THE RELATIONSHIP OF ANTIBODIES TO AUTOLOGOUS HEPATO-CELLULAR ANTIGENS AND LIVER DAMAGE IN EXPERIMENTAL ANIMALS

A THESIS

BY

Austin U. Sargent, M.D.C.M.

Submitted to the Faculty of Graduate Studies and Research in Partial Fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Experimental Medicine, McGill University.

April 1965
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER I</th>
<th>THE PROBLEM OF CHRONIC LIVER DISEASE</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER II</td>
<td>MECHANISMS OF IMMUNOLOGICALLY-INDUCED TISSUE INJURY</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PART I: THE IMMUNE RESPONSE</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>PART II: IMMUNOLOGICALLY-INDUCED TISSUE INJURY</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>A. Mechanisms of Immune Cellular Lysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. The Effect of Antibody on Mammalian Cells in the Absence of Complement</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2. The Effects of Antibody and Complement on the Mammalian Cell</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>B. Antigen-Antibody Complexes as Pathogenic Agents</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>1. The Arthus Reaction</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2. Serum Sickness</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>C. Tissue Damage Associated With the 'Delayed' or 'Cellular' Type of Immunity</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>a) The invasive necrotic lesion</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>b) The vascular necrotic lesion</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>c) The massive necrosis</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>PART III: TOLERANCE, AUTO-IMMUNITY AND TISSUE DAMAGE</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>A. Auto-Immune Reactions to Normally Inaccessible Antigens</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>B. Breakdown of Tolerance by Cross-Reacting Antigens</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>C. Breakdown of Tolerance Induced by Altered 'Self' Antigens</td>
<td>54</td>
</tr>
</tbody>
</table>
D. Breakdown of Tolerance by an Altered Immune Apparatus

E. The Concept of Physiogenic Auto-Antibodies

CHAPTER III MATERIALS AND METHODS

A) Source and Preparation of Organ Extracts and Sera

B) Preparation of Antigens

C) Immunization Procedures

D) Absorption of Antisera for Use in Haemagglutination Experiments and Fluorescent Microscopy

E) Tanned Red Cell Haemagglutination Technique

F) Inhibition of Haemagglutination

G) Fluorescent Microscopy

CHAPTER IV RESULTS

A) Specificity of Rabbit Anti-Rat Liver Sera for Rat Hepato-Cellular Antigens

1. Cross Reactions of Rabbit Anti-Rat Liver Sera with Rat Serum Proteins and Red Cells

2. Cross Reactions of Rabbit Anti-Rat Liver Sera with Rat Heart, Kidney and Lung

Organ Specificity of Rat Hepato-Cellular Antigens as Determined by the Use of Rabbit Anti-Rat Liver Serum

B) Organ Specificity of Rat Hepato-Cellular Antigens as Determined by the Use of Goat Anti-Rat Liver Serum

C) Species Distribution of the Rat Organ Specific Hepato-Cellular Antigens

Species Specificity of Hepato-Cellular Antigens
D) Localization of the Organ Specific Hepato-Cellular Antigens in the Rat as Demonstrated by Indirect Fluorescent Microscopy

E) Attempts to Break Tolerance to the Hepato-Cellular Antigens of the Rat by Immunization with Emulsions of Rabbit or Rat Livers

1. Breakage of Tolerance to Rat Hepato-Cellular Antigens

2. Cross Reactions of Rat Anti-Rabbit and Rat Anti-Rat Liver Sera

F) Detection of Circulating Organ Specific Hepatic Antigens after Injection of the Hepato-Cellular Toxin Thioacetamide

G) Attempts to Induce the Formation of Auto-Antibodies to Hepato-Cellular Proteins by Injection of Thioacetamide

CHAPTER V DISCUSSION

SUMMARY

CLAIMS TO ORIGINALITY

FIGURES

BIBLIOGRAPHY
ACKNOWLEDGEMENTS

The author wishes to acknowledge with gratitude the stimulating encouragement and guidance of his research directors, Drs. Bram Rose and Maxwell Richter.

Dr. Jeffrey Myers is thanked for performance of the experiments involving fluorescent microscopy and for examining and photographing sections of livers obtained from the various experimental animals. His suggestions and constructive criticism were always appreciated.

The members of the Division of Immunochemistry and Allergy Research, Royal Victoria Hospital, and especially Miss R. Gerber who supplied expert technical assistance, are thanked for their co-operation.

The work in this thesis was supported by grants from the Canadian Cancer Society and a fellowship from the Canadian Arthritis and Rheumatism Society.
Chapter I

THE PROBLEM OF CHRONIC LIVER DISEASE

Prometheus, creator of mankind, stole fire from the chariot of the sun and in defiance of the all-powerful Zeus gave it to mankind. Zeus, enraged, had Prometheus chained to a mountain pillar where greedy vultures tore at his liver all day, year in and year out. There was to be no end to Prometheus' punishment since each night his liver grew whole again (1).

This Greek myth serves as an apt analogy for the clinical problem of liver disease. Although no ravaging vultures have been cited in the aetiology of cirrhosis, the repeated episodes of hepatic destruction and regeneration prescribed by Zeus as punishment for Prometheus are well-recognized clinical phenomena.

Laennec first introduced the term 'cirrhosis' of the liver in 1869. At present it is used to describe that state which is characterized by progressive diffuse chronic inflammation, accompanied by fibrosis, and with destruction and regeneration of the parenchymal cells (2, 3). However, despite the fact that the morphological, physiological and clinical features characteristic of hepatic cirrhosis are well known, its aetiology remains controversial. It is the fourth most frequent cause of death in North America today (3), but accepted aetiological factors contribute to its pathogenesis in less than 50 per cent of cases (4, 5). Haemachromatosis,
hepatolenticular degeneration, hepatic steatosis and cardiac cirrhosis constitute the only forms of cirrhosis known to result from the presence of persistent offending aetiological agents (4, 5). The contributory effects of chronic viral infection (6) and of malnutrition and alcohol intake (7) have yet to be established as definitive aetiological agents in the production of chronic liver disease.

Uncertainty exists as to the very nature of the cirrhotic process. Is it a primary disorder of the hepatic parenchymal cell, with reactive connective-tissue formation, or is it "primarily a mesenchymal lesion, inflammatory in character, in which the epithelial alterations are secondary to the mesenchymal changes" (3)? This latter concept would tend to identify cirrhosis with chronic hepatitis. Popper quotes Himsworth's suggestion to discard the term cirrhosis and to designate as 'fibrosis' the sclerosed appearance of the liver, "leaving us with the entity chronic hepatitis with fibrosis" (3).

In the absence of a demonstrable persistent aetiological agent, the nature of the progressive character of cirrhosis remains uncertain. Over the past decade, increasing attention has been drawn to those aspects of chronic liver disease that reflect activity of the body's immune system. In one of the earliest published reports speculating on the role of antibody in the pathogenesis of chronic liver disease, Eaton and his colleagues wrote: "It is conceivable that an antigenic complex liberated from the liver tissue in the acute phase of
hepatitis, preceding the appearance of icterus, gives rise to an antibody which in turn reacts with more antigen in vivo. This consideration would by no means disprove the infectious nature of hepatitis because the observed phenomena may only be a secondary reaction to parenchymal liver damage produced by the virus or other self-propagating agents." (8). In regard to chronic hepatitis, they further speculated that "The liver antibody, once produced, may combine with additional antigen in situ in the liver cells and cause further damage after the acute infectious process has subsided. This might be the explanation for some fatal cases in which the disease is greatly prolonged...." (8). Others have commented on the considerable alterations in serum proteins that occur in acute or chronic liver disease (4, 9). Particularly striking changes may occur in those serum proteins associated with antibody activity, i.e., the immunoglobulins. In acute hepatitis they increase early in the course of the disease and return to normal when the active inflammation subsides (4). Not infrequently, their failure to return to normal, or their further increase in concentration, is associated with the progressive form of the disease and heralds the ominous transition from acute to subacute hepatitis (4). Thus serial electrophoretic determinations of the immunoglobulins have a definite diagnostic value (4).

It is reasonably certain that the plasma cell is responsible for the synthesis of immunoglobulins (10-13). Fahey has demonstrated that the rate of production of these
serum proteins is the major factor determining their concentration in the serum (14). Increase in gamma-globulin synthesis occurs following antigenic stimulation which, if prolonged, will result in elevation of their serum level (14). In view of Haven's reported findings of enhanced antibody-producing capacity in a series of patients with chronic liver disease (15-17), the possibility arises that the hypergammaglobulinaemia seen in this condition reflects the continuing presence of intense antigenic stimulation. Using tetanus toxoid and alum-precipitated diphtheria toxoid as the antigens, Havens found that patients with chronic liver disease produced amounts of antitoxin two to three times greater than those produced by a control group of patients with extrahepatic disorders (15-17).

Good, in a series of similar investigations, studied the immune response in six patients with severe chronic hepatitis, extreme hypergammaglobulinaemia and extensive plasma-cell infiltration of the liver (18). In response to the injection of typhoid H and O antigens and the somatic antigen of paratyphoid B, he noted vigorous, though not abnormal, antibody response. He concluded that, although the synthesis of the immunoglobulins was grossly increased in these patients, there was no evidence of gross immunological hyperactivity (18). However, Good felt that the distribution of plasma cells around the margins of the parenchymal nodules in the diseased liver might represent the morphological reflection of intense antigenic stimulation. Similar findings in patients with chronic liver disease, marked hypergammaglobulinaemia and...
plasma-cell infiltration of the liver have been described by Kunkel et al. (19, 20), Mackay (21), and Sherlock (22).

Popper recently provided evidence strongly suggesting that, in chronic liver disease, mesenchymal cells of the liver may undertake synthesis of the immunoglobulins (23–25). Immunofluorescent studies of liver biopsies obtained from patients with portal and postnecrotic cirrhosis show gamma globulin to be present within the hepatic mesenchymal cells (23–25). In support of the contention that these cells were actively synthesizing gamma globulin, Popper demonstrated that the cytoplasm of the reactive cells contained a prominent endoplasmic reticulum and was rich in ribonucleic acid (2, 25). Both of these characteristics conventionally are associated with cells actively synthesizing protein (2, 25). Further indirect evidence indicating the active production of gamma globulin by the reticulo-endothelial cells of the liver is the fact that, in primary biliary cirrhosis, and in a single case of Waldenström's macroglobulinaemia complicated by viral hepatitis, fluorescent microscopy has revealed that many of these cells contain 19 S macroglobulins (25). This is consistent with the known increase in the 19 S immunoglobulins in both conditions (25). It is significant that, in hypergammaglobulinaemic states without associated hepatic pathology, fluorescent microscopy failed to demonstrate cells containing gamma globulin within the liver. The antibody nature of this 'locally formed gamma globulin' has not been established. In particular, whether it represents antibody to hepatocellular proteins is not known, but
it has been adequately demonstrated that patients with chronic liver disease produce antibody to a wide variety of autologous cellular components (26–28).

An analogous problem to that encountered in chronic liver disease occurs in chronic nonspecific thyroiditis. Both conditions are progressive in character and, like most forms of cirrhosis, chronic nonspecific thyroiditis was until recently a disease of unknown aetiology (29, 30). The serum of patients with either condition is characterized by hypergammaglobulinaemia, and infiltrations of plasma cells and lymphocytes, and actual formation of germinal centres, demonstrated in the thyroid gland (29, 30).

Chronic nonspecific thyroiditis in man and experimental thyroiditis in animals represent well-defined models of autoimmune disease (30–37). To a substantial degree, experimental thyroiditis fulfils the criteria necessary to establish involvement of immune reactions in the pathogenesis of the tissue lesions:

1) The antigen is known (31, 32, 34, 35).
2) The antibodies present can react with the autologous antigen (31, 34).
3) It has been reported that the disease can be transferred with immunologically competent cells obtained from afflicted animals, but not with immune serum (32, 36).
4) The expected development of the disease, following immunization with an emulsion of thyroglobulin and Freund's adjuvant, can be prevented by the administration of immunosuppressive drugs (34, 35).
Recent work by Mellors and others on thyroid tissue obtained from patients with chronic nonspecific thyroiditis has demonstrated vigorous production of auto-antibodies to thyroglobulin in the plasma cells infiltrating the gland in this condition (30, 37). Thyroglobulin and gamma-globulin, or thyroglobulin and "its specific antibody", were demonstrated within the thyroid and were considered to represent the presence of immune complexes (30). Thus, in chronic nonspecific thyroiditis there exist within the thyroid gland the major components of an immune response - the antigen, the specific auto-antibody, and apparent immune complexes (30). This constitutes strong evidence that immune reactants contribute to the cycle of pathogenic events which, once initiated, presumably lead to the gradual self-destruction of the gland (30, 32, 34).

The present project was undertaken to investigate whether similar immunologically mediated tissue damage might be induced experimentally in the liver. The organ and species distribution of the hepatocellular antigens was to be determined. Once the organ specificity of the hepatocellular antigens had been established attempt could then be made to break immune tolerance to these antigens. Should this prove possible, the effects of the resultant auto-immune response on the integrity of the hepatic parenchymal cells could be evaluated.

To provide a basis for discussion of the results of this study, a review of the literature will be presented, selecting as examples the various types of immunologically mediated tissue damage.
INTRODUCTION

Largely as a result of the classical experiments of Ehrlich and Morgenroth, the terms 'antigen' and 'foreign' have become synonymous \(^{38}\). On the basis of their experiments the classical theories of antibody production have traditionally denied to autologous cells and macromolecules the ability to elicit an immune response from the antibody-forming tissues of the host from which these cells and macromolecules were derived \(^{39 - 41}\).

Ehrlich and Morgenroth showed that the sera of goats immunized with homologous red blood cells regularly developed haemolysins that were active against all goat red cells except those obtained from the specific immunized animal \(^{38}\). This finding led them to make the clear distinction between an 'isolysin', capable of haemolyzing the homologous red cells, and an 'autolysin', capable of haemolyzing cells obtained from the donor of the immune serum.

In his English translation of Ehrlich's Collected Studies of Immunity, Bolduan wrote: "It is therefore of the highest pathological importance to determine whether the absorption of its own body material can excite reactive changes in the organism, and what the nature of the change is. If an
organism when injected with blood cells of a foreign species always produces a specific haemolysin for each of these species, it must surely be following a natural law; it is improbable that the law which applies in any particular number of cases should be suspended in the case of blood cells of the same individual. It cannot be doubted that the organism seeks a way out of this difficulty by means of certain regulating contrivances whose determination will be of the highest importance"(38, p.25).

Reference is made to the results of Ehrlich's experimental work with goats as an example of the general law that autolysins are not capable of existing within an organism and it is postulated that an 'anti-autolysin' might play a part in neutralizing autolysins if and when they formed (38). Later, the celebrated phrase "horror autotoxicus" is mentioned: "We pointed out that the organism possesses certain contrivances by means of which the immune reaction, so easily produced by all kinds of cells, is prevented from reacting against the organism's own cellular elements and so gives rise to autotoxins .... Investigations made by us have confirmed this view, so that one might be justified in speaking of a "horror autotoxicus" of the organism. These contrivances are naturally of the highest importance for the existence of the individual" (38). In referring to the possibility of auto-intoxication occurring in man, Ehrlich and Morgenroth made the point that,"Only when the internal regulating contrivances are no longer intact can great dangers arise"(38).

This concept, proposed over sixty years ago, can hardly be faulted even today. Though the internal regulating contrivances may not be those they visualized, the suggestion that their
breakdown could lead to "great dangers" is certainly correct.

The dire consequences of Ehrlich's dictum found ready confirmation in the clinical case reports published in France at the turn of the century. Widal, Abrami and Brule (42) probably provided the first accurate description of "l'ictère hemolytique acquis". The characteristic auto-haemagglutination of the patient's red blood cells was stressed by these workers. Legendre and Brule (43) reported a patient acutely ill with jaundice and haemoglobinuria, whose serum contained an auto- and an iso-haemolysin; both of these disappeared as the patient recovered.

Metalnikoff's work, performed in the early 1900s, provided the first indication that experimental immunization with autologous tissue components could evoke an immune response in the organism from which the tissue was derived (44). By immunizing an animal with its own sperm she demonstrated the presence of an auto-antibody to the immunizing preparation. Clinicians and researchers of this period were not impressed by these reports, not sensing their worth or interest. Therefore, whereas clinical and experimental observations suggesting that disease could occasionally result from an immunological reaction to an individual's own tissue antigens are almost as old as immunology itself, these reports were largely ignored until revived and reviewed by Dameshek and Schwartz in 1940 (45).

In 1949 Burnet and Fenner speculated on why such reactions are the exception rather than the rule, and questioned the mechanism by which the antibody-forming cells of the body are so
effectively prevented from reacting to the myriad of potential antigens surrounding them. They pointed out that, in order for the immune apparatus of the body not to respond to autologous antigens, it must somehow be capable of distinguishing 'self' from 'not self' (46). By whatever mechanism the self-recognition is achieved, the end result is an absence of immune response to the autologous antigen by the antibody-forming tissues (47-51): therefore the immunologically competent cells of the body are said to be 'tolerant' of the neighbouring cells and proteins that, collectively, constitute the tissues of the organism.

