hind feet of normal rats had very similar thicknesses (± 0.05 mm).

**Statistical Methods**

Standard errors of the means and P values were calculated using the Student's t-test.
RESULTS

Humoral and Cell-Mediated Immune Responses Induced in Adult Wistar Rats by Different Doses Given Daily of a Cyanogen Bromide Digest of Flagellin

Experiments were carried out to determine the ability of different doses of CNBr digested flagellin to induce (a) antibody production to flagellin, (b) delayed-type hypersensitivity to flagellin, and (c) immunological tolerance to flagellin both at the humoral and cell-mediated level. These determinations were made in two separate strains of Wistar rats (strains W and J).

The experimental design is presented in Fig. 1. Adult rats were injected intraperitoneally daily for 27 days with different doses of a CNBr digest of flagellin. On day 28, animals were bled and their antibody titres estimated. Also, at this time point, the levels of delayed-type hypersensitivity induced by the different doses of the CNBr digest were determined by eliciting rats with 100 µg of flagellin in saline into the right hind footpads (see Materials and Methods). This high dose (100 µg) of flagellin was used, as not only did it efficiently elicit delayed hypersensitivity, but it also provoked a good antibody response to flagellin and therefore tested animals for antibody tolerance. Antibody titres were measured at weekly intervals following flagellin challenge (up to day 56). The levels of delayed-type hypersensitivity were again determined in animals on day 56 by the injection of 0.5 µg of flagellin in saline.
Fig. 1. An experimental design to test the capability of CNBr digested flagellin to induce antibody formation, delayed-type hypersensitivity and immunological tolerance. The same experimental design was used for both strain W and strain J Wistar rats.
into the right hind footpads. A dose of 0.5 µg of flagellin elicited hypersensitivity reactions just as efficiently as 100 µg of flagellin (i.e., day 28 challenge) (3).

(a) Immune Responses in Strain W Wistar Rats

Adult, strain W, Wistar rats (seven per group) were injected intraperitoneally daily for 27 days with amounts of a CNBr digest of flagellin varying in ten-fold dilution steps from 100 µg to 10 fg (fg = femtogram). The experimental protocol was as described above (Fig. 1).

Fig. 2 presents the antibody titres and delayed-type hypersensitivity responses (24 hr footpad swellings) of rats following 27 daily injections of different doses of the CNBr digest of flagellin. Significant delayed-type hypersensitivity was induced by doses of the CNBr digest ranging from 10 fg to 1 ng/day and 1 µg to 100 µg/day, the strongest delayed responses being induced by 100 fg/day and 100 µg/day (Fig. 2). It is noteworthy that doses of antigen as low as 10 fg/day could induce significant delayed-type hypersensitivity. In contrast, only the larger doses of antigen (100 ng to 100 µg/day) produced detectable antibody. This result is at variance with an earlier study in strain W Wistar rats, where it was demonstrated that doses of the CNBr digest as low as 10 pg could induce detectable primary antibody (4). This variation is probably due to the presence of variable amounts of undigested flagellin in different preparations of the CNBr
Fig. 2. Antibody titres (■) and delayed-type hypersensitivity responses (□) of strain W Wistar rats which had been injected intraperitoneally daily for 28 days with varying amounts of a CNBr digest of flagellin. Antibody titres were estimated on day 28 and delayed-type hypersensitivity was elicited on the same day by the injection of 100 µg of flagellin in saline into the hind footpads, The broken line represents the 24 hour footpad swelling of control rats which were elicited with 100 µg of flagellin in saline. Vertical bars represent standard errors of the means.
digest of flagellin.

The rats which had been pretreated with the CNBr digest of flagellin (see Fig. 2) were challenged with 100 µg of flagellin. The immune status of these animals four weeks after flagellin challenge is presented in Fig. 3. The antibody responses showed two zones of antibody tolerance separated by a region where enhanced antibody formation occurred (Fig. 3). A dose of 100 µg/day of the digest induced high zone antibody tolerance (0.05 > P > 0.01) whereas optimum low zone tolerance was induced by 100 fg/day (0.05 > P > 0.01). These results confirm earlier antibody tolerance studies in strain W Wistar rats (4). In contrast, both high and low zone antibody tolerance were accompanied by enhanced delayed-type hypersensitivity. Furthermore, significant suppression of delayed-type hypersensitivity was observed (0.01 > P > 0.001) when an enhanced antibody response existed (i.e., 10 ng and 100 ng/day treatments). Thus, in this rat strain there appears to be a striking "mirror-image" relationship between humoral and cell-mediated immunity.