The central problem in auto-immunity and auto-immune disease obviously concerns the mechanism by which tolerance to 'self' is achieved. Before one can discuss the conditions in which auto-immunity may occur, it is necessary to consider briefly the theories of antibody production and the phenomenon of tolerance to autologous antigens. Unfortunately a diversity of opinion exists concerning certain fundamental aspects of the immune response. At the present time the major point of controversy centres on whether the specificity of the antibody is determined by specific instructions imparted by the antigen to the antibody-forming cells, or whether the unstimulated immunologically mature animal already possesses the necessary information for the synthesis of antibody of any conceivable specificity. In this latter instance, antibody specificity is not conferred on the antibody-forming cell by the antigen; rather, the appropriate specificity already exists within the cell and
its synthesis is stimulated by the presence of the corresponding antigen (47–51). Burnet's theory has as its core this selective role of the antigen in the development of the immune response. He postulates that the unstimulated animal possesses the genetic information necessary for the synthesis of antibody of every conceivable specificity, this information being so distributed among the antibody-forming cells of the body that each can form only one or, at the most, a limited number of antibodies (51, 52). The antibody-forming tissue is thus composed of individual clones, or groups of cells, each of which is characterized by the specificity of the antibody it is potentially capable of synthesizing. According to Burnet, the presence of antigen in the immediate environment of the mature clones of antibody-forming cells serves to stimulate that clone with the corresponding antibody-forming potential to proliferate and synthesize antibody (41, 51).

"The "internal regulating contrivances" postulated by Ehrlich to account for the lack of reactivity to autologous antigens are visualized by Burnet as consisting of a process of immunological homeostasis which ensures that those clones of immunologically competent cells committed to react with autologous antigens will be eliminated from the organism. This may take the form of an inhibition of the reactive cell at some stage in its development, or actual destruction of the particular clone (41, 47, 48, 51, 52). Medawar states that, "In respect to an autologous antigen, the animal remains in a state of retarded development with one particular faculty excised
from its normal repertoire of immune response." (53) For the state of tolerance to persist it appears necessary that the immune apparatus be continually exposed to the antigen (53). Removal of the antigen from the tissues of the body results in a selective loss of tolerance to that antigen (54). Two additional possibilities may theoretically terminate immune tolerance to a particular antigen: 1) an escape of the previously tolerant cell from inhibition (55); and 2) the stimulation to activity of new, immunologically competent cells arising from a precursor pool in the thymus or elsewhere within the body (52).

Regardless of the mechanism by which tolerance operates, the fact that it does occur and can be verified experimentally is no longer in doubt. In the development of blood-group chimeras in humans (56) and in cattle (57), nature has provided spontaneously occurring examples of the toleration of a foreign antigen in the circulation of the host. Owen's studies of the 'immuno-genetic' consequences of the vascular anastomoses that exist between the chorionic vessels of the placentas of bovine twins revealed for the first time that cells antigenically distinct from those of the host could be tolerated by it without a detectable immunological reaction. These studies suggested that the induction of a state of tolerance to a foreign antigen is a function of the period in the animal's existence when parental contact with the antigen is first established (48, 52, 53). Woodruff's studies of chimerical blood groups in a pair of twins suggested that a similar mechanism exists in
man (56). In the pioneer experimental work of Billingham, Brent and Medawar (58), the relationship between the age of the animal when first exposed to an antigen and the subsequent development of acquired immune tolerance was demonstrated to extend into the immediate postnatal period.

If the state of acquired immune tolerance is in fact due to a selective suppressive effect exerted by the foreign antigen upon the developing immune system of the host, it should be possible to render autologous proteins antigenic by removing them from contact with the host during this period. The elegant experiment of Triplett (54) established that tolerance to autologous proteins is developed during embryonic life and is not genetically determined. He isolated the pituitary from the frog (*Hyla regilla*) after it had become capable of self-differentiation but before it could secrete its own organ-specific hormones. The gland was allowed to grow and differentiate in another frog of the same species until it attained maturity and was able to secrete its own specific hormones, and then was transplanted as an autograft into the original donor. In a statistically significant proportion of cases the animal rejected its own pituitary. Control experiments ruled out the possibility that the antigens of the graft had changed or that the animal had lost its ability to accept autografts.

As data on experimentally induced tolerance accrue, it becomes increasingly apparent that such tolerance may easily be abrogated. Weigle has shown that a stable, experimentally-induced state of tolerance to bovine serum albumin may be terminated by
the injection of a serologically related foreign serum albumin (55). Assuming that experimentally induced and naturally occurring tolerance are the same, it appears that the potential for immune reactivity to the tolerated antigen continues to exist in the tolerant animal.

The breakdown of tolerance to autologous proteins is now well documented: the development of antibodies to thyroglobulin in some apparently normal people (59, 60), and of the numerous antibodies directed to cytoplasmic and nuclear components of the cell in patients with one of the collagen diseases, amply confirms this (39, 61, 62).

The state of tolerance and its breakdown provide a well-documented frame of reference through which to visualize the development of an immune response to one's own tissue antigens (63). Before considering the various ways by which tolerance may be circumvented and a state of auto-immunity engendered, it is necessary to define those agents that mediate the body's immune response.

THE IMMUNE RESPONSE

PART I:

Two qualitatively different types of immune response occur in the presence of antigen. Primitive reticular cells within the germinal centres of the lymphatic tissue are stimulated to differentiate, and a system of cells evolves that possesses a specific reactivity for the determinants of the stimulating
antigen (12, 64). This capacity to react with the antigen may be manifested by the synthesis and secretion of a specific antibody or may result in the presence of cells possessing the ability to react with the antigen in the 'delayed' or 'cellular' type of immune response (12, 64). Within the lymphatic tissues, therefore, the presence of antigen has stimulated the primitive reticular cells to differentiate along one of the several pathways they might have taken (12, 65). The term 'immunologically competent cells' was coined by Medawar to designate those cells responsible for the specific component of the immune response - "That component which varies from one response to another depending on the nature of the antigen which calls it forth" (64). Lymphocytes and plasma cells almost certainly constitute immunologically competent cells (11, 12, 65, 66).

The presence of antigen, whether in the form of bacteria, viruses, fungi, homografts, or certain chemical compounds, may serve to stimulate the development of 'lymphoid' or 'mononuclear' cells which are capable of mediating the 'delayed' or 'cellular' type of immune response (67-71). These cells, presumably derived from precursors within the lymphatic tissues (65, 72-74), are capable of reacting specifically with the exciting antigen (67). Such cells develop within 24 to 48 hours of the introduction of antigen into the tissues of the sensitized host (39, 68). It has been generally assumed that the presence of circulating antibody is not necessary to the development of a 'delayed' reaction (68, 70, 71). Reactivity, specific for the exciting antigen, may be passively transferred
by the use of 'sensitized lymphoid cells' (68, 71) or, in man, with the extracts of leukocytes derived from a sensitized individual (132,133). Recent work reported by several authors suggests that the small lymphocyte constitutes the major component of the 'delayed' response (65, 68, 71, 72, 74).

Procedures which depress the circulating lymphoid cells also suppress the 'delayed' response, the degree of suppression corresponding roughly with the total lymphocyte count (75-76).

The immunologically competent plasma cell almost certainly is not concerned in delayed hypersensitivity reactions, its function being restricted solely to the synthesis of gamma globulin (10, 12, 13, 39, 71, 72, 74).

The gamma-globulin fraction has recently been shown to comprise a family of proteins which, though linked together by common antigenic determinants, exhibit important differences in antigenicity, molecular weight, and electrophoretic mobility (10). By means of immuno-electrophoresis, the classical gamma-globulin fraction of Tiselius can be resolved into three distinct components: the gamma II, gamma IA, and gamma IM fractions (10). Collectively, this family of proteins is referred to as the 'immunoglobulins', a term used by Waldenström to stress the common biological function these proteins shared (i.e., the ability to react with antigen in an immune reaction) and which obviates the necessity of referring to them solely on the basis of their electrophoretic mobility (77). The structural sub-units responsible for the antigenic properties of the immunoglobulins have been elucidated by use of proteolytic
enzymes and reducing agents. Each immunoglobulin may be visualized as comprising a basic monomeric unit made up of two identical polypeptide chains (10). These chains (designated 'L' chains) are present in all three immunoglobulins and possess the antigenic determinants responsible for the common antigenicity exhibited by this family of proteins (10). Each individual immunoglobulin is characterized also by the presence of two polypeptide chains ('H' chains) which are unique for that particular protein and are responsible for the distinctive antigenic properties of the individual members of the immunoglobulin family.

Recent work has shown that the synthesis and structure of the polypeptide chains that make up these proteins are controlled by well-defined genetic laws, and, like the haptoglobulins and the transferrins, differ from one individual to another (10, 78.-81). It is known that there are two genetic loci which express themselves on different polypeptide chains. One locus on the 'L' chain is designated as the Inv or Gm II factor; it controls synthesis of the polypeptide chains common to all of the immunoglobulins (10, 78.-81). The other locus so far identified is located on the 'H' chain of the gamma II globulin and is termed the Gm I factor. Analogous loci controlling the synthesis of the 'H' chain of the gamma I A and gamma I M globulins have not yet been demonstrated, but it is presumed that they must exist (10), and they have been tentatively designated Gm III and Gm IV factors, respectively.

In summary, therefore, it would appear that the three
immunoglobulins not only possess a common biological function, but also are closely related to each other by virtue of a group of common polypeptide chains whose structure and synthesis is controlled by a single genetic locus. Further, the individuality of each member is due to the characteristics of its 'H' chain, the structure of which is thought to be controlled by a genetic locus distinct for each individual member (10, 79).

Results of genetic studies on the protein products of plasma cells indicate that these cells possess a limited capacity to express genetic information (79, 82). Confirmation of these observations would provide strong evidence in support of Burnet's theories of antibody production.

IMMUNOLOGICALLY INDUCED TISSUE INJURY

PART II:

The humoral and cellular mediators of the body's immune response have been discussed, and it is timely now to review the mechanisms by which host tissue may be injured when immune reactions either specifically or nonspecifically involve autologous antigens.

A. MECHANISMS OF IMMUNE CELLULAR LYSIS

A series of important experiments conducted by Green and Goldberg and recently summarized by the latter (83) clarify
our knowledge of the complex mechanism of immune lysis as it applies to erythrocytes and nucleated mammalian cells. An identical immune mechanism is operative for both cell types (83).

1. The Effect of Antibody on Mammalian Cells in the Absence of Complement:

In electron-microscopy studies with ferritin-labelled antibodies to erythrocytes and Krebs mouse ascites-tumour cells, Green and Goldberg and their co-workers demonstrated fixation of antibody to surface antigens of the cell membrane of the tumour cell (83). A series of focal evaginations of the cell membrane appeared but the viability of the cell was not otherwise disturbed (83, 84). Indeed, these workers demonstrated that mammalian cells can be grown in a significant concentration of antibody and still survive. When complement was absent the ferritin-labelled antibody did not appear capable of directly penetrating the cell membrane, entering the cell interior by a process of pinocytosis, so that the small amount of antibody demonstrated within the cell was always enclosed within a membrane. While not directly impairing the viability of the cell, the fixation of antibody and the subsequent change in conformation of the cell membrane may render that cell more susceptible to agglutination. Goldberg postulates that this may severely handicap those cells whose function depends upon such surface activities as pinocytosis and motility. Similarly, cells which are continually filtered through the reticulo-endothelial system might be selectively sequestered if their surface charge, conformation and agglutinability have been altered (83). Therefore,
whereas the attachment of antibody to the cell membrane may not be directly damaging to the cell, it may nevertheless effectively impair the cell's function or cause its removal from circulation.

2. The Effects of Antibody and Complement on the Mammalian Cell:

The addition of complement to a suspension of mammalian cells and their specific antibody appears to induce a permeability defect in the cell membrane which allows for the development of a marked alteration in the osmotic gradient between the intracellular and extracellular fluids. There is simultaneously a rapid loss of intracellular K+ and other small molecules from the cell, and a net intake of water. As a result, the cell swells, further increasing the permeability of the membrane. This permits the eventual loss of vital ribonucleic acid and protein from the cell interior, and at the same time allows antibody to enter the cell and attach itself to elements of endoplasmic reticulum (83). It appears, therefore, that the combination of antibody and complement combine to kill the cell by the induction of a permeability defect in the cell membrane and consequent osmotic lysis.

Green and Goldberg (83) have analyzed in detail the mechanisms of the observed permeability defect. It is well known that normally the balance of intracellular cation is a result of two processes: a) the passive diffusion of K+ out of, and Na+ into, the cell, under the influence of an electrochemical gradient, and b) the active transport of K+ into and Na+ out of the cell by means of an energy-dependent cation pump (85). The
high intracellular colloidal osmotic pressure of the mammalian cell is effectively balanced by the cation pump, which serves to maintain total cellular ionic concentration at a level lower than that of extracellular fluids and keeps the cell iso-osmotic with its surroundings (85). The immediate effect of the addition of antibody and complement is to increase the passive diffusion of these ions intracellularly, thus overwhelming the cation pump and upsetting the balance between intra- and extracellular cations (83). Within two to three minutes of the addition of complement to the antibody-coated cell, 90 per cent of its intracellular K+ is lost to the surrounding medium and an equivalent amount of Na+ has entered the cell (83); within 10 minutes, 75 per cent of the cellular amino acids and ribonucleotides are to be found in the suspending medium. In contrast, the intracellular ribonucleic acid and protein leak much less readily. Green and Goldberg presume that the almost instantaneous equilibration of ions and small molecules occurs either through 'holes' actually produced by the combined action of antibody and complement, or through enlargement of pre-existing 'pores' in the cell membrane. In any event, the net effect of these exchanges is to increase the total osmotic pressure of the cell in relation to that of the surrounding medium, with the result that water enters the cell along an osmotic gradient created by the differences in colloidal osmotic pressure. Immune lysis is, therefore, a special form of colloid osmotic lysis (83). The osmotic lysis that develops from the combined action of antibody and complement on the cell membrane differs from that produced by such nonimmunological
agents as salicylates, bacterial toxins, and primiquine, solely
in the magnitude of the permeability defect induced in the cell
membrane (83).

The above data have been wholly derived from an in
vitro system characterized by the presence of a high concentration
of heterologous antibody directed against the structural antigens
of cells freely suspended in solution. These conditions are
highly artificial, but Goldberg suggests that the information
derived may nevertheless be usefully applied to immune reactions
involving cells incorporated into body tissues (83). It is his
opinion that, in vivo, the nucleated cell may be partially
protected from immune cytolysis by virtue of its incorporation
into organized tissue. The protection afforded the cell in these
circumstances may be due to a number of factors, which vary from
one tissue to another. In a relatively avascular tissue, the
necessary concentration of antibody and complement may be attained
only with great difficulty. The unhindered osmotic swelling
observed to occur in vitro may be restricted in vivo by the
close physical proximity of adjacent cells and connective tissue
as well as by the presence of a significant concentration of
protein in the extracellular fluid (83). Although the
permeability defect produced in the cellular membrane by the
attachment of antibody and complement has been shown to be
irreversible in vitro, it is conceivable that, if osmotic
swelling is restricted, repair of the membrane might be affected
and death of the cell prevented. Thus it is Goldberg's opinion
that, should immune cytolysis occur in vivo, it probably proceeds
at a slower rate than that observed in vitro.

The fixation of antibody to the surface of the cell and its subsequent lysis in the presence of complement may occur as readily with iso- or auto-antibodies as with hetero-antibodies (83, 86). It has been demonstrated that the reactive antigen need not even be a native component of the cell. In this instance the cell functions as an indifferent bystander, innocently involved in an immune reaction. Shulman has recently presented evidence indicating that such a mechanism is responsible for the thrombocytopenic purpura that occasionally follows quinidine therapy or the haemolytic anaemia sometimes associated with the administration of Stibophen (87, 88). These drug reactions had previously been explained by a theory which suggested that the drug, having reacted and combined with the cell surface, acted as a hapten which then served as stimulus for the production of antibody reactive with the drug-cell complex (88). Such a theory no longer appears tenable to Shulman (87, 88). He suggests that immune complexes of antigen (i.e., the drug complexed to some as yet unidentified component of the serum proteins) and antibody are nonspecifically absorbed on to the surface of the cell which then undergoes lysis in the presence of complement. The absorption of the immune complex, even though immunologically non-specific, nevertheless is selective for a particular cell type (87, 88). This selectivity appears to depend upon the immunochemical characteristics of the antibody. Shulman reports that the antibodies associated with quinidine-induced thrombocytopenic purpura are 7 S
immunoglobulins, whereas those associated with Stibophen-induced haemolytic anaemia are of the 19 S variety (87).

B. ANTIGEN-ANTIBODY COMPLEXES AS PATHOGENIC AGENTS

At the turn of century, von Pirquet made some amazingly astute observations on the analogies existing between the clinical and immunological events in serum sickness (89). He observed that the signs and symptoms of serum sickness developed in relation to the appearance in the systemic circulation of antibody to the injected foreign protein, and suggested, therefore, that serum sickness was due to the action of a toxic compound formed by the interaction of host antibody and foreign protein (89). Lacking the necessary immunological techniques, however, von Pirquet was unable to define the pathogenic agents mediating the tissue lesions and could not substantiate this theory.

Within the past twenty years the availability of purified antigens and more-sophisticated immunological techniques has made it possible to define exactly the pathogenic agent of serum sickness. Hawn and Janeway injected bovine serum albumin intravenously and succeeded in producing glomerulonephritis, arteritis and endocarditis identical to the states produced by Rich and Gregory when they injected horse serum intravenously into rabbits (90). Germuth's important study precisely analyzed the relationship between the intravenous injection of bovine serum albumin and the development of tissue lesions (91). He demonstrated conclusively that lesions developed during the
immune phase of antigen elimination, at a time when soluble antigen-antibody complexes were present in the circulation. The tissue lesions regressed when the antibody circulated freely. Thus it was shown that the toxic compounds suggested by von Pirquet to account for the signs and symptoms of serum sickness comprise complexes of antigen and antibody. The experiments of Rich (92), Janeway (90), Germuth (91) and Dixon (93 - 96) have combined to demonstrate that the interaction of antibody and antigen may result in the formation of macromolecular immune complexes which, if soluble, can circulate in the host, and upon further reaction with serum factors or cells act as an inflammatory stimulus and injure any tissue in which they are deposited, either fortuitously or by design.

The mechanism by which immune complexes cause tissue damage has been clarified by the recent experiments of several workers independently. Considerable evidence has accrued to suggest that the biological activity of the complexes depends upon the attainment of an optimal ratio of antigen to antibody (94, 97, 98). For immune complexes to manifest maximal biological activity, it is necessary that the concentration of the antigen component of the complex be $10^4$ times that of the antibody (94, 97). Complexes formed under these conditions appear to exert a greater systemic anaphylactogenic effect if given intravenously, and a greater phlogogenic effect if given locally, than complexes formed in antibody excess, equivalence, or extreme antigen excess (93 - 96). To quote Dixon: "An explanation for the greater effect of complexes formed in moderate antigen excess is that they are capable both of reacting
with complement and of being soluble and able to diffuse throughout the interstitial fluids of the tissues and thereby attaining an intimate relationship with the various cellular elements. Complexes of other compositions do not possess these properties....." (94).

Ishizaka and his co-workers, in a series of well-conceived studies, defined some of the molecular characteristics responsible for the biological activity of the immune complexes (99). The pathogenicity of the antigen-antibody complexes does not depend upon the antigenic component, rather reflecting the source and immunochemical characteristics of the antibody (94, 99). Active complexes could be formed only from human, rabbit and guinea-pig gamma globulin; horse or chicken gamma globulin was ineffective. Only those antibodies capable of fixing complement and binding to tissues were effective in inducing "complex reactions in vivo" (94, 99). This activity was found to be localized to the 'H' chain of the antibody molecule (10, 94, 99). Ishizaka demonstrated that biologically active complexes manifested a change in their property of optical rotation relative to the unreacted antibody molecule. He proposed that, by virtue of its union with antigen, the configuration of the antibody molecule was altered, probably in the 'H' chain, and that this conferred on the antibody the ability to fix complement, to induce a phlogogenic stimulus, and to acquire a new optical rotation (99). If an alteration in the configuration of the antibody molecule is all that is required to endow that molecule with biological activity, it
should be possible to induce this property by means other than antigen-antibody reactions. Both Ishizaka and Christian have demonstrated that chemically or heat-aggregated human or rabbit gamma globulin developed the capacity to fix complement (99, 100). Ishizaka further showed that some of these aggregates possess the ability to fix to tissue, and if injected locally, to induce increased vascular permeability (99). In these reactions the artificially aggregated gamma globulin acted qualitatively and quantitatively in a fashion similar to that of the immune complexes. These observations serve to confirm Ishizaka's suggestion that the biological activity of the complexes is due to the altered configuration of the antibody molecule.

Antigen-antibody complexes produce their biological activity by interacting with certain humoral and cellular elements of the host (94 - 96). The intensity of the resultant tissue reaction is a function of the concentration of the immune complexes (94 - 96). Large local concentrations are capable of inducing an acute, haemorrhagic, vascular necrosis with polymorphonuclear infiltration, identical in all respects to that seen in the Arthus reaction (94). In the presence of lesser concentrations, the inflammatory reaction induced by the presence of immune complexes is decreased, but some vascular injury still occurs, and Dixon suggests that complexes may react with certain serum constituents to cause the liberation of such soluble substances as the active components of complement (e.g., esterases), fibrinolysin, and anaphylatoxin. These agents themselves may be capable of producing vascular injury without the direct
intervention of immune complexes (94). Various cellular constituents of the host may be affected, directly or indirectly, by the presence of antigen-antibody complexes. Degranulation of mast cells (101), agglutination of leukocytes and platelets (102, 103) with resultant release of lysosomes from the former (104, 105) and histamine from the latter (106, 107), contraction of smooth muscle, production of increased vascular permeability and endothelial proliferation (108), and the attraction of polymorphonuclear leukocytes by chemotaxis (109): all of these mechanisms may evolve from the presence of antigen-antibody complexes. Tissue injury mediated by immune complexes probably is the result of one or more of the cellular effects (94).

Immune complexes are extremely vulnerable to phagocytosis and to digestion by polymorphonuclear leukocytes and macrophages (94), which effectively terminate the phlogogenic stimulus induced by the complex and neutralize their biological activity (94). Tissues rich in reticule-endothelial macrophages are therefore extremely resistant to 'complex' induced tissue injury (94). In certain tissues of the body, however, complexes may accumulate and may be sequestered from contact with phagocytic cells. In such preferred sites, an acute phlogogenic reaction does not occur; rather, the complexes stimulate a chronic response on the part of local fixed cells that may be equally degenerative and inflammatory (94).

Various examples of tissue lesions, experimentally induced by the presence of antigen-antibody complexes, will be discussed in the following section.
1. The Arthus reaction:

The subcutaneous injection of antigen into an animal which possesses circulating antibody against that antigen causes a typical Arthus reaction to develop, characterized by a local, acute, necrotizing vasculitis (39). Formation of relatively large amounts of immune precipitates in the vessel walls is essential to the full development of the lesion (110–113). For this to occur, it is necessary for the antigen (or antibody) to be in the circulation of the host when the antibody (or antigen) is injected locally (39). The exact site of union of antigen and antibody is not known for sure. Some workers have presented evidence to suggest that precipitation occurs within the vessel wall (110, 112), whereas others state that union occurs intravascularly within the lumen of the vessel (112). In either case, antigen-antibody complexes concentrate within the vessel wall and it is there that maximal inflammation and tissue necrosis develop (113). Polymorphonuclear leukocytes accumulate rapidly in and around the involved blood vessels, and masses of agglutinated leukocytes and platelets appear within the lumen of the vessels. These cellular effects result from the chemotactic influence of antigen-antibody complexes as well as from the ability of the complexes to induce agglutination of leukocytes and platelets (102, 103). The agglutinated platelets probably contribute to the furtherance of tissue damage by initiation of the clotting mechanism and release of histamine (106). The role of the clotting mechanism in the pathogenesis of the Arthus reaction is supported by the observation that the
intravenous injection of heparin or Warfarin sodium will decrease the intensity of the reaction (113).

The severity of the Arthus reaction correlates well with the level of circulating antibody (113), but the primary factor governing the toxicity of immune complexes in this reaction is their ability to attract neutrophils. Animals rendered neutropenic by treatment with nitrogen mustard fail to develop the morphological features characteristic of the Arthus reaction, despite the demonstrable presence of antigen-antibody complexes in and around the blood-vessel walls (113). The polymorphonuclear leukocytes play a varied role in the Arthus reaction; in addition to mediating the inflammatory reaction and accompanying tissue necrosis, they are responsible for effective termination of the reaction by phagocytosis of the immune complexes (111, 113).

2. Serum Sickness:

The continued presence in the circulation of a foreign protein provides the stimulus necessary for the development of serum sickness. The presence of the foreign protein stimulates the production of antibody, which initially combines with the circulating antigen in an environment of antigen excess and allows the formation of circulating, soluble, antigen-antibody complexes. (94) With the continued formation of antibody, the zone of antigen-antibody equivalence is approached and the immune complexes become larger and less soluble; at this time they are rapidly phagocytosed and removed from the circulation.
by the reticulo-endothelial system (93). Simultaneously with the detectable appearance of soluble antigen-antibody complexes in the circulation a sharp reduction occurs in the level of serum complement, and acute inflammatory lesions appear in the heart, kidneys, arteries and joints (94). Differing cellular responses may be observed in these lesions, but prominent in most are endothelial proliferation, variable polymorphonuclear infiltration, and increased vascular permeability (94). With the incorporation of all available antigen into immune complexes, and their subsequent removal from the circulation, all of the inflammatory lesions resolve rapidly, leaving only occasional minute scars (94).

Using fluorescent microscopy, Dixon and his co-workers studied the cardiovascular and renal lesions found in serum sickness; they demonstrated the presence of antigen, host complement, and gamma globulin (presumably specific antibody) at the site of the tissue lesions (94). The fact that arteritis, glomerulitis and endocarditis can be induced by the intravenous infusion of immune complexes prepared in vitro provides additional support for the theory that they act as the pathogenic agent in the production of these tissue lesions (114).

The transitory nature of serum sickness limits its usefulness as a conceptual or practical model for the study of those progressive diseases the early stages of which it so closely resembles (e.g., lupus erythematosus, rheumatic fever, rheumatoid arthritis, glomerulonephritis). If the immunological complexes are involved in the pathogenesis of
chronic disease, it seems likely that they must be present in the circulation for prolonged periods of time. Dixon attempted to create such a situation by injecting rabbits daily with a heterologous serum protein (94, 115), thus establishing a condition of continuing antigen excess, which ensured an internal environment favourable to the continued presence of soluble circulating immune complexes (94, 115). The daily administration of the heterologous serum protein produced in the recipient rabbits three broad patterns of antibody response, which were designated as follows. **Group 1** Very large - the animal having an excess of antibody in the circulation at all times and no circulating soluble complexes, **Group 2** Non-existent - in which case antigen was found in the circulation in considerable amounts without the presence of antigen-antibody complexes, **Group 3** Relatively small in relation to the antigenic exposure, with the result that the antibody combined with the injected antigen in an antigen-excess environment and formed soluble antigen-antibody complexes which persisted in the circulation for most of the interval between antigen injections (94). The morphological changes induced in host tissue as a result of daily antigen-antibody reactions depended primarily upon the critical ratio of antigen to antibody, and little, if at all, upon the amount of antibody produced or the immunological character of the antigen (94).

The rabbits in **Group 1** passed rapidly from an environment of antigen excess to one of antibody excess, experiencing only briefly the conditions necessary for production of soluble circulating immune complexes. Typical
serum sickness developed during this period, characterized by acute inflammatory lesions in the heart, blood vessels, and kidneys. As expected, the lesions were of a transitory nature and disappeared rapidly as soon as the animal had established an environment of permanent antibody excess. Because of the excess of antibody in the circulation, further daily injection of antigen resulted in its incorporation into insoluble complexes of antigen and antibody, which were promptly removed from the circulation by the reticulo-endothelial system.

In Group 2: (no antibody response to the daily injection of foreign serum protein) the tissues showed no evidence of disease, indicating lack of toxicity associated with the presence of circulating antigen alone (94 - 96).

The animals in Group 3 (production of too little antibody to cause its elimination from the circulation, but sufficient to induce the formation of soluble, circulating, immune complexes), developed a chronic progressive glomerulonephritis (94 - 96). Morphologically the renal lesion appeared initially as a membranous glomerulonephritis associated with little endothelial proliferation. As visualized by immunofluorescence, concentrations of antigen, host gamma globulin, and complement, could be demonstrated in the thickened basement membrane. Electron microscopy revealed a lumpy, dense deposit along the outer aspects of the basement membrane, corresponding to the antigen, gamma globulin, and complement demonstrated by the Coon technique. The complexes deposited in the basement membrane persisted for up to one year after cessation.
of antigen administration, and, as long as they were there, renal malfunction persisted (94 - 96). The continuing circulation of soluble immune complexes failed to induce lesions in any other organ - in contradistinction to the findings in acute serum sickness, in which acute inflammatory lesions develop in multiple organs.

The vulnerability of the kidney to the presence of circulating immune complexes may be related to its extensive blood flow and the filtering function normally performed by this organ (94). Dixon suggests that complexes of antigen and antibody may be able to traverse the basement membrane and once there be retained. either along its outer margins or between the basement membrane and the epithelial cells. In this location the complexes appear to be sequestered from tissue or circulating cells capable of degrading them (94); and their continued presence is associated with proteinuria, suggesting that they interfere with the function of the basement membrane and/or epithelial cells (94). In this situation it would appear that deposition of complexes in a sequestered site is capable of interfering with the function of adjacent structures (e.g., the bone marrow) without involvement of other factors (e.g., inflammatory reaction) (94).
C. TISSUE DAMAGE ASSOCIATED WITH THE 'DELAYED' OR 'CELLULAR' TYPE OF IMMUNITY

The pathogenic mechanisms responsible for the tissue destruction that so frequently accompanies the 'delayed' or 'cellular' type of immune response are less clearly understood than those mediated by humoral antibody or antigen-antibody complexes. Those tissue lesions generally considered to be the result of 'delayed' immunity include such diverse entities as the bacterial or tuberculin type of allergy, contact allergy, homograft reaction, and the lesions that accompany certain experimentally induced auto-immune diseases (70, 71), such as experimental auto-immune encephalomyelitis and experimental auto-immune thyroiditis. Despite their differing aetiologies, these lesions exhibit the same basic morphological characteristics: vasodilatation; and perivascular infiltration of mononuclear cells located specifically in relation to the distribution of the sensitizing antigen, e.g., subcutaneous injection of old tuberculin results in a tissue reaction characterized by a mononuclear-cell infiltration of the dermis, and contact sensitivity in a mononuclear-cell infiltration restricted to the epidermis, whereas a vascular homograft of skin has mononuclear cells throughout the graft (70, 71). The perivascular infiltrative cells are of varying morphology and their exact identity remains obscure. Waksman prefers to use the noncommittal term 'mononuclear cells' to describe the cellular infiltrate (71); Gell, on the other hand, claims that they have the appearance of small or large lymphocytes (73) -
an opinion shared by Gowans and his colleagues who, using radioactive materials, have presented very convincing evidence in favour of the small lymphocyte's primary role in initiating homograft reaction (116, 117).

In an attempt to identify the events that follow initiation of a 'delayed' response (e.g., the subcutaneous injection of old tuberculin, the placement of a skin homograft, or the injection of an emulsion of brain and Freund's adjuvant), careful histological studies have revealed the presence of a massive perivascular accumulation of mononuclear cells in the antigen-containing parenchyma (70). At present the evidence strongly favours the hypothesis that, in response to the presence of the specific antigen, the mononuclear cells located within the regional lymph nodes and in the blood stream actively proliferate and disseminate (65, 70). The ensuing tissue damage appears entirely secondary to the presence of the cells and exhibits three quite distinct morphological forms; these have been designated (1) the invasive necrotic lesion, (2) the vascular necrotic lesion, and (3) massive necrosis (70).

a) The invasive necrotic lesion may appear in homograft reactions (70, 118) and in several other types of 'delayed' reactions, such as experimental auto-immune encephalomyelitis and thyroiditis (70, 71). The homograft reaction is characterized by a rapid, massive invasion of the graft by mononuclear cells of host origin which appear first within the capillaries of the dermis and finally in the epidermis (118). Billingham and others have stated that these cells are derived from circulating,
'sensitized' lymphoid cells that actively migrate through the blood-vessel walls to attain a close relationship with the epidermal cells of the graft (65, 71, 119). During the course of rejection there is an intimate association between infiltrating mononuclear cells and epidermal cells of the graft, terminating before the appearance of cellular damage. Other types of inflammatory cells do not appear until graft rejection is well advanced (118).

b) The vascular necrotic lesion, initially described by Gell (73), in its simplest form consists of impregnation of the vessel wall with 'fibrinoid' and an exudation of polymorphonuclear cells superimposed upon the typical infiltration of mononuclear cells. In the extreme reaction, actual necrosis of the vessel wall and of contiguous parenchyma may develop. Waksman noted that, when this reaction occurs in experimental encephalomyelitis, the 'fibrinoid' is, or contains, plasma proteins and its presence precedes the appearance of polymorphonuclear cells (71). He therefore suggests that the infiltrating mononuclear cells, on coming in contact with the specific antigen, release an agent which affects vascular tone and permeability, allowing leakage of plasma constituents and sometimes of formed cellular elements and "leads to the subsequent chemotactic attraction of polymorphonuclear cells" (71). Since vasodilatation occurs in the initial stages of the 'delayed' reaction, it is probable that the vaso-active agent is released early in the course of the immune reaction (70, 71, 121). The exact nature of this agent remains a point of controversy. On
the basis of his experiments Voisin maintains that the vaso-active substance is not histamine (121). Inderbitzen, however, claims to have shown an accumulation of histamine at the sites of 'delayed' reactions, correlating with the presence of mononuclear cells (122). As evidence in favour of the role of histamine as a mediator of the vascular reactions associated with 'cellular' sensitivity, Waksman cites the work of Vivera and Fisher, who claim to have demonstrated the accelerated synthesis of histamine in lymphoid cells actively sensitized to the purified protein derivative of tuberculin (71). Johanosky recently reported that buffy-coat cells obtained from rabbits with 'delayed' sensitivity release both a pyrogen and a factor capable of evoking an inflammatory response when they are injected into living tissue after having been in contact with the antigen in vitro (120). These observations certainly suggest that sensitized mononuclear cells involved in 'delayed' reactions might, when exposed to the antigen, release a substance which induces an inflammatory reaction and increases vascular permeability.

c) **The massive necrosis** so characteristic of the tuberculin reaction (70) is thought by Waksman to be of ischaemic origin and may be attributed partially to the accumulation of occluding aggregates of mononuclear cells within the vessel lumen (71). He further contends that the release of vaso-active agents, induced by the immune response, contributes to the ischaemia by producing vasodilatation, venous stasis, and arterial vasoconstriction (71).
The role of the chemical mediators of the vascular changes that accompany 'delayed' sensitivity have been mentioned; the pathogenic role played by the infiltrating mononuclear cells remains to be discussed further, however. In this regard, first to be considered is the nature of the agent that mediates the immunological specificity of the 'delayed' reaction. Paramount is the fact that this type of immune reactivity cannot be transferred with serum but only with mononuclear cells obtained from sensitized animals (71). It is postulated, therefore, that 'sensitized' cells capable of accumulating and reacting with the specific antigen wherever it is to be found are responsible for mediating the 'delayed' reaction (65, 71). Mononuclear cells capable of reacting specifically with the homograft have been found in the blood stream and regional lymph nodes of graft recipients (119). The lymphocyte is almost certainly one of these cells (65, 116). Further evidence supporting the role of the mononuclear cell in graft rejection has been elicited by several experimenters working independently, the results of their work suggesting the necessity for actual contact of the lymphocyte with the cells of the homograft to ensure its destruction (116, 118, 123, 124, 125).