(b) Immune Responses in Strain J Wistar Rats

The experimental protocol was identical to that described in the preceding section (see Fig. 1), although the 10 fg dose of the CNBr digest of flagellin was not included in this experiment. Fig. 4 presents the antibody titres and delayed-type hypersensitivity responses of strain J Wistar rats which had been pretreated with different doses of the CNBr digest of flagellin and then
Fig. 3. Antibody titres (■) and delayed-type hypersensitivity responses (□) of strain W Wistar rats injected daily for 28 days with varying amounts of the CNBr digest of flagellin (Fig. 2) and then challenged with 100 µg of flagellin in saline. The antibody titres represent the mean of the 7, 14, 21 and 28 day post challenge titres. Delayed-type hypersensitivity was elicited 28 days after the flagellin challenge. The broken line represents the antibody and delayed hypersensitivity responses of control rats which were injected only with 100 µg of flagellin in saline. Vertical bars represent standard errors of the means.
Fig. 4. Antibody titres (■) and delayed-type hypersensitivity responses (□) of strain J Wistar rats injected daily for 28 days with varying amounts of the CNBr digest of flagellin and then challenged with 100 µg of flagellin in saline. The antibody titres represent the mean of the 7, 14, 21 and 28 days after the flagellin challenge. The broken line represents the antibody and delayed hypersensitivity responses of control rats which were injected only with 100 µg of flagellin in saline. Vertical bars represent standard errors of the means.
challenged with 100 µg of flagellin in saline.

In contrast to strain W rats antibody tolerance to flagellin was not induced in strain J Wistar rats by any dose of the CNBr digest of flagellin (Fig. 4). Furthermore, the inverse relationship between humoral and cell-mediated immunity, seen so clearly with the W rat strain, was not so clear cut with the J strain. For example, with certain doses of the CNBr digest (10 ng and 100 ng/day) both humoral and cell-mediated immunity were higher than control animals. Despite these results, suppression of delayed-type hypersensitivity was associated with heightened antibody titres (i.e., 1 µg/day dose of digest), whereas the lowest antibody responses were accompanied by high delayed reactions (i.e., 1 pg to 1 ng/day doses).

Comparison of the Humoral and Cell-Mediated Immune Responses to Flagellin in Strain J and Strain W Wistar Rats

Experimental data presented in the preceding section clearly demonstrated that strain J and strain W Wistar rats differed in their immunological responsiveness to flagellin. Experiments were carried out to further investigate these differences in responsiveness. Groups of adult strain J and strain W Wistar rats (7-8 per group) were injected into the flanks with 1 µg of either flagellin or acetoacetylated-flagellin (16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) (2) in saline. Thirty-five days later delayed-type hypersensitivity and secondary antibody responses were elicited by the injection of 1 µg of flagellin in saline.
into the right hind footpads. Control groups of rats which had been injected with saline alone were also challenged with flagellin at this time. Footpad swelling was determined as described in the preceding section. Antibody titres were measured at weekly intervals during the experiment.

Table 1 compares the immune responses to flagellin of strain J and strain W Wistar rats. From this comparison the following observations were made:-

(a) The primary antibody responses of the two rat strains were similar. At all time points both strains produced similar primary antibody titres to flagellin. Furthermore, both strains failed to produce any primary antibodies when immunized with acetoacetyl-flagellin.

(b) Both rat strains expressed comparable levels of delayed-type hypersensitivity when injected with either flagellin or acetoacetyl-flagellin.

(c) In contrast, strain J rats gave a significantly higher and more prolonged secondary antibody response to flagellin than strain W rats.

(d) Acetoacetyl-flagellin induced antibody tolerance to flagellin in strain W Wistar rats but failed to produce antibody tolerance in strain J animals.

In addition, multiple doses of a cyanogen bromide digest of flagellin induced higher levels of delayed-type hypersensitivity in strain J rats (peak response = 6.9) than in strain W rats (peak response = 4.0) (compare Fig. 3. and 4).
TABLE 1
COMPARISON OF THE IMMUNE RESPONSES TO FLAGELLIN PRODUCED BY STRAIN J AND STRAIN W WISTAR RATS