If lymphocytes are truly sensitized cells capable of accumulating at the site of the specific antigen, then experimental verification of this should be possible by the use of sensitized lymphocytes labelled with $^3$H thymidine. Experimental studies in animals subjected to passive transfer of 'delayed' hypersensitivity with labelled lymphoid cells
obtained from sensitized donors revealed that the great majority (80% - 90%) of the infiltrating cells were of host and not donor origin (126, 127, 128). The proportion of labelled donor cells in the 'delayed' reactions ranged from only two to eight per cent (126). In experiments from three different laboratories, no evidence could be found for the specific accumulation of sensitized mononuclear cells concentrated at the site of a 'delayed' reaction, since comparable numbers of labelled cells also accumulate at the site of an immunologically unrelated 'delayed' reaction (125, 127). Conversely, Najarian and Feldman reported a small but definite accumulation of sensitized cells at the site of antigen localization (129).

The recent work of David and his co-workers has modified the interpretation of these results by emphasizing the extraordinary effect one 'sensitized' cell can have upon a large population of mononuclear cells. They showed that as little as 2.5% of the lymphoid cells need be sensitized for the rest of the cells to behave as though they were derived from a sensitized animal (130, 131).

An alternative possibility exists that actively sensitized cells derived from a donor with 'delayed' hypersensitivity could transfer to the recipient's mononuclear cells some factor that could confer sensitivity on them and cause their accumulation at the sites of antigen location (68). In this regard, Lawrence has demonstrated the presence of a 'transfer factor' in man (132, 133), but it has not yet been
found in animals.

As a result of the work of Boyden and his colleagues it has been suggested that, during the course of immunization, certain antibodies are produced (cytophilic antibodies) that are capable of attaching to mononuclear cells, and then are responsible for mediating the specificity of the 'delayed' reaction (134). The relationship of such an antibody to 'delayed' immunity has not been established, but the recent observations of Rosenau and Moon (125, 135) and of Koprowski and Fernandes (136) indicate that such a mechanism may be operating. These workers have reported independently that lymphocytes derived from sensitized animals are capable of damaging their specific target cells in tissue culture. Identical results could be obtained by incubating 'normal' lymphocytes with immune serum (125).

In summary, the proposition that living mononuclear cells mediate the events that characterize immune reactions of the 'delayed' type represents the most reasonable hypothesis concerning the pathogenesis of this reaction (65). Whether or not humoral antibodies also are concerned in this reaction cannot at present be ascertained.
PART III:

The problems to be discussed in this final section are the relationships between immune tolerance, auto-immunity, and tissue damage. Brent and Medawar have suggested that the biological *raison d'etre* of tolerance is to prevent the organism's immunologically competent cells from responding to the antigenic challenge presented by neighbouring cells and proteins (63). Consideration of whether the properties of tolerance qualify it to fulfil this function must be preceded by a definition of what constitutes an auto-immune reaction.

Auto-immunity may be broadly defined as an immune response resulting from stimulation of host immunologically competent cells by antigenic material derived from host tissues. This definition may be extended to encompass those immune reactions that may develop in response to the presence in the host tissues of a genetically foreign protein possessing antigenic determinants in common with certain autologous cells. Auto-immune reactions are not restricted to a specific category of immune reactivity or a particular chemical class of antigens (63). The same diverse spectrum of immune reactions discussed previously in regard to antigens of foreign origin may be elicited equally well by autologous antigens (63). The heterogeneity of the host antigens and the variety of immune reactions that theoretically they are capable of provoking co-exists with a mechanism of
tolerance that normally prevents any immune response from developing to these antigens (63): "There is no known property of tolerance that would disqualify it from acting on behalf of native antigens to prevent auto-immune reactions from occurring" (63).

It was implied in the definition of auto-immunity that the reaction was mediated by a normal immune apparatus. There is evidence to suggest, however, that - at least in man - auto-immune reactions probably never occur in the presence of a completely normal immune system (49). The breakdown of the normal homeostatic mechanism of tolerance is being intensively investigated to ascertain the role that this may play in the pathogenesis of a variety of clinical conditions (55, 138 - 141). Certain well-established models of auto-immune disease exist, and experimental data derived from them indicate that, in certain circumstances, immune tolerance to autologous proteins may be broken, thus allowing for the development of a state of auto-immunity (49, 55, 63).

Several examples of these will now be considered.

A. AUTO-IMMUNE REACTIONS TO NORMALLY INACCESSIBLE ANTIGENS

It has long been known that the body's immune mechanism may fail to recognize as 'self' such antigens as lens and uveal-tract protein, spermatozoa, thyroglobulin, and central-nervous-system tissue (142): the parental administration of these tissue antigens is fully capable of provoking production of auto-antibodies in the immunized animal (142). These tissues are
distinguished from others in the body by virtue of the unique antigenic determinants that each possesses (39, 142). Such antigens are designated 'organ-specific', and as such may occur in the corresponding organ of various species, e.g., central-nervous-system tissue (142, 143), testes (144), thyroglobulin (31, 34). Alternatively the organ-specific antigen may be restricted to a particular organ of a particular species, e.g., the pancreas (145). Present evidence suggests that the potential auto-antigenicity of organ-specific antigens is a result either of their localization behind a basement or cellular membrane - which can constitute an important, perhaps absolute, barrier to the passage of antigenic material - or that the organs in which they are located lack lymphatic drainage (142). In both instances it is probable that these antigens never reach the immune system of the host in sufficient amounts persistent enough to allow for the induction of tolerance to them (49, 142). This interpretation is consistent with the fact that experimental work shows the brain, lens protein, spermatozoa, etc., to serve as effective immunizing agents in the same animal from which they were derived initially (142).

The experimental work of Metchnikoff (44) has been confirmed by many workers. Guyer (146), McCartney (147), and Kennedy (148) claim to have produced auto-antibodies to sperm and testicular degeneration in experimental animals, by parenteral administration of a suspension of sperms or whole testicular tissue. Whereas such immunizing procedures gave rise to the production of auto-antibodies, it was not until
many years later, with the advent of adjuvants, that testicular damage could regularly be produced (39, 49, 142, 149, 150, 151). Freund (152), having perfected a technique of Pasteur (153), was consistently successful in inducing aspermatogenesis and orchitis by immunizing guinea pigs with homologous testes emulsified in an adjuvant consisting of a water-and-oil mixture of *Mycobacterium butyricum*, Bayol F, and Arlacel-A40* (154, 155). The adjuvant-tissue-emulsion technique was employed by Freund and others to enhance antibody production (156–159) and to increase the antigenicity of tissue components that normally were poorly antigenic (39, 150, 151, 159).

The mechanism by which adjuvant enhances antibody production and increases the antigenicity of tissue antigens has been discussed by many workers. Freund considered that the dispersal of antigen in the water-and-oil emulsion enabled it to remain longer at the site of injection, thereby prolonging the interval of antigenic stimulation (158). Also, adjuvant may allow more efficient dissemination of antigen to the body's antibody-forming cells (156, 160, 161). It has been demonstrated that, following the administration of adjuvant, a marked hyperplasia of the reticulo-endothelial system occurs, with increase in size of lymph nodes and spleen (156) and with conspicuous germinal centres visible within the hyperplastic lymphatic follicles (156, 157). Coons and others have

*Obtainable from Difco, Canadian Laboratories, Montreal, Quebec.*
demonstrated extensive proliferation of plasma cells within these glands (162, 163). It is not surprising, therefore, that animals immunized with antigen emulsified in Freund's adjuvant produce an amount of antibody approximately five times the amount produced in controls immunized with antigen alone (156). In addition to increasing the antibody-producing capacity of the immunized animal, adjuvant is capable also of procuring maturation of the animal's immune system. Neonatal rabbits immunized with an antigen-adjuvant emulsion produce antibody at a significantly earlier age than those who receive antigen alone (157, 164).

Although it is known that adjuvant may enhance the antigenicity of tissue components that normally are poor antigens, the mechanism of this is still a matter of some controversy (150, 151, 159). Burnet views the effect within the context of his clonal-selection theory (49), which postulates that, during neonatal life, immunologically competent cells arise which are reactive with all types of antigen. 'Clones' of cells reactive with 'self components' normally are eliminated or suppressed (49), and Burnet considers that the abnormal conditions imposed on the host by the use of adjuvant cause immunologically competent cells to arise, possessing the same capacity as embryonic ones to react with native tissue and thus inducing an auto-immune reaction (49).

The production of tissue injury by parenteral administration of certain organ-specific antigens emulsified in Freund's adjuvant has been well documented, and its
pathogenesis is known to involve an immune reaction by the host to certain of his own tissue antigens. The criteria for establishing that a tissue lesion is the result of an immunological reaction has been clearly defined (142):

1) The specificity of the immune reaction for a well-defined antigen must be demonstrated.

2) Administration of antigen must be followed by a suitable latent period before the appearance of antibodies or 'sensitized cells'.

3) This reaction must be amenable to passive-transfer experiments, utilizing either immune serum or 'sensitized cells'.

4) The reaction must be susceptible to suppression by immunosuppressive drugs or by experimental induction of tolerance to the specific antigen.

5) Finally, progression of the lesion may correlate with dermal sensitivity of the delayed type to the intracutaneous injection of the same antigen.

Experimental auto-immune encephalomyelitis, thyroiditis, and aspermatogenesis fulfil these criteria to varying degrees.

The specificity of the immune reaction that mediates the pathogenesis of the experimentally induced aspermatogenesis and orchitis is clearly illustrated by the fact that immunization with an emulsion of adjuvant and prepubertal testes, which lacks the organ-specific antigen (165), cannot produce orchitis or induce an immune response (165). On the other hand, immunization with adult testicular tissue effectively produces both immune
response and tissue lesion (165). In a classical example of the antigenicity of an autologous tissue and the specificity of the immune reaction it is capable of evoking, Kabat and his co-workers immunized monkeys with nervous tissue derived from their own brains after lobectomy (166); after a suitable latent period the monkeys developed encephalitis. Similarly, immunization with heterologous, homologous or autologous thyroid tissue results in the development of thyroiditis and the production of antibodies specific for thyroglobulin (31, 32, 34). The organ antigens are well documented in three examples of experimental auto-immune disease - thyroiditis, encephalitis, and orchitis - and the specificity of the immune reaction is illustrated by the restriction of tissue lesions to those organs containing the antigens present in the immunizing emulsion. Thus, parenteral administration of an alcoholic extract of brain, which possesses antigens in common with those of testes, can induce both encephalitis and aspermatogenesis in the immunized animal (165).

Passive-transfer experiments confirm that immune reactions are involved in the pathogenesis of these experimental disease states (31 - 34, 167, 168). The tissue-destroying immune reaction (generated by the parenteral administration of thyroglobulin, brain, or testicular tissue, emulsified in Freund's adjuvant) can be passively transferred, by way of immunologically competent cells derived from afflicted animals, to normal recipients of the same inbred strain.

The development of encephalitis or thyroiditis can be
prevented by administration of 6-mercaptopurine at the time of, or immediately after, immunization with thyroid or brain tissue (35, 171). The failure of the immunized animal maintained on this drug to develop encephalitis or thyroiditis does not appear to result from the production of leukopenia, general debility, or any nonspecific toxicity associated with its use. Rather, existing evidence suggests that this agent exerts its protective effect(s) by suppressing humoral or cellular components of the immune response (169 - 171).

Finally, the progress of experimentally induced orchitis, thyroiditis and encephalitis correlates well with dermal sensitivity of the 'delayed' type elicited by the intracutaneous injection of the immunizing antigen (34, 142, 143).

In summary, it seems evident that immunological reactions mediate the pathogenesis of these experimentally induced auto-immune diseases. They represent valuable experimental models documenting the destructive effects which may arise from auto-immune reactions.

B. BREAKDOWN OF TOLERANCE BY CROSS-REACTING ANTIGENS

Although by definition immunological reactions are highly specific (39, 159, 172), it is not unexpected that exposure to a foreign antigen may result in production of antibodies capable of cross-reacting with certain tissue constituents of the host possessing antigenic determinants in common with the foreign antigen. Such a mechanism is postulated
by Kaplan to account for the pathogenesis of rheumatic heart disease (173). The data upon which he bases his hypothesis were derived from a series of well-conceived experiments which can be summarized as follows (173 - 181):

a) Immunofluorescence studies revealed that the sera of patients with rheumatic fever or rheumatic heart disease commonly exhibited an affinity for the constituents of human heart (173, 176). The serum factors manifesting this affinity consisted of the 7S or 19 S component of the immunoglobulins (176). These antibodies exhibited a reactivity for several different components of the myofibres: some sera stained the subsarcolemmal rim of the sarcoplasm, which Kaplan terms 'subsarcolemmal-sarcoplasmic staining'; others stained the edge of the myofibres, and were said to have produced a pattern of intermyofibrillar staining; and some sera exhibited homogeneous staining, termed by him a 'diffuse sarcoplasmic' pattern (176). Kaplan also demonstrated the presence of an antibody reactive with autologous heart, in sera obtained from rheumatic and non-rheumatic patients two weeks after cardiotomy (173). In control sera obtained before operation, specific immunofluorescence was not observed or, when seen, was of lesser intensity (173).

b) Deposits of bound gammaglobulin were visualized by immunofluorescence in a significant number of biopsies obtained from the auricular appendages of patients with rheumatic heart disease (174, 175). These deposits were widely distributed in the myocardium, mainly in the sarcolemma and subsarcoplasm of
the myofibres. The staining was observed, but to a lesser extent, in the walls of blood vessels and in interstitial connective tissue. Kaplan states that tissue sites containing bound gamma-globulin showed evidence of the histochemical alteration characteristic of fibrinoid (i.e., increased affinity for eosin, increased periodic-acid-Schiff reaction, and intense metachromasia with toluidine blue). Deposition of the gamma-globulin was specific, for neither albumin nor fibrinogen could be demonstrated in these locations. Gamma-globulin was not observed in the Aschoff bodies. In none of the material studied was it possible to demonstrate a correlation between the presence of circulating auto-antibodies to the heart and the presence of bound gamma-globulin (173, 174, 176).

c) In studies designed to identify the antigen(s) in heart tissue reactive with the auto-antibodies just described, Kaplan demonstrated by means of complement-fixation, immunofluorescence, and flocculation techniques, the presence of organ-specific heart antigens (177). As observed by immunofluorescence these antigens were mainly localized to the myocardial cells, between the myofibrils, producing in Kaplan's words, "an intermyofibrillar pattern of staining". Such a pattern, he feels, is compatible with the possibility that the antigen originates from the sarcoplasmic reticulin or the mitochondria of the myocardial cell (177). Immunization of rabbits with a homogenate of homologous heart tissue, emulsified in an adjuvant of alumina gel, resulted in the production of circulating auto-antibodies and of foci of myocardial necrosis (178). It was Kaplan's opinion that the circulating auto-
antibodies were involved in the pathogenesis of these cardiac lesions, since deposits of bound gammaglobulin could be demonstrated in foci within the cardiac myofibres. Bound gammaglobulin was widespread in those lesions with marked degeneration and fibrosis (178). However, he was unable to demonstrate the antibody nature of the bound gamma-globulin (173, 178).

d) More recently Kaplan has demonstrated a mutual sharing of antigens between mammalian heart and the cell wall of certain group-A streptococci (174, 179, 181). The immunological relationship between the bacterial cell-wall antigen and the heart tissue constituents was demonstrated, using both anti-streptococcal and anti-heart sera (173, 174, 179, 181). In a significant percentage of sera obtained from patients with recent streptococcal infection, he detected antibody to the streptococcal cell-wall antigen, which cross-reacted with the constituents of cardiac myofibres (180). Immunofluorescence showed the myocardial constituents cross-reactive with the streptococcal cell-wall antigen to be localized to the sarcolemmal or subsarcolemmal sarcoplasm of the cardiac myofibres. As noted previously, immunofluorescence studies had indicated an intermyofibrillar distribution for the organ-specific antigen of heart tissue, indicating that the two heart antigens probably are not related (177, 180). In the majority of cases absorption of the clinical sera with human heart, but not with other human organs, abolished the affinity for streptococcal antigen (180).
The significance of this cross-reactive antibody remains to be determined. Kaplan postulates that it may represent the pathogenic mechanism responsible for production of rheumatic fever and rheumatic heart disease (173, 179, 180). The fact that bound gamma globulin locates in the cardiac myofibres in rheumatic heart disease, and that circulating auto-antibodies to the constituents of cardiac myofibres exist in the sera of these patients, prompts him to believe that these antibodies might become fixed in vivo. If that were so, the deposits of bound gamma-globulin could be derived from either antibody formed in response to the heart-related, cross-reactive antigen of the streptococcus or from activation of a true autoimmune mechanism subsequent to the release of the organ-specific heart antigen. The release of this antigen could result from an initial insult inflicted upon the heart by the cross-reactive antistreptococcal antibody. It is perhaps significant that antibody reactive with both sets of antigens could be demonstrated in the sera of patients with rheumatic heart disease (180). Direct proof of Kaplan's hypothesis would require identification of deposits of gamma-globulin in the heart as auto-antibody, and the demonstration of its cytotoxicity. To date this has not been achieved (173, 174, 180).

C. BREAKDOWN OF TOLERANCE INDUCED BY ALTERED 'SELF' ANTIGENS

The alteration of autologous cells and macromolecules by physical, chemical, or infectious processes, or through the
action of drugs, sometimes modifies these tissue antigens so that they lose their immune tolerance (142, 182). The altered 'self antigen' then may elicit an immune response with the production of antibodies and sensitized cells capable of reacting with the 'modified self antigen', and at times cross-reacting with the original unmodified host component (142, 182). Lawrence regards this sequence of reactions as possibly the pathogenic mechanism responsible for production of auto-immune disease (182). It is his theory that alterations in host-cell constituents, sufficient to render them foreign and thus capable of procuring an immune reaction, may be produced by bacteria with a preference for intracellular residence (e.g., tubercle bacilli, brucellae), by viruses that require it, and by fungi or other as yet undefined tissue-altering agents. Lawrence refers to this as the 'self plus X' theory of auto-immunity (182). The conversion of 'self' constituents to 'foreign' initiates an immune response directed against 'self' plus 'X' wherever they are found in combination - an immune response that is viewed by Lawrence as being of the 'delayed' type (182). He postulates that the immune reaction provoked by the altered native antigen induces in the leukocytes of the sensitized host, in a manner still undefined, the formation of a soluble, dialyzable factor which is then capable of mediating future immune reactions generated by the presence of the stimulating antigen (132, 133). Lawrence terms this agent the 'transfer factor' (133). To date, all attempts to identify it have succeeded only in defining what it is not. The transfer factor is capable of endowing a normal recipient with the specific
sensitivity of the sensitized donor, and thus transferred sensitivity may persist for months or for years. The activity of the transfer factor is unaffected by treatment with desoxyribonuclease, ribonuclease, or trypsin (132, 133). It is neither conventional antigen nor antibody. Lawrence favours the interpretation that the transfer factor represents an intermediate product resulting from the interaction of antigen and a specific cell population of the immunized subject (133). Thus, the transfer factor may activate immunologically competent cells, endowing them with the requisite immune specificity enabling them to react with the sensitizing antigen (133).

D. BREAKDOWN OF TOLERANCE BY AN ALTERED IMMUNE APPARATUS

Thus far the experimental development of an autoimmune state has been considered solely within the context of the potential auto-antigenicity of autologous proteins. There is much evidence to suggest, however, that the fundamental factor in the development of auto-immunity is not the potential auto-antigenicity of the host's cells and proteins, but rather a basic alteration in the antibody-forming tissues themselves (49, 66, 150). Burnet has suggested that auto-immune disease in man is associated with the presence of what he calls "forbidden clones" of immunologically competent cells (47, 48, 49, 50). These clones consist of lymphoid cells differing from the other lymphoid cells of the body in that they have an inherited capacity to react with an antigen naturally present
in the body and to resist the normal homeostatic processes that should ensure the absences of such clones (47, 48, 49).

As mentioned earlier, a proliferation of immunologically competent cells occurs within the germinal centres of the lymph node in response to antigenic stimulation (65, 66, 183). Whether by genetic or other mechanisms, the presence of antigen results in a 'conditioning' of these cells that imparts to them the functional capacity to react with an immune response to the stimulating antigen (65). The presence of antigen has resulted in the development of a 'clone' of 'committed' or 'conditioned' lymphocytes that are then incapable of any other line of development (65). Such cells do not normally proliferate; instead they may exhibit a very slow cyclical turnover or manifest an extremely long life-span which has been estimated by Yoffey as up to 11 years (65).

Under the stimulus of a viral infection or neoplastic change, the lymphoid cells may proliferate (184). These cells are identical with or markedly similar to normal, immunologically competent lymphocytes (66, 185), and are capable of being stimulated by antigenic determinants present within the tissues of the host (184, 185). These cells make up what Burnet calls 'forbidden clones' of immunologically competent lymphoid cells; he postulates that such cells are responsible for the production of auto-immune disease whenever it occurs in man (49).

Substantial support for the theory that disordered immune mechanism plays the primary role in the pathogenesis of auto-immune disease lies in a consideration of haematological complications associated with a benign or malignant proliferation
of the cells which comprise the reticulo-endothelial system (i.e., in tumours composed of, or closely related to, normal immunologically competent cells).

It is well known that, in primary atypical pneumonia, lymphocytic proliferation occurs and is associated in some cases with the development of a considerable concentration of cold haemagglutinins (66, 185). The studies of Leddy et al. have shown these cold haemagglutinins to possess the attributes of true auto-antibodies, reacting with the I antigen of the patient's own red blood cells (186). A possible explanation for the presence of these abnormal antibodies may be found in the proliferation of lymphoid cells that accompanies the viral infection, giving rise to 'forbidden clones' of immunologically competent lymphoid cells capable of reacting with autologous red cells (66, 185). Dacie considers that there is no firm evidence in support of the alternative hypothesis that the auto-immune haemolytic state is due to a viral-induced change in red-cell antigenicity (185).

Green suggests that the lymphocytes of patients with chronic lymphocytic leukaemia, although presumably abnormal, may retain immunological competence (187). If so, the normal red-cell antigens of the host may not be recognized as 'self' by these neoplastic cells, resulting in production of antibodies to the red cell and induction of auto-immune haemolytic anaemia (187). Dameshek has advocated a similar theory in discussing the mechanisms of development of the haemolytic anaemia that commonly accompanies various lymphoproliferative disorders (188).
He cites the frequency with which auto-immune haemolytic anaemia develops in these disorders and its infrequency in myeloproliferative disorders as evidence in favour of such a hypothesis (66): approximately 20 per cent of patients with chronic lymphocytic leukaemia develop, at some time during the course of the disease, auto-immune haemolytic anaemia (66, 185).

The patient with generalized lymphoproliferative disease (whether benign or malignant) may be considered as having incorporated into his tissues abnormal lymphoid cells possessing the potential capacity to respond to the antigens present in neighbouring autologous cells and proteins (66). Starting from results obtained by Billingham, Brent, and Medawar in their initial experiments (189), Simonsen with others has developed an experimental model to demonstrate the adverse affects that accrue to the host when it tolerates within its tissues genetically foreign, immunologically competent lymphoid cells (190, 191). He has observed that spleen cells derived from an unrelated adult donor, when inoculated into immunologically incompetent embryonic chicks, produced hepatosplenomegaly with distinct pathological lesions in these and other organs, and acute haemolytic anaemia with positive direct Coombs test (190). Histological studies of the affected spleens indicated replacement of the normal cell population to a varying extent by proliferating lymphoid cells of donor origin (190). Thus it appeared that the pathogenesis of the haemolytic anaemia and the hepatosplenomegaly was developed from an attack on the host tissues by the proliferating, foreign, lymphoid cells
(191, 192), and the condition was therefore designated the 'graft versus host reaction', or 'runting syndrome'. The experiments of Aisenberg and Waksman have verified that immunological competence of the recipient animal is not necessary for the development of the runting syndrome (193). These workers produced widespread tissue damage by injecting foreign lymphoid cells into rats rendered immunologically incompetent by thymectomy at birth. In this state the rats were incapable of responding to and rejecting the foreign cells, which therefore survived and proliferated within their tissues.

The similarity between the runting syndrome and what are commonly referred to as the collagen diseases has been stressed by Ziff and his co-workers, who produced joint, heart, kidney and chronic skin lesions by injecting lymphoid cells into adult rats rendered tolerant to these cells (137 - 139). The role of the grafted cells in the pathogenesis of this experimentally induced multi-organ disease was illustrated by the specific sequence of events manifested by the cutaneous lesions. The latent period observed between inoculation of the foreign lymphoid cells and the appearance of skin lesions was typical of that seen in a primary immune response and could be shortened significantly by the use of lymphocytes obtained from donors pre-immunized to the recipient's skin (137 - 139). Finally, the runted animal could accept a homograft of skin derived from the donor of the lymphocytes while, at the same time, rejecting an autograft of its own skin (137 - 139). A mild to moderate polyarthritis developed in approximately 50
per cent of the runted rats, having the histological characteristics of a subacute or chronic synovitis. Fibrinoid necrosis, infiltration with mononuclear cells, and occasional destruction of cartilage were observed. Inflammatory changes in the heart were noted in a high percentage of cases. Grossly, the heart was enlarged, with petechial haemorrhages and with nodules on the valves. Histologically, the valvular changes were characterized by oedema, infiltration with mononuclear cells, and some degree of fibrosis (137 - 139).

It is unnecessary to stress that these experiments represent highly artificial situations in which the immunologically competent cells were not derived from the host, but were grafted into him by artificial manipulation. However, this experimental approach does illustrate the results that might be expected to occur when immunologically competent lymphoid cells undergo 'mutations', and give rise to the equivalent of the genetically foreign lymphoid cells just described in the 'graft versus host reaction'. Such a situation has been postulated by Burnet (49) and Dameshek (66) to explain the pathogenesis of systemic lupus erythematosus.

Patients with lupus erythematosus appear to possess a fundamental abnormality of the immune system that permits the formation of auto-antibodies and delayed hypersensitivity to a diverse group of widely distributed tissue components, some of which are not even good antigens, e.g., desoxyribonucleic acid, and the circulating proteins of the clotting complex (50, 142,194, 195). The sera of these patients may exhibit a battery of
antibodies against the cellular components of the blood (red cells, leukocytes, and platelets) (185), against some circulating proteins involved in the clotting reaction (195, 196), and against nuclear and cytoplasmic constituents of nucleated cells (197, 198). These antibodies will react as readily with autologous as with homologous material, as has been demonstrated by the occurrence of the LE-cell phenomenon when both components of the reaction were derived from the same patient (199). Friedman et al. showed recently that patients with lupus erythematosus may exhibit cutaneous reactions of the delayed type to the injection of autologous white cells (200), and similar findings have been reported for autologous desoxyribonucleic acid (201). Burnet underlines the fundamental nature of the immunological abnormality that permits both the formation of auto-antibodies and delayed hypersensitivity to these cellular constituents (49). He points out that lymphocytes are liable to be broken down under a variety of stress conditions, with the result that constituents of the cell nuclei are almost certainly always present in the immediate environment of lymph nodes, where immunological processes are initiated and implemented (50). Therefore, of all the autologous tissue components, the immune apparatus should be most fully tolerant to the constituents of the cell nuclei (50). Yet, under the conditions existing in lupus erythematosus, antibodies and delayed hypersensitivity to these very constituents appear.

It is becoming increasingly certain that genetic factors are involved in the expression of this unusual propensity for immune reactivity observed in patients with systemic lupus
and the collagen diseases in general (49, 202 - 206). The evidence for this is based on numerous family studies which have revealed that, in comparison with control groups, there is a statistically significant increase in the frequency of collagen diseases and serological abnormalities in the relatives of patients with rheumatoid arthritis, lupus erythematosus, scleroderma and dermatomyositis (202 - 205). To paraphrase Epstein: "We have a group of diseases which runs in families associated with abnormal auto-antibodies which also run in families" (206). In addition to these abnormal antibodies, the asymptomatic relatives of the afflicted patient may exhibit quantitative abnormalities in their immunoglobulins, such as agammaglobulinaemia, hypogammaglobulinaemia, a selective hypergammaglobulinaemia of the myeloma or macroglobulin type, or diffuse hypergammaglobulinaemia (205). The results of family studies indicate, therefore, that there is a familial predisposition to the collagen diseases as well as to abnormalities in immunoglobulin synthesis (205), and it is conceivable that a genetically induced abnormality of the immune system may be involved in their pathogenesis (205).

The first experimental evidence of the influence of genetic factors on the normal functioning of the immune apparatus has been the demonstration in a highly inbred strain of mice of a spontaneously occurring auto-immune disease markedly similar to that observed in systemic lupus erythematous (49, 50, 207, 208). It is Burnet's opinion that the clinical and pathological features manifested by these
animals (haemolytic anaemia with positive Coombs test, circulating antinuclear antibodies and LE factor, hepatosplenomegaly, the development of a membranous nephritis with wire-loop lesions, lymphoid hyperplasia, and alopecia) are consistent with the development of 'forbidden clones' of immunologically competent cells (49, 50). He postulates that these cells arise by the somatic mutations of lymphoid cells, in animals genetically predisposed to the appearance of such clones. This genetic susceptibility appears to be due to the presence of a single autosomal gene (208). Confirmatory evidence in support of the theory that the pathological changes observed in these rats are due to genetic factors is the observation that the relative incidence of the various lesions may be altered by cross-breeding experiments (208). In the cross-bred progeny, the haemolytic anaemia - initially almost uniformly present - can to a considerable extent be suppressed and replaced by a clinical syndrome closely resembling lupus nephritis (208). Virtually 100 per cent of these animals now exhibit symptoms of renal failure resulting from glomerular lesions of the type seen in lupus nephritis (208). The inherited character of the disease is further emphasized by the fact that the incidence of positive LE-cell tests occurring in female mice is twice that seen in the male (208). It is of importance to note that, once present, the auto-immune condition persists throughout the animal's lifetime (49), which supports Burnet's contention that the homeostatic mechanism that normally prevents the appearance of immunologically competent cells reactive with native antigens is over-ridden (49). Direct evidence for the existence of
abnormal, immunologically competent cells in the afflicted animals has been obtained by means of passive-transfer experiments. These experiments demonstrate that spleen cells obtained from rats having haemolytic anaemia with a positive Coombs test could successfully transfer this disorder upon their injection into asymptomatic rats of the same strain (49, 50, 207, 208).

E. THE CONCEPT OF PHYSIOGENIC AUTO-ANTIBODIES

In the sera of the Gila monster, a viper noted for its potent venom, Tyler noted the presence of a globulin with anti-venom activity (209). This globulin could be detected only in the sera of adult snakes which, upon attaining full maturity, had acquired the capacity to secrete the poisonous substance. Tyler suggested that this globulin, with the characteristics of an auto-antibody, served to protect the snake from the lethal venom it now possessed. The capacity to secrete the venom, presumably acquired after full immunological competence had been attained, no doubt prevents the development of tolerance to it. As a result, the adult snake responds to the appearance of the venom with an auto-immune response which is not destructive to the organism but rather serves a biologically useful purpose (63).

Hints that analogous situations may occur in certain human disease has been cited by Miescher in regard to hypersensitivity reactions involving uveal-tract antigens (210).
He quotes the observations of Woods, that patients who manifest
dermal sensitivity of the 'delayed' type to the subcutaneous
injection of uveal-tract antigens are susceptible to the
development of sympathetic ophthalmia but that those who
develop complement-fixing antibodies to these same antigens do
not develop the condition (210).

Miescher himself has recently reported the presence of
an antibody to autologous lysosomes in sera obtained from
patients with infectious hepatitis (211). This antibody, when
tested in vitro in the presence of complement, served to
increase the stability of lysosomes to toxic agents. Incubation
of lysosomal granules with the antibody effectively blocked
their lysis when subsequently they were exposed to vitamin A.
The experimental conditions cited here are highly artificial
of course, but a theoretical case can be made for the beneficial
effects of such an antibody. Stabilization of the intracellular
lysosomes, such as produced by the antibody in vitro, might
occur in vivo and retard the release of their potentially
harmful enzymes into the cell sap or the extracellular fluid
after viral-induced liver injury (211, 212).