<table>
<thead>
<tr>
<th>STRAIN OF WISTAR RATS</th>
<th>PRIMING ANTIGEN (1 µg in saline)</th>
<th>PEAK PRIMARY ANTIBODY (35 day)</th>
<th>DELAYED-TYPE HYPERSENSITIVITY&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CHALLENGE (1 µg in saline)</th>
<th>SECONDARY ANTIBODY TITRES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 day</td>
</tr>
<tr>
<td>Strain J</td>
<td>Flagellin</td>
<td>100</td>
<td>2.3 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Flagellin</td>
<td>8,700</td>
</tr>
<tr>
<td>Strain J</td>
<td>Acetoacetyl-flagellin</td>
<td>&lt;2.5</td>
<td>4.2 ± 0.4</td>
<td>Flagellin</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Strain J</td>
<td>Nil</td>
<td>&lt;2.5</td>
<td>0.4 ± 0.2</td>
<td>Flagellin</td>
<td>2.5</td>
</tr>
<tr>
<td>Strain W</td>
<td>Flagellin</td>
<td>160</td>
<td>2.3 ± 0.5</td>
<td>Flagellin</td>
<td>3,580</td>
</tr>
<tr>
<td>Strain W</td>
<td>Acetoacetyl-flagellin</td>
<td>&lt;2.5</td>
<td>3.7 ± 0.3</td>
<td>Flagellin</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Strain W</td>
<td>Nil</td>
<td>&lt;2.5</td>
<td>0.3 ± 0.2</td>
<td>Flagellin</td>
<td>&lt;2.5</td>
</tr>
</tbody>
</table>

Rats were primed in the flanks and delayed-type hypersensitivity and secondary antibody responses were elicited in the hind footpads with flagellin (1 µg) 35 days later.

<sup>a</sup> Footpad swellings (1/10th mm) 24 hr following eliciting dose of flagellin.

<sup>b</sup> Standard error of mean.

<sup>c</sup> 16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$ (2).

<sup>d</sup> Significant difference between Strain J and strain W response ($P < 0.01$).
From these results it was concluded that strain J Wistar rats give a higher immune response to flagellin than do strain W rats. This difference became evident when animals were treated with multiple doses of antigen.

**Ability of Cyanogen Bromide Digested Flagellin to Induce Immunological Tolerance in Neonatal Rats**

Cyanogen bromide digested flagellin was injected into strain J Wistar rats in amounts of 1 µg, 3 times weekly, beginning within 24 hr of birth. Following this injection schedule, animals were challenged into the flanks with either 1 µg of flagellin in FCA or 1 µg of acetoacetyl-flagellin in FCA (16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) (2). Control groups of rats which had not been pretreated with the CNBr digest were similarly challenged. Four weeks following the FCA challenges delayed-type hypersensitivity was elicited by the injection of 0.5 µg of flagellin in saline into the right hind footpads. Footpad swelling was determined as described earlier. Antibody titres were measured prior to FCA challenge and at weekly intervals for 4 weeks post challenge. The prechallenge and 28 days post challenge titres are presented in Table 2.

No antibody was detected in the rats following 8 week of injection with CNBr digested flagellin (Table 2). Pretreatment of rats from birth with CNBr digested flagellin completely suppressed their antibody response to a subsequent challenge of flagellin. Similarly,
<table>
<thead>
<tr>
<th>INITIAL COURSE OF INJECTIONS(^a)</th>
<th>ANTIBODY TITRE BEFORE SECOND INJECTION</th>
<th>SECOND INJECTION (1 µg in FCA)</th>
<th>ANTIBODY TITRE (28 day)</th>
<th>DELAYED-TYPE HYPERSENSITIVITY (28 day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µg CNBr digest</td>
<td>&lt;2.5</td>
<td>Flagellin</td>
<td>&lt;2.5</td>
<td>0.25 ± 0.2(^b)</td>
</tr>
<tr>
<td>NIL</td>
<td>&lt;2.5</td>
<td>Flagellin</td>
<td>5120</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>1 µg CNBr digest</td>
<td>&lt;2.5</td>
<td>Acetoacetyl-flagellin(^c)</td>
<td>&lt;2.5</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>NIL</td>
<td>&lt;2.5</td>
<td>Acetoacetyl-flagellin(^c)</td>
<td>&lt;2.5</td>
<td>7.0 ± 0.5</td>
</tr>
</tbody>
</table>

\(^a\) Strain J Wistar rats injected three times weekly from birth for 8 week prior to challenge.

\(^b\) Represents 24 hr footpad swelling (1/10th mm) following elicitation with 0.5 µg of flagellin in saline. Standard errors of means are included.