Paterson has demonstrated that a striking inverse
relationship exists between the occurrence of complement-
fixing auto-antibody to brain tissue and the development of
auto-immune encephalomyelitis (E.A.E) (213, 214) and his
experiments showed that this auto-antibody is capable of
modifying the course of the experimentally induced disease
(212, 213). Lewis rats immunized with brain tissue develop a
particularly severe form of E.A.E. which is invariably lethal within 28 days. Complement-fixing antibody to brain tissue cannot be demonstrated in this strain of rats. In marked contrast, the Wistar strain develop a transitory type of auto-immune encephalitis, from which the majority recover. Complement-fixing antibodies to nervous tissue can be demonstrated in the sera of most of the immunized animals (213, 214, 215). The protective effects exerted by these sera are dramatically demonstrated by their ability to prevent the development of encephalitis when this is passively transferred to Lewis rats immunized with an emulsion of brain tissue and Freund's adjuvant (214).

Coombs has described the presence of an auto-antibody to complement which he has designated 'conglutinin' (216); it is composed of the 7S and 19S components of the immunoglobulins (217). Elevated levels of conglutinin in the sera have been found in a variety of infections in which bacterial organisms and antibody are likely to interact with the consumption of complement (217), and Coombs has demonstrated that the presence of conglutinin actually benefits the organism by facilitating the removal of bacteria (216).

Kunkel has recently drawn attention to the striking analogy existing between the rheumatoid factors and conglutinin (217). He points out that the formation of both may arise from the presence of circulating complexes of foreign antigen and its specific antibody. In support of this contention, rheumatoid factor has been demonstrated in the sera of patients with certain
chronic infections in which circulating complexes of foreign antigen and antibody are most likely to be present, e.g., subacute bacterial endocarditis (217, 218). The role of these complexes in the production of rheumatoid factor has been indicated by the demonstration that, in subacute bacterial endocarditis, when the septicaemia subsides the rheumatoid factor can be detected no longer in the circulation (217, 218). Rheumatoid factors, in common with conglutinin, may therefore represent auto-antibodies which are beneficial to the host by facilitating the removal of potentially harmful immune complexes; but the pertinent question of just what the rheumatoid factor is reacting to, or protecting against, remains unanswered (217).

It is ironic that the concept of auto-immunity which was, initially, so foreign to the historical roots of immunology, should now show evidence of a partial return to the traditional concepts which envisaged the immune reaction as fulfilling a protective function. In the over-all context of the pathogenesis of various disease states therefore, auto-antibodies can no longer be considered as an irrelevant part of an immune response (215).
Wistar rats, adult albino New Zealand rabbits, cats and mongrel dogs were exsanguinated and the livers as well as other required organs were removed. Autopsy material no more than six hours old was used as a source of human liver.

The individual livers were repeatedly washed in ice cold saline and then finely minced with scissors and passed through a tissue press (obtained from Harvard Apparatus Co. Inc. Dover, Mass.) to remove the connective tissue and vascular components. Examination of the resultant liver preparation by light microscopy revealed that it was composed almost entirely of parenchymal cells. This liver preparation was then placed in flasks and shell frozen in a bath of ethanol and dry ice and lyophilized by means of a Vertis A 40 lyophilizer. The lyophilized liver was subsequently sealed in glass vials and stored in a desiccator until required. Other rat organs required for the experiments (kidney, heart, lung, intestinal tract) were prepared and stored in a similar fashion.

Upon exsanguination of the animals the bloods were allowed to clot and the serum removed and pooled according to species. The latter were then either stored in sealed glass vials at -20° C, or placed in flasks and shell frozen in ethanol
and dry ice and lyophilized. The lyophilized sera were stored in glass vials in a desiccator until required.

E. PREPARATION OF ANTIGENS:

1. Liver Homogenates Employed for Immunization:

Twenty milligrams of lyophilized liver was homogenized in 0.5 ml of saline with 10 complete strokes of a Potter homogenizer. The liver homogenate was added to an equal volume of a mixture consisting of 17 parts of paraffin oil (Bayol F), three parts Arlacel A and 1 mg of killed lyophilized mycobacterium butyricum. Collectively, these three agents constitute Freund's complete adjuvant (obtained ready-made from Difco Laboratories, Detroit, Mich.). In order for this method of immunization to be most efficient it is important that the antigen and adjuvant be thoroughly emulsified (220). Incomplete emulsification reduces the efficiency of the method to a great extent (223). To ensure the complete emulsification of the liver homogenate in Freund's adjuvant, these two materials were repeatedly transferred between two 10 ml luer lock syringes connected by a single double hubbed needle. The final product proved to be a true water and oil emulsion as indicated by the fact that a drop placed on water did not spread.

2. Organ Antigens Employed for Haemagglutination Experiments:

Five mg's of lyophilized liver were suspended in 1 ml of phosphate buffered saline pH 7.2 (100 ml saline + 24 ml of
0.15 N KH$_2$PO$_4$ + 76 ml of 0.15 N Na$_2$ HPO$_4$, henceforth referred to as PBS) and thoroughly homogenized with 10 slow complete strokes of a Potter homogenizer. The resultant homogenate was then centrifuged at three thousand RPM for 10 minutes. The supernatant was decanted and diluted with PBS to a concentration previously determined to be optimal for the sensitization of the tanned red cells employed in the haemagglutination experiments. The sensitized cells were then employed in Boyden's tanned red cell haemagglutination technique to detect the presence and specificity of anti-liver antibodies.

C. IMMUNIZATION PROCEDURES

1. Raising of Antisera to Heterologous Livers:

Antisera to livers obtained from Wistar rats, mongrel dogs, cats and humans were prepared in groups of three adult albino New Zealand rabbits. To enhance the antigenic potency of the liver emulsion an additional 1 mg of dried killed mycobacterium butyricum was added to each ml of the immunizing preparation. One ml of the emulsified liver was injected into multiple sites in the footpads of each rabbit using a #21 gauge needle. These injections were repeated at intervals of 10 days for a period of 40 days. Ten days after the fourth and final injection the animals were bled and their sera tested for their capacity to agglutinate tanned red cells sensitized with saline extracts of the immunizing antigen(s).
2. **Raising of Antisera in Goats to Rat Liver:***

Two goats were immunized at intervals of two weeks with 20 mgs of lyophilized rat liver emulsified in complete Freund's adjuvant. Due to the susceptibility of the goat to develop severe lesions after subcutaneous administration of Freund's adjuvant, the immunizing injections were given intramuscularly. Each goat received a total of 4 injections. Two weeks after the final injection the animals were bled and the sera collected.

3. **Raising of Antisera in Wistar Rats to Homologous Rat Liver and Heterologous Rabbit Liver:***

The preparation and injection of the liver emulsions followed the procedure outlined for the immunization of rabbits with heterologous livers. The immunizing schedule was, however, varied to allow for a total of six injections with an interval of 4 weeks between the 4th and 5th injections. The last two injections were administered subcutaneously into the flanks. One week following the final injection the animals were exsanguinated and the individual sera collected and stored at -20°C. The livers and kidneys were removed and processed according to the method previously described. The organs were lyophilized and stored in sealed glass vials in a desiccator until required.
D. ABSORPTION OF ANTISERA FOR USE IN HAEMAGGLUTINATION EXPERIMENTS AND FLUORESCENT MICROSCOPY

The following procedures were undertaken to establish the specificity of the anti-liver sera for hepato-cellular antigens.

1) Citrated rat blood was added to an equal volume of an aliquot of anti-rat liver serum diluted tenfold in PSS. The suspension was incubated at room temperature for two hours and centrifuged at 1500 RPM for 10 minutes. The supernatant was then removed and subsequently employed in Section A, Experiment II of Chapter IV.

2) Twenty mgs of lyophilized rat heart, kidney, lung or serum proteins were added to a tenfold dilution of anti-rat liver serum previously absorbed with rat blood. The antiserum and the lyophilized organ were carefully homogenized with 10 slow complete strokes of the Potter homogenizer. The resultant homogenate was allowed to stand at room temperature with intermittent shaking for 2 hours. It was then centrifuged at 3,000 RPM for 20 minutes and the supernatant removed and employed to demonstrate the organ specificity of the hepato-cellular antigens. (Section A, Experiment IV, Chapter IV). This procedure was followed in all subsequent experiments which required the absorption of anti-liver sera with lyophilized organs or serum proteins.
F. THE TANNED RED CELL HAEMAGGLUTINATION TECHNIQUE

In 1951 Boyden demonstrated that the treatment of red cells with a dilute solution of tannic acid produced a change in the surface properties of these cells which rendered them capable of absorbing certain protein antigens (219). Such red cells may then be agglutinated by antibodies specific for the protein coating the cell (219). The indirect or passive haemagglutination technique, developed by Boyden, has today become an accepted, powerful, sensitive tool, for the detection of small amounts of circulating antibody (220).

Preparation of Reagents:

Boyden's method for the indirect or passive haemagglutination test was followed throughout (219). Fresh defibrinated sheep blood was centrifuged for 10 minutes at 1500 RPM and the serum discarded. The red cells were then washed 3 times in 0.9 percent physiological saline (PSS). A 2.5 per cent suspension of the washed cells was prepared in PSS and mixed with an equal volume of 1:20,000 dilution of tannic acid, freshly prepared from a 1:100 stock solution of tannic acid. The mixture of red cells and tannic acid was incubated at 37°C for 10 minutes. The tannic acid treated cells (referred to as tanned cells) were washed twice in PES and resuspended in the same medium to make a final cell concentration of 2.5 per cent. In Section B of this chapter the preparation and extraction of the soluble organ antigens for use in haemagglutination experiments
was described. Sensitization of the tanned cells was accomplished by thoroughly mixing them with an equal volume of a PBS extract of lyophilized heart, liver, kidney or lung respectively. Each mixture was then incubated for 15 minutes at 37°C after which time the cells were again centrifuged, washed twice in diluent (normal rabbit serum diluted hundredfold in saline) and resuspended in the same medium to make a final cell concentration of 0.5 per cent.

The concentration of each organ extract required to optimally sensitize the tanned cells varied with each of the organ preparations used and was determined by trial and error. Aliquots of tanned red cells were sensitized with a 1:10, 1:20, 1:40, 1:80 or 1:160 dilutions of a PBS extract of one of the lyophilized organs. The concentration of organ extract used to sensitize the cells which gave the highest haemagglutinating titer, when added to serial, twofold dilutions of the specific antiserum, were used in all subsequent experiments.

Anti-liver sera employed in the haemagglutination experiments were first decomplemented by heating to 56°C for 30 minutes. An equal volume of sheep red cells were then added to the decomplemented antisera. The red cell-antiserum suspension was allowed to incubate at room temperature for 30 minutes, at which time it was centrifuged at 1500 RPM for 10 minutes and the supernatant removed. As has already been described in Section D of this chapter all antisera were routinely absorbed with lyophilized serum proteins obtained from the same animal.
species that supplied the antigens used in the preparation of the antisera. Similarly, when required, the anti-liver sera were absorbed with lyophilized homogenates of rat kidney, heart, lung or liver before use in haemagglutination experiments or fluorescent microscopy.

Procedure:

Twofold serial dilutions of anti-liver sera, were made in 1 ml volumes of diluent, in rows of lipped, round-bottomed 10 x 75 mm test tubes. A volume of 0.05 ml of the sensitized cell preparation was then pipetted into each tube. The tubes were gently shaken to disperse the cells and the ensuing antigen-antibody reaction was allowed to proceed at room temperature for 10 to 16 hours at which time the haemagglutinating titer of the antiserum was read. The titer of the antiserum was expressed as the reciprocal of the highest dilution of antiserum which gave a positive agglutination pattern (see Figure 1). All experiments were performed in duplicate.

Controls for each experiment consisted of demonstrating the absence of agglutination when a) untreated cells were suspended in diluent or antiserum; b) when sensitized cells were suspended in diluent; and c) when tanned cells were suspended in antiserum. A positive control eliminated the experiment.
F. INHIBITION OF HAEMAGGLUTINATION

For inhibition experiments the above procedures were modified as follows:

Twofold serial dilutions of test serum were prepared in 0.5 ml volumes in a series of test tubes. 0.5 ml of anti-rat liver serum, diluted to a concentration of 5 haemagglutinating units/ml was added to each of these tubes.* After incubation at room temperature for 30 minutes, cells sensitized with rat liver were added. The absence of haemagglutination indicated the presence in the test serum of antigens identical to those employed to sensitize the cells (220) (Figure 2).

G. FLUORESCENT MICROSCOPY

Antisera to rat and rabbit gamma globulin conjugated with fluorescein isothiocyanate were obtained from Hyland Laboratories, Los Angeles, California.

Saline insoluble homogenates of guinea pig liver were used to absorb the non-specific fluorescence from fluorescein conjugated anti-rabbit or anti-rat gamma globulin. The precipitates were prepared from guinea pig livers finely minced in ice cold PSS, ground in a Vertis Homogenizer and

* 1 Haemagglutinating Unit = the maximum dilution of antiserum capable of agglutinating sensitized tanned red cells.
centrifuged at 1500 g for 15 minutes at 4°C. The supernatant was decanted and the precipitate suspended and washed 3 times in cold PSS and then resuspended in an equal volume of PBS. Two ml of this suspension were centrifuged in a Spinco preparatory ultracentrifuge at 15,000 RPM for 20 minutes. The resultant precipitate was again resuspended in PBS and centrifuged at the same speed for an additional 20 minutes. Following this, the supernatant was decanted, the tubes drained by inversion in a test tube rack, and the precipitate frozen and stored in the centrifuge tubes at -20°C. When required the saline insoluble guinea pig liver precipitates were thawed and used to absorb the non-specific fluorescence from fluorescein conjugated anti-rabbits or anti-rat gamma globulin (221). A volume of 0.5 ml of conjugated antiserum was added to each centrifuge tube containing the liver homogenate. The contents were thoroughly mixed by means of a wooden applicator stick and incubated in a water bath for 15 minutes at 37°C. Following incubation the tubes were centrifuged at 15,000 RPM for 20 minutes in a Spinco preparatory ultracentrifuge and the supernatant, containing the conjugated antiserum, removed with a Pasteur pipette. Due to the instability of the absorbed conjugated antiserum, the foregoing procedure was always performed immediately before the actual experiment (221).

Immediately following exsanguination, the liver, heart, kidneys and lungs were removed from Wistar rats. Sections of tissue, 2 mm or less in thickness, were obtained from each organ and processed, embedded and stored according to the method of
Sainte-Marie (222). After fixation in ethanol at 4°C overnight, the tissue slices were dehydrated by immersion at hourly intervals in 4 successive baths of ethanol, followed immediately by immersion in 3 successive baths of xylene. After the last change of xylene the tissue sections were removed from the cold room and allowed to reach room temperature. The sections were then incubated at 56°C in 3 or 4 successive baths of paraffin. This procedure produced well-infiltrated specimens. The blocks could then be stored in a desiccator at 4°C until required, or cut into sections and without removing the paraffin stored in the same manner.

At 4°C the paraffin sections were cleared by passing the microscope slides through two changes of xylene, one of absolute ethanol and three of 95 per cent ethanol. Hydration was completed by washing the slides twice in cold phosphate buffer (0.1M pH 7.4) for 3 to 5 minutes. Following this, the slides were removed from the cold room and allowed to reach room temperature at which time the antiserum was applied with a Pasteur pipette (221).

In the direct fluorescent antibody technique, the conjugated rabbit or rat anti-gamma globulin was applied directly to the microscope slide. The slide was then placed in a saturated atmosphere at room temperature for 30 to 45 minutes. At the end of this time the slides were washed in three changes of PBS, the excess buffer removed and the slides mounted in buffered glycerol (221) (9 parts glycerol plus 1 part PBS). The coverslips were then sealed with nail polish and examined under the fluorescent microscope.
For the indirect or sandwich technique, unconjugated rabbit or rat anti-rat liver serum was applied in the same manner as the conjugated antibody in the direct technique (221). Following the last wash, fluorescein labelled goat anti-rabbit or anti-rat gamma globulin was applied. The remainder of the procedure was identical to that described for the direct technique (221).

Controls for the fluorescent antibody technique consisted of demonstrating the absence of specific staining when:

a) Tissues not containing the antigen(s) in question were substituted for tissues containing the antigen(s).

b) Normal rabbit or rat serum was substituted for the antiserum in the indirect technique, or fluorescein conjugated normal rabbit or rat gamma globulin was substituted for the conjugated anti-gamma globulin in the direct technique.

c) The unconjugated antiserum was omitted in the indirect technique.

d) Unlabelled antiserum was applied to the tissues before the labelled antiserum was applied (direct technique).

e) The specific antiserum was incubated with the antigen(s) prior to the staining procedure.

All tissues were examined under a Reichert Zetopan microscope. For fluorescent antibody studies, an Osram HBO 200 watt pressure mercury lamp was employed for illumination. A primary ultraviolet passing and heat absorbing filter with maximum transmittance at 360 nm (Schott and Genossen, UG1/1.5mm + BG12/2mm) and an orange-yellow secondary filter (Schott and Genossen, OG5/1mm + OG1/1.5 mm) were used in conjugation with a bright ground condenser.
Chapter IV

RESULTS

A. THE SPECIFICITY OF RABBIT ANTI-RAT LIVER SERA FOR RAT HEPATO-CELLULAR ANTIGENS

Procedures and Results:

1. Cross Reactions of Rabbit Anti-Rat Liver Sera with Rat Serum Proteins and Red Cells

As described in the preceding chapter antisera to the parenchymal cell antigens of rat liver were prepared in adult albino New Zealand rabbits. The rabbit anti-rat liver sera were first tested for their ability to agglutinate aliquots of tanned cells sensitized with rat serum proteins or a saline extract of rat liver.* Aliquots of anti-rat liver sera were also added to saline suspensions of rat red cells. Table I and all subsequent tables record the average results obtained from individual experiments performed in duplicate and then repeated on two or more occasions.

*(Table I)
**TABLE I**

Cross Reactions of Rabbit Anti-Rat Liver Sera:

<table>
<thead>
<tr>
<th>Anti-Serum</th>
<th>Haemagglutinating Titer* Obtained With:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Liver Sensitized TRBC:**</td>
<td>Rat Serum Sensitized TRBC:</td>
<td>Untreated Rat RBC:</td>
</tr>
<tr>
<td># 74</td>
<td>172,840</td>
<td>172,840</td>
</tr>
<tr>
<td># 76</td>
<td>172,840</td>
<td>172,840</td>
</tr>
<tr>
<td># 3</td>
<td>81,920</td>
<td>81,920</td>
</tr>
</tbody>
</table>

* The titer of the antisera, in this and all subsequent experiments, is expressed as the maximum dilution capable of agglutinating the sensitized cells.

** TRBC refers in this and all subsequent tables to sheep cells treated with tannic acid.

Absorption of the antisera with citrated rat blood abolished their capacity to agglutinate either rat red cells or tanned cells sensitized with rat serum proteins. In all cases the absorbed immune sera retained their capacity to agglutinate cells sensitized with a saline extract of rat liver (Table II).
Cross Reactions of Rabbit Anti-Rat Liver Sera:

<table>
<thead>
<tr>
<th>Anti-Serum Absorbed With Rat Blood:</th>
<th>Haemagglutinating Titer Obtained With:</th>
<th>Rat Liver Sensitized TRBC:</th>
<th>Rat Serum Sensitized TRBC:</th>
<th>Untreated Rat RBC:</th>
</tr>
</thead>
<tbody>
<tr>
<td># 74</td>
<td></td>
<td>40,960</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># 76</td>
<td></td>
<td>40,960</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># 3</td>
<td></td>
<td>40,960</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In all subsequent experiments, only rabbit anti-rat liver sera freed of all cross reacting antibodies to the cellular and serum protein components of rat blood were employed.

2. Cross Reactions of Rabbit Anti-Rat Liver Sera with Rat Heart, Kidney, and Lung

Aliquots of rabbit anti-rat liver sera, absorbed with rat blood, were examined for their capacity to agglutinate cells sensitized with saline extracts of rat heart, kidney or lung. Table III reveals that cells sensitized with antigens derived from any one of the three organs were agglutinated by the antisera, though the haemagglutinating titers were reduced in comparison to those recorded using cells sensitized with rat liver.
## TABLE III

**Cross Reactions of Rabbit Anti-Rat Liver Sera:**

<table>
<thead>
<tr>
<th>Anti-Serum</th>
<th>Haemagglutinating Titer for Cells Sensitized With:</th>
<th>Rat Liver</th>
<th>Rat Kidney</th>
<th>Rat Heart</th>
<th>Rat Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td># 74</td>
<td></td>
<td>40,960</td>
<td>10,240</td>
<td>2560</td>
<td>1280</td>
</tr>
<tr>
<td># 76</td>
<td></td>
<td>40,960</td>
<td>20,480</td>
<td>10,240</td>
<td>5120</td>
</tr>
<tr>
<td># 3</td>
<td></td>
<td>20,480</td>
<td>5120</td>
<td>1280</td>
<td>690</td>
</tr>
</tbody>
</table>

The capacity of the antisera to agglutinate cells sensitized with these same organ extracts was assessed after absorption of aliquots of immune sera with a lyophilized homogenate of rat heart, lung or kidney, respectively. After absorption with rat heart or lung, only cells sensitized with rat kidney or liver antigens were agglutinated. Antisera absorbed with rat kidney were found to react specifically with cells sensitized with the immunizing hepatocellular antigens (Table IV). The specificity of these immune sera for liver antigens was confirmed by indirect fluorescent microscopy (Section D).
### TABLE IV:

**Organ Specificity of Rat Hepato-Cellular Antigens as Determined by the Use of Rabbit Anti-Rat Liver Serum**

<table>
<thead>
<tr>
<th>Absorbing Serum: Antigen:</th>
<th>Haemagglutinating Titer for Cells Sensitized With:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Liver:</td>
</tr>
<tr>
<td># 74 Not Absorbed</td>
<td>40,960</td>
</tr>
<tr>
<td># 74 Rat Heart</td>
<td>40,960</td>
</tr>
<tr>
<td># 74 Rat Lung</td>
<td>40,960</td>
</tr>
<tr>
<td># 74* Rat Kidney</td>
<td>40,960</td>
</tr>
<tr>
<td># 74 Rat Liver</td>
<td>0</td>
</tr>
</tbody>
</table>

* In a later experiment aliquots of this serum were used as the unconjugated middle layer in the indirect fluorescent antibody technique (Section D).

Further verification of the specificity of rabbit anti-rat liver sera for hepato-cellular antigens were obtained by absorbing individual aliquots with a lyophilized homogenate of one of the following rat organs: intestinal tract, brain, spleen and skeletal muscle. In all instances the capacity of the immune serum to effect the agglutination of the liver sensitized cells was unimpaired (Table V).
**TABLE V**

**Organ Specificity of Rat Hepato-Cellular Antigens as Determined by the Use of Rabbit Anti-Rat Liver Serum:**

<table>
<thead>
<tr>
<th>Serum:</th>
<th>Absorbing Antigen:</th>
<th>Haemagglutinating Titer for Cells Sensitized With Rat Liver:</th>
</tr>
</thead>
<tbody>
<tr>
<td># 74</td>
<td>Not Absorbed</td>
<td>40,960</td>
</tr>
<tr>
<td># 74</td>
<td>Rat Intestine</td>
<td>40,960</td>
</tr>
<tr>
<td># 74</td>
<td>Rat Muscle</td>
<td>40,960</td>
</tr>
<tr>
<td># 74</td>
<td>Rat Brain</td>
<td>40,960</td>
</tr>
<tr>
<td># 74</td>
<td>Rat Spleen</td>
<td>40,960</td>
</tr>
<tr>
<td># 74</td>
<td>Rat Liver</td>
<td>0</td>
</tr>
</tbody>
</table>

**B. ORGAN SPECIFICITY OF RAT HEPATO-CELLULAR ANTIGENS AS DETERMINED BY THE USE OF GOAT ANTI-RAT LIVER SERUM:**

**Procedures and Results:**

1. **Cross Reactions of Goat Anti-Rat Liver Serum:**

   In an attempt to circumvent the possibility that rabbits might be tolerant, and therefore unresponsive, to certain antigens present in rat liver, several goats were immunized with a lyophilized homogenate of rat liver emulsified in complete Freund's adjuvant. The immune sera were employed in a series of experiments similar to those just described using rabbit anti-rat liver sera. In all instances, identical results were
obtained (Table VI).

**TABLE VI**

**Organ Specificity of Rat Hepato-Cellular Antigens as Determined by the Use of Goat Anti-Rat Liver Serum:**

<table>
<thead>
<tr>
<th>Absorbing Antigens:</th>
<th>Rat Liver</th>
<th>Rat Kidney</th>
<th>Rat Lung</th>
<th>Rat Heart</th>
<th>Rat Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Blood:</td>
<td>81,920</td>
<td>10,240</td>
<td>640</td>
<td>10,240</td>
<td>0</td>
</tr>
<tr>
<td>Rat Blood +</td>
<td>81,920</td>
<td>10,240</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat Blood +</td>
<td>81,920</td>
<td>10,240</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat Blood +</td>
<td>81,920</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat Blood +</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
G. SPECIES DISTRIBUTION OF THE RAT ORGAN

SPECIFIC HEPATO-CELLULAR ANTIGENS:

Procedures and Results:

1. Cross Reactions of Rabbit and Goat Anti-Rat Liver Sera:

Rabbit and goat anti-rat liver sera rendered specific for liver antigens by absorption with rat kidney and serum proteins were used to determine the species distribution of the organ specific hepato-cellular antigens. For this purpose aliquots of anti-rat liver sera were examined for their capacity to agglutinate cells sensitized with saline extracts of lyophilized dog, cat, rabbit and human livers respectively. It is apparent from Table VII that maximal antibody activity was recorded when the antisera were added to cells sensitized with the specific immunizing antigen(s). Analogous results were obtained when this experiment was repeated with antisera to dog, cat, rabbit and human livers (Table VII).
### TABLE VII

**Species Distribution of Hepato-Cellular Antigens:**

<table>
<thead>
<tr>
<th>Anti-Serum*:</th>
<th>Haemagglutinating Titer for Cells Sensitized With:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Liver:</td>
<td>Rabbit Liver:</td>
</tr>
<tr>
<td>Rabbit Anti-Rat Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 74</td>
<td>40,960</td>
<td>2560</td>
</tr>
<tr>
<td>Goat Anti-Rat Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 1</td>
<td>81,920</td>
<td>5120</td>
</tr>
<tr>
<td>Rat Anti-Rabbit Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 3</td>
<td>5,120</td>
<td>5120</td>
</tr>
<tr>
<td># 7</td>
<td>10,240</td>
<td>40,960</td>
</tr>
<tr>
<td>Rabbit Anti-Human Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 52</td>
<td>10,240</td>
<td>5120</td>
</tr>
<tr>
<td>Rabbit Anti-Cat Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 71</td>
<td>10,240</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit Anti-Dog Liver:</td>
<td></td>
<td></td>
</tr>
<tr>
<td># 5</td>
<td>3200</td>
<td>0</td>
</tr>
</tbody>
</table>

*In each instance the immune serum had first been absorbed with the serum proteins of the organ donor.*
The capacity of selected anti-liver sera to agglutinate cells sensitized with the immunizing liver preparation were then assessed following absorption of the antisera with lyophilized liver homogenates obtained from different animal species. In all instances the immune sera retained their capacity to agglutinate cells sensitized with their specific hepato-cellular antigens (Table VIII).

### TABLE VIII

**Species Specificity of Hepato-Cellular Antigens:**

<table>
<thead>
<tr>
<th>Anti-Serum: Absorbing Liver:</th>
<th>Haemagglutinating Titer for Cells Sensitized With:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Liver:</td>
</tr>
<tr>
<td>Rabbit Anti-Rat Liver:</td>
<td></td>
</tr>
<tr>
<td># 74</td>
<td>Not Absorbed</td>
</tr>
<tr>
<td># 74</td>
<td>Dog</td>
</tr>
<tr>
<td># 74</td>
<td>Cat</td>
</tr>
<tr>
<td># 74</td>
<td>Rat</td>
</tr>
<tr>
<td>Rabbit Anti-Dog Liver:</td>
<td></td>
</tr>
<tr>
<td># 5</td>
<td>Not Absorbed</td>
</tr>
<tr>
<td># 5</td>
<td>Rat</td>
</tr>
<tr>
<td>Rabbit Anti-Cat Liver:</td>
<td></td>
</tr>
<tr>
<td># 71</td>
<td>Not Absorbed</td>
</tr>
<tr>
<td># 71</td>
<td>Rat</td>
</tr>
</tbody>
</table>
D. LOCALIZATION OF THE ORGAN SPECIFIC HEPATO-CELLULAR ANTIGENS IN THE RAT AS DEMONSTRATED BY INDIRECT FLUORESCENT MICROSCOPY:

Procedures and Results:

Sections of rat liver were studied by indirect fluorescent microscopy utilizing rabbit anti-rat liver serum (No. 74), as the unstained middle layer. This antiserum, absorbed with rat kidney and serum protein, had previously been demonstrated by haemagglutination to react specifically with liver antigens (Table IV). The immune serum consistently demonstrated an affinity for the cytoplasm of the hepatic parenchymal cells. The nuclear components of the cell, as well as the adjoining portal spaces and connective tissue septa were devoid of specific immuno-fluorescence. When sections of rat kidney, lung or heart were substituted for the liver, specific immunofluorescence was not observed.

E. ATTEMPTS TO BREAK TOLERANCE TO THE HEPATO-CELLULAR ANTIGENS OF THE RAT BY IMMUNIZATION WITH EMULSIONS OF RABBIT OR RAT LIVERS:

Procedure and Results:

1. Breakage of Tolerance to Rat Hepato-Cellular Antigens:

   As described in detail in the chapter on methods two groups of twelve Wistar rats were immunized with lyophilized homogenates of rat and rabbit liver respectively. Upon completion of the immunizing schedule, the sera were harvested *Figure III
by exsanguinating the animals and specimens of liver and kidney were obtained for use as antigens in future haemagglutination experiments and for study by light and fluorescent microscopy.

Each rat serum was assessed for its capacity to agglutinate cells sensitized with saline extracts of lyophilized rat liver. As recorded in Table IX sera obtained from both groups of rats agglutinated the sensitized cells.

**TABLE IX**

*Breakage of Immune Tolerance to Rat Hepato-Cellular Antigens Following Immunization with Rat or Rabbit Livers:*

<table>
<thead>
<tr>
<th>Anti-Serum:</th>
<th>Haemagglutinating Titer for Cells Sensitized with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Anti-Rat Liver:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat Liver</td>
</tr>
<tr>
<td></td>
<td>pooled:</td>
</tr>
<tr>
<td>No. 1</td>
<td>20,480</td>
</tr>
<tr>
<td>No. 2</td>
<td>20,480</td>
</tr>
<tr>
<td>No. 3</td>
<td>20,480</td>
</tr>
<tr>
<td>No. 4</td>
<td>640</td>
</tr>
<tr>
<td>Rat Anti-Rabbit Liver:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat Liver</td>
</tr>
<tr>
<td></td>
<td>pooled:</td>
</tr>
<tr>
<td>No. 1</td>
<td>10,240</td>
</tr>
<tr>
<td>No. 7</td>
<td>20,480</td>
</tr>
<tr>
<td>No. 4</td>
<td>40,960</td>
</tr>
<tr>
<td>No. 3</td>
<td>5,120</td>
</tr>
</tbody>
</table>
2. Cross Reactions of Rat Anti-Rabbit and Rat Anti-Rat Liver Sera:

The specificity of the rat anti-rat and rat anti-rabbit liver sera for rat hepato-cellular antigens was supported by their inability to agglutinate cells sensitized with rat kidney (Table X). Cells sensitized with rabbit kidney were however agglutinated by the rat anti-rabbit liver sera (Table X). An analogous series of results were obtained when rabbit anti-rat liver serum No. 74 was examined for its ability to agglutinate aliquots of cells sensitized with rabbit liver and kidney respectively. (This anti-serum has previously been demonstrated to agglutinate cells sensitized with rat kidney (Table III)).
TABLE X

Cross Reactions of Anti-Liver Serum with Heterologous, Homologous and Autologous Liver and Kidney:

<table>
<thead>
<tr>
<th>Anti-serum:</th>
<th>Haemagglutinating Titer for Cells Sensitized With:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Anti-Rat Liver:</td>
<td>Rat Liver:** Rat Kidney: Rabbit Liver: Rabbit Kidney:</td>
</tr>
<tr>
<td>No. 1</td>
<td>10,240 0 0 N.T.*</td>
</tr>
<tr>
<td>No. 2</td>
<td>20,960 0 640 N.T.</td>
</tr>
<tr>
<td>No. 8</td>
<td>40,960 0 10,240 N.T.</td>
</tr>
<tr>
<td>Rat Anti-Rabbit Liver:</td>
<td></td>
</tr>
<tr>
<td>No. 3</td>
<td>2,560 0 10,280 640</td>
</tr>
<tr>
<td>No. 1</td>
<td>2,560 0 20,480 80</td>
</tr>
<tr>
<td>No. 7</td>
<td>10,240 0 40,960 640</td>
</tr>
<tr>
<td>Rabbit Anti-Rat Liver: ***</td>
<td></td>
</tr>
<tr>
<td>No. 74</td>
<td>40,960 10,240 320 0</td>
</tr>
</tbody>
</table>

*N.T. = Not Tested.

**Autologous Tissue.

*** This serum was obtained after ten injections of rat liver given over a period of eight months.
3. Cross Reactions of Rat Anti-Rat Liver Serum with Heterologous Livers:

The ability of the rat anti-rat liver sera to agglutinate cells sensitized with saline extracts of lyophilized dog, cat, rabbit and human liver was investigated. All sera tested consistently failed to agglutinate cells sensitized with human liver, though a capacity to agglutinate cells sensitized with dog, cat and rabbit liver was exhibited by some immune sera (Table XI).

**TABLE XI**

<table>
<thead>
<tr>
<th>Anti-Serum:</th>
<th>Haemagglutinating Capacity for Cells Sensitized With:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Anti-Rat Liver:</td>
<td>Rat Liver:</td>
</tr>
<tr>
<td>No. 8</td>
<td>40,960</td>
</tr>
<tr>
<td>No. 2</td>
<td>20,480</td>
</tr>
<tr>
<td>No. 1</td>
<td>10,240</td>
</tr>
</tbody>
</table>

4. Immunofluorescent Studies with Rat Anti-Rat Liver Serum:

Sections of rat liver were studied by indirect fluorescent microscopy utilizing, as the unstained middle layer, rat anti-rat and rat anti-rabbit liver serum. A pattern of diffuse cytoplasmic staining was seen, identical to that observed with the rabbit anti-rat liver serum. Though the intensity of the fluorescent staining was reduced, a similar picture was
obtained when autologous liver was substituted for the isologous specimen.