\(^c\) 16.8 acetoacetyl groups/mole, \(K_{rel} = 6.8 \times 10^{-3}\) (2).
pretreatment with the CNBr digest induced complete "cellular immunity tolerance" as these animals were unable to produce detectable delayed-type hypersensitivity against either flagellin or acetoacetyl-flagellin. Thus, CNBr digested flagellin simultaneously induces tolerance in neonatal rats at the level of both humoral and cell-mediated immunity. In contrast, the same preparation in adult rats produces immunological tolerance only at either the humoral or cell-mediated level.

**Desensitization of Delayed-Type Hypersensitivity to Flagellin**

To our knowledge, no other laboratory has demonstrated that suppression of antibody formation can be accompanied by heightened levels of delayed-type hypersensitivity (for further details see Discussion). It may well be, however, that in earlier studies, desensitization of delayed-type hypersensitivity by persisting antigen could have occurred. To test this possibility, an experiment was carried out to determine whether flagellin could desensitize animals which were already hypersensitive to flagellin.

Adult, strain J, Wistar rats were sensitized in the flanks with 1 µg of acetoacetyl-flagellin (16.8 acetoacetyl groups/mole, $K_{rel} = 6.8 \times 10^{-3}$) (2) in FCA and 10 days later delayed-type hypersensitivity was elicited by the injection of 0.5 µg of flagellin in saline into the right hind footpads. Desensitizing doses of flagellin (1 µg to 1 mg in 0.5 ml saline) were injected intravenously at the same
time as the eliciting antigen. Footpad swelling was determined as described in the Materials and Methods. Control rats were injected into the flanks with FCA-saline and elicited with flagellin 10 days later. It was found that animals hypersensitive to flagellin could be readily desensitized by the intravenous injection of flagellin (Fig. 5). As little as 1 µg of flagellin produced partial desensitization and doses of 10 µg or more completely eliminated the delayed response.

DISCUSSION

It was originally demonstrated by Mitchison (5) and Dresser (9,10) that antibody tolerance could be induced in adult animals by the injection of antigen at two different dose levels. Intermediate doses of antigen usually resulted in enhanced antibody titres rather than in antibody tolerance. These two regions of antibody tolerance have been termed "high zone" and "low zone" tolerance according to the relative amounts of antigen required to induce the tolerant state (5). More recently it has been reported by several laboratories that high and low zone antibody tolerance can be induced by a range of antigens (11). However, although the antibody responses in these systems have been extensively investigated the cell-mediated immune responses have been largely neglected.

Data presented in this paper indicates for the first time that both high and low zone antibody tolerance to
Fig. 5. Desensitization of delayed-type hypersensitivity to flagellin in strain J Wistar rats. For experimental details see text. The broken line represents the response of control animals which had been primed with FCA-saline.
flagellin may be accompanied by enhanced levels of delayed-type hypersensitivity. In addition, it was observed that an enhanced antibody response can be associated with suppressed delayed-type hypersensitivity. This "mirror-image" relationship between humoral and cell-mediated immunity was very striking in strain W Wistar rats but was not quite so clear cut in strain J animals. From these results it was concluded that frequently, but not invariably, an inverse relationship exists between humoral and cell-mediated immunity in adult rats.

From the data presented in this paper it could be argued that all doses of the CNBr digest of flagellin induced similar levels of delayed-type hypersensitivity but that high levels of serum antibody effectively masked the delayed response by competing with hypersensitive cells for antigen. Two lines of evidence have already been reported suggesting that this interpretation is unlikely. (a) The administration of large doses of hyperimmune serum into rats hypersensitive to flagellin failed to mask the elicitation of delayed responsiveness (3). (b) Peritoneal cells from rats with high antibody titres to flagellin were unable to transfer delayed-type hypersensitivity (3). If, in fact, antibody had masked delayed hypersensitivity, then these cells should have transferred delayed responsiveness.

Although to our knowledge, no other laboratory has demonstrated such a striking inverse relationship between humoral and cell-mediated immunity, there have been some indications that this phenomenon may exist with other
antigens. For example, it has been observed that "antibody tolerance" to tuberculin (12) and lysozyme (E. Benjamini - personal communication) in adult animals was accompanied by normal levels of delayed-type hypersensitivity. On the other hand, numerous investigators (13-22) have reported that pretreatment of adult animals with antigen in saline can suppress delayed hypersensitivity and this suppression is usually accompanied by "control" (13-21) and sometimes by heightened (22) levels of antibody.

In contrast, there have been several reports of "immunological tolerance" occurring in adult animals at the level of both humoral and cell-mediated immunity (13, 16, 17, 19-23). However, in these systems complete tolerance was only induced by the administration of large quantities of antigen which was slowly eliminated and it would be expected that such antigen persisting in the serum would both desensitize delayed-type hypersensitivity and neutralize serum antibodies. In these experiments, it has been particularly useful that flagellin is very rapidly eliminated from the circulation (24) and only minute amounts of antigen are needed to immunize (25). Thus, it is unlikely that delayed-type hypersensitivity or serum antibody would be masked by persisting flagellin. As additional evidence for this interpretation, it was demonstrated that flagellin can desensitize animals which are hypersensitive to flagellin when both desensitizing and eliciting doses of antigen are injected simultaneously.
(Fig. 5). It should be noted that as little as 1 µg of flagellin induced partial desensitization and doses of 10 µg or more completely abrogated the delayed response. Similar desensitization results have been reported for several other antigens (26,27).

Many workers would agree that there is no ideal method for measuring delayed-type hypersensitivity. For example, inhibition of macrophage migration works well with some antigens but not with others. In our experience, footpad swelling has been found to be a reliable and reproducible assay method. It must be pointed out, however, that frequently the increases in footpad thickness are not large and considerable practice is necessary to obtain reproducible results. It may be that the small differences obtained is the reason why others have not observed the relationship described in this paper between antibody production and delayed-type hypersensitivity.

In this study two strains of Wistar rats (strain W and J) were investigated which significantly differed in their immunological responsiveness to flagellin. It was found that strain J Wistar rats had a greater potential to respond to flagellin than strain W rats both at the humoral and cell-mediated level. However, these differences in responsiveness only became apparent when rats were injected with multiple doses of antigen. In addition, adult antibody tolerance to flagellin could be induced in strain W rats but not in strain J animals (Table 1). These
results could be interpreted as indicating that strain J rats possess a greater number of immunocompetent cells to flagellin than strain W rats (28). The additional observation that strain J rats required a 100 fold higher dose of the CNBr digest than strain W rats to both suppress and enhance delayed hypersensitivity was consistent with this interpretation (compare Fig. 3 and Fig. 4). In other words, if immunocompetent cells compete with one another for antigen, then the greater the number of immunocompetent cells present in an animal the higher the dose of antigen required to induce immunological responses.

One of the most remarkable features of this study was the demonstration that as little as 10 fg/day of a CNBr digest of flagellin induced significant delayed-type hypersensitivity in strain W Wistar rats (Fig. 2). During the 28 day course of daily injections this dose is equivalent to injecting between $10^6$ and $10^7$ molecules of degraded flagellin. It is likely that substantially less than this amount of antigen would reach the lymphoid system. The mechanism by which such small amounts of antigen can mediate immunological effects is uncertain, although it has been proposed in an earlier publication that antigen localized in lymphoid follicles may play a role (4).

In this paper data was presented confirming the observation that a fundamental difference exists between neonatal and adult tolerance (3). Rats injected with the
CNBr digest of flagellin from birth were rendered tolerant to flagellin at the level of both humoral and cell-mediated immunity. In contrast, the CNBr digest in adult animals produced immunological tolerance only at either the humoral or cell-mediated level. This difference between neonatal and adult tolerance has been observed in both strain J (Table 2) and strain W (3) Wistar rats.

A hypothesis was proposed by Parish (3) to explain the inverse relationship between humoral and cell-mediated immunity. Briefly, it was postulated that antigen mediated cell-cell interaction between 'B' (bursa equivalent) and 'T' (thymus-derived) cells is essential for the induction of antibody formation and suppression of delayed hypersensitivity. In contrast, providing the binding energy of antigen to separate immunocompetent cells reaches a certain threshold, antibody tolerance will be induced in 'B' cells and cell-mediated immunity in 'T' cells. This hypothesis can readily accommodate the observation presented in this paper that both high and low zone antibody tolerance can be accompanied by enhanced cell-mediated immunity. Firstly, it would be anticipated that low doses of the CNBr digest of flagellin would preferentially induce delayed-type hypersensitivity and antibody tolerance, as the probability of small quantities of antigen inducing cell-cell interaction is low, whereas this amount of antigen would probably attain the "threshold energy of binding" required to activate cells. Secondly,
higher doses of antigen would be expected to favour cell-cell interaction and therefore antibody formation. Finally, it would be predicted that very high doses of antigen would produce enhanced cell-mediated immunity and antibody tolerance, as at this dose level antigen molecules would tend to compete with one another for receptors on immunocompetent cells.
REFERENCES


8. LIEW, F.Y., 1971. The detection and production of macrophage cytophilic antibody to different antigens in different laboratory animals. Immunology, in press.