F. DETECTION OF CIRCULATING ORGAN SPECIFIC HEPATIC ANTIGENS AFTER INJECTION OF THE HEPATO-CELLULAR TOXIN THIOACETAMIDE:

Procedure and Results:

An aqueous solution containing eighty milligrams of the hepato-cellular toxin, thioacetamide, was administered in a single intra-peritoneal injection, to each of twenty Wistar rats. The animals were then bled from the jugular vein at twelve hour intervals and the sera were stored at minus 10°C. As described in the section on methods, doubling dilutions of each serum specimen was made in a series of fourteen tubes. A constant quantity of a rabbit anti-rat liver serum was then added to each of the tubes. After incubation at room temperature for one hour, cells sensitized with a saline extract of rat liver were added. The presence of liver specific protein(s) released into the circulation as a result of the thioacetamide induced hepato-cellular injury was indicated by the absence of haemagglutination, since the organ specific liver antigens present in the test serum were capable of completely neutralizing the antibody in the test tube. The inhibition produced by the experimental rat serum was compared to that obtained when a saline extract derived from a known weight of lyophilized rat liver was substituted for the rat serum.
The results listed in Table XII indicate the presence of hepato-cellular protein(s) in serum obtained twelve hours after the injection of the hepato-toxin. The inhibitory capacity of the individual rat sera reached a maximum at twenty-four to forty-eight hours, after which a rapid decline occurred. Serum obtained at seventy-two to ninety-six hours after thioacetamide administration was incapable of inhibiting the specific liver anti-liver system.

**TABLE XII:**

Detection of Circulating Liver Proteins After Thioacetamide Injection:

Inhibiting Capacity of Individual Rat Serum**

<table>
<thead>
<tr>
<th>Time of Bleeding: *</th>
<th>0 hours</th>
<th>12 hours</th>
<th>24 hours</th>
<th>36 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 0 0 0</td>
<td>0 7 8 5 5 6 0</td>
<td>7 7 8 6 6 4</td>
<td>10 8 0 8 8 2 9</td>
<td>6 9 9 8 0 4</td>
<td>0 0 0 0 0 0 0</td>
<td>0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

* Expressed as hours after the administration of thioacetamide.

** Expressed as the number of tubes in which haemagglutination was inhibited. The 10 tube inhibition produced by a saline
extract obtained from 10 mgs of lyophilized rat liver remained constant throughout the experiment.

The preceding experiment was repeated and the protocol duplicated in all respects except that groups of four rats were killed at intervals of twelve, twenty-four, thirty-six, forty-eight and ninety-six hours following the injection of thioacetamide. In all cases the serum was saved for use in haemagglutination inhibition experiments and sections of liver processed for histological examination. A comparison could then be made between the morphological evidence of necrosis visible in the liver and the degree of inhibition produced by serum obtained from the same animal. Table XIII compares the average inhibition produced by serum obtained from each individual member of the five groups of rats with the degree of parenchymal cell necrosis present in the corresponding livers. Arbitrarily this has been graded from zero to plus three.

**Table XIII**

| Correlation Between Inhibiting Capacity of Rat Serum and Parenchymal Cell Necrosis: |
|--------------------------------------|-----------------|-----------------|
| Inhibiting Capacity of Rat Serum:*   | Hepato-Cellular Necrosis | Hours After Thioacetamide Injection: |
| Group A: 5 tubes**                   | normal liver     | 12 hours        |
| Group B: 7 tubes                     | necrosis, 2+     | 24 hours        |
| Group C: 7 tubes                     | necrosis, 3+     | 36 hours        |
| Group D: 7 tubes                     | necrosis, 1+     | 48 hours        |
| Group E: 0 tubes                     | normal liver     | 96 hours        |

*The average value obtained from 5 individual serum.

**Number of tubes in which haemagglutination was inhibited.
As noted in the accompanying photographs* taken at twelve hours the liver parenchyma appeared normal despite the fact that sera obtained from these animals contained hepato-cellular antigens as indicated by the neutralization of the anti-liver serum in the test tube. At twenty-four hours extensive areas of necrosis were visible around the central vein. The necrosis reached a maximum at approximately thirty-six hours. At forty-eight hours these areas were infiltrated by mononuclear cells and the necrotizing process was markedly reduced. Four days after the injection of the hepato-toxin the liver parenchyma appeared virtually normal.

G. ATTEMPTS TO INDUCE THE FORMATION OF AUTO-ANTIBODIES TO HEPATO-CELLULAR PROTEINS BY INJECTION OF THIOACETAMIDE.

A group of rats were given a single intra-peritoneal injection of thioacetamide and then bled at 1, 2, 3, 5, 7, 9 and 12 days intervals. At the time of the last bleeding the animals were sacrificed and in a number of cases specimens of their livers were saved for study by direct fluorescent microscopy.

The first serum specimen was tested for the presence of circulating hepato-cellular proteins. Subsequent sera were evaluated for their capacity to agglutinate cells sensitized with a saline extract of rat liver. Though haemagglutination inhibition demonstrated the presence of hepato-cellular proteins in the sera of all test animals, the *Figures V to IX
majority of these sera failed to agglutinate liver sensitized tanned cells (Table XIV).

**TABLE XIV**

Detection of Circulating Auto-Antibodies to Rat Liver After the Injection of Thioacetamide

<table>
<thead>
<tr>
<th>Sera</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>80</td>
<td>80</td>
<td>x*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* x = animal died.

Examination by direct fluorescent microscopy of eight livers obtained 12 days after the administration of thioacetamide failed to reveal the presence of tissue bound rat gamma globulin. The livers of the two animals whose sera agglutinated the sensitized cells were not available for study by fluorescent microscopy.
Chapter V.

DISCUSSION

The present investigation of the organ-specific hepato-cellular antigens was initiated with the aim of furthering our understanding of these proteins and the possible role they may play in the pathogenesis of chronic liver disease of unknown aetiology i.e., post necrotic cirrhosis, lupoid hepatitis and plasma cell hepatitis 18 - 22). Recent developments in the field of auto-immunity have unequivocally confirmed that organ-specific antigens contribute to the pathogenesis of experimental auto-immune encephalomyelitis, orchitis and thyroiditis (142). It is of no exaggeration to say that the emphasis currently placed on the role of auto-immune reactions in the pathogenesis of chronic non-specific thyroiditis (29, 30, 31, 34) evolved directly from the demonstration by Witebsky and his colleagues, showing highly antigenic, organ-specific proteins present within the thyroid that are capable of producing an auto-immune response (31, 34). The investigation of organ-specific antigens, established principally as a result of the work of Witebsky (224), has therefore become of increasing practical importance.

In the historical summary of this thesis it was suggested that the occurrence of hypergammaglobulinemia in patients with chronic non-specific thyroiditis and the infiltration of their thyroids by lymphocytes and plasma cells reflected the occurrence of an immune reaction involving autologous thyroid antigens and immunologically competent cells
Attention was drawn to the analogous occurrence of hypergammaglobulinemia and plasma cell infiltrations of the liver in certain cases of chronic liver disease of unknown aetiology. If immune mechanisms are involved in the pathogenesis of chronic liver disease, then a knowledge of the antigenic structure of the liver, similar to that which proved so fruitful in furthering our understanding of thyroiditis, is essential. Such information can be ascertained by immunization of a heterologous animal with liver (142). A study of the immune serum obtained by this procedure provides valuable information as to the number, type and organ-specificity of the antigens present within the immunizing preparation (142, 172). This information is of great importance, for it is a well-documented fact that the majority of conditions in which auto-immunization occurs involve potent organ-specific antigens (142). The development of auto-immune reactions to poorly-antigenic, non-organ-specific antigens is an exceptional finding (225).

The use of anti-organ antisera as an instrument to study the antigenicity and organ-specificity of tissue antigens possesses certain limitations which must be fully realized, in order that the results be correctly interpreted. 1. It is possible that an animal immunized with a heterologous tissue might contain within its own organs antigens in common with those of the immunizing preparation. The phenomenon of immune tolerance would therefore prevent an immune response from developing to the mutually shared antigens. 2. An antigen may be present in the immunizing preparation in too low a
concentration to stimulate an immune response. 3. Alternatively, the immune serum may contain the specific antibody but the respective antigen may be present in too low a concentration to be detected. 4. Where haemagglutination is employed to detect antigen-antibody reactions, the tissue antigens may not be able to adequately sensitize the cell due to competition with other tissue antigens or proteins present in higher concentration (226).

In the present study the extremely sensitive haemagglutination technique of Boyden was employed to detect the organ- and species-specificity of various anti-liver sera. Haemagglutination had been found by Witebsky and his co-workers to represent a sensitive and reproducible technique for the detection and study of organ-specific antigens (232, 233). The work of Perlmann (228), which established the soluble microsomal proteins as the antigens responsible for the production of anti-liver antibodies (when animals were immunized with whole liver homogenate), suggested that we would experience little difficulty in sensitizing tanned cells with saline extracts of liver. Fluorescent microscopy was employed to confirm the results obtained by haemagglutination, to localize the antigen(s) within the hepatic cell and to detect the presence of antibodies to any insoluble hepato-cellular antigen(s) which otherwise would not have been revealed by haemagglutination. In all instances a high degree of correlation was found to exist between the two methods and in no case was a reaction found by haemagglutination that was not confirmed by fluorescent microscopy.
The rat was ideally suited as a choice for the experimental animal. Its tissue lacks the complicating presence of the Forsmann antigen (39). Further, the animals were available as an inbred strain, though no attempt was made to prove their genetic identity by the exchange of skin homografts. Finally there exist a number of studies on the antigens which comprise the rat hepatic cell (227 - 230).

The microsomal fraction of the parenchymal cell of rat liver has been shown by Henle et al. to contain tissue-specific antigens (227). Employing rabbit anti-rat liver microsomal sera, they demonstrated by slide agglutination and to a lesser extent by complement fixation, the presence of organ- and species-specific hepato-cellular antigens. Perlmann and D'Amelio had shown that antisera to a homogenate of rat liver contained antibodies reactive with the microsomal and mitochondrial components of the hepatic cell (228). By immunodiffusion in agar gel these workers demonstrated a minimum of 5 antigens within the microsome of the cell. It was their opinion that these antigens constituted the bulk of protein extractable from the endoplasmic reticulum of the hepatic cell, and that they appear to function as the predominant stimulators of antibody production when rat liver was injected into a heterologous animal (228). Vogt demonstrated the formation of precipitin bands in agar gel when soluble extracts of rat liver or kidney microsomes were reacted with anti-rat liver, microsomal antisera (230). Absorption of the immune sera with kidney microsomes rendered the antisera specific for
liver microsomes (230).

It was our opinion that these studies had not rigorously established the organ-specificity of the hepato-cellular antigens, based upon the following considerations: 

1. Vogt had demonstrated the organ-specificity of the immune sera after absorption with only one organ i.e., kidney. Furthermore, it should be emphasized that the absence of precipitin lines in agar gel is not indicative of the absence of antibody. To the contrary, it has been demonstrated that antiserum may be rich in non-precipitating antibody which can be detected in high titer by haemagglutination (238). 

2. In demonstrating the organ-specificity of hepato-cellular antigens Henle had relied upon complement fixation and the relatively insensitive slide agglutination reaction. Today the classical concept that complement fixation is a specific characteristic of antigen-antibody reactions is no longer accepted (99). The work of Ishizaka has shown that heat or chemically aggregated gamma globulin can bind complement in a fashion identical to that which occurs when antigen and its specific antibody interact (99). The recent work of Beall (231) suggests that such a mechanism is responsible for the auto-immune complement fixation reaction described by Gajdusek (26). In this reaction, fixation of complement was observed when extracts of human liver (or other tissues) were added to selected human sera (26). Gajdusek had assumed that the fixation of complement reflected the presence of auto-antibodies reacting with antigens present in the tissue extract. However, on the basis of his work, Beall suggests
caution in interpreting these reactions as indicative of antigen-antibody interaction. He has found that the antigenically-active fraction of human liver extract used in the auto-immune complement fixation reaction, to be proteins which behave similarly to serum 7S gamma globulin on salt fractionation, electrophoresis and anion exchange chromatography. As these proteins were not precipitated by an antiserum to 7S gamma globulin, it was Beall's opinion that they represented proteins similar to, but not immunologically identical with, 7S gamma globulin (231). He therefore suggested that the observed complement fixation reaction resulted not from antigen-antibody interaction but from aggregations of normal tissue proteins and serum gamma globulin (231). Suspicion that the auto-immune complement fixation reactions were not due to antigen-antibody interaction was further enhanced by Beall's inability to detect such reactions by haemagglutination. He therefore concluded that it seems reasonable to advise skepticism in the interpretation of complement fixation as an indicator of antigen-antibody interaction when tissue extracts are employed (231). Finally, it is of interest to note that in an extensive table listing known organ-specific antigens, Waksman records the presence of 'characteristic' hepato-cellular antigens but makes no claim to their organ-specificity (142).

The initial experiments demonstrated the extreme heterogeneity of the antigens which comprise the immunizing emulsion of rat liver. The antisera, while consistently agglutinating cells sensitized with rat liver, also agglutinated
to a much lower titer cells sensitized with rat heart, kidney, lung and serum proteins. The relative distributions of those antigens which rat liver shares with rat heart, lung and kidney were established by the absorption experiments. It appeared that lung and heart share a common group of antigens with the kidney and liver, while the kidney contains those antigens in common with the liver which are not present in either lung or heart. As indicated by both haemagglutination and fluorescent microscopy, absorption of the immune serum with a lyophilized homogenate of rat kidney and serum proteins freed the antiserum of all cross-reacting antibodies and conferred on it strict specificity for the antigens present within the cytoplasm of the hepatic parenchymal cell. The specific reaction with rat liver resisted all absorption except that with the homologous rat liver. Absorption with rat liver promptly removed all antibody activity from the immune serum. Indirect fluorescent microscopy indicated that the immune serum reacted with antigens localized to the cytoplasm of the hepatic parenchymal cell, suggesting that we are dealing with a group of antigens similar to those described by Vogt and Perlmann (228, 230).

The results obtained from the study of the species distribution of the organ-specific hepatocellular antigens suggest that each animal species is characterized by the presence of species-specific hepatic antigens, in addition to which there exists a group of antigens which cross species barriers and are present in all species investigated.

It was then investigated whether it was possible to
break immune tolerance to the organ-specific hepatocellular antigens of the rat by immunizing rats with serologically-related rabbit liver or homologous rat liver. The immunization of rats with either of the two liver preparations resulted in the production of antibodies reactive with autologous rat liver. By indirect fluorescent microscopy the antisera diffusely stained the cytoplasm of the hepatic parenchymal cell. The reaction observed with autologous tissue was identical to, but of lesser intensity than, that obtained with homologous rat or heterologous rabbit liver.

Burnet defines an auto-antibody as a globulin which will react specifically with an antigen obtained from the tissues of the animal producing the antiserum (49). If this definition is accepted then the antibody against autologous rat liver present in the serum of the immunized rat can justifiably be considered as an auto-antibody.

The auto-antibody to rat liver manifested strict organ-specificity as indicated by both indirect fluorescent microscopy and haemagglutination. Cells sensitized with rat kidney were not agglutinated by the immune serum, nor was kidney tissue stained when antiserum containing the auto-antibody was employed as the unconjugated middle layer in indirect fluorescent microscopy. It is significant, however, that the rat anti-rabbit liver sera were capable of agglutinating cells sensitized with rabbit kidney, in a manner analogous to that observed with rabbit anti-rat liver sera.

The data therefore suggests that immunization of rats
with rat or rabbit liver procured a breakdown of tolerance to autologous hepatic antigens. The breach in tolerance appears to be selective for the organ-specific components of the liver. The more widely distributed antigens which the liver shares with autologous heart, lung and kidney are not involved in the process. These findings would tend to support and confirm the experiments which demonstrated the presence of such liver-specific antigens, and suggest that under appropriate conditions they may be capable of eliciting the formation of auto-antibodies.

The mechanism whereby immunization with rat or rabbit liver causes the production of auto-antibodies is of course completely unknown. In view of the intimate association of the hepatic parenchymal cell with elements of the reticuloendothelial system, it is extremely unlikely that auto-antibody formation results from the failure of immune tolerance due to inadequate access of the hepato-cellular antigens to the antibody forming system during foetal life.

From the experiments with thioacetamide it is evident that hepato-cellular antigens released into the circulation as a result of toxic injury to the hepatic parenchymal cells have resulted in the formation of auto-antibody in only two out of twenty rats. The significance of these two positive sera remains obscure and will require further investigation. The recent work of Weir certainly suggests that carbon tetrachloride induced hepato-cellular necrosis results in the formation of complement fixing auto-antibodies to liver (234).

Weigle has shown that it is possible to break a stable,
experimentally-induced state of tolerance to BSA in rabbits by the injection of a serologically related foreign serum albumin (235). The break in tolerance was specific for BSA. Kaplan has demonstrated the presence of antibody reactive with autologous organ-specific heart antigens in the sera of rabbits immunized with an emulsion of heterologous heart (177, 178). Similarly, Steblay has shown that sheep which were immunized with heterologous, but not homologous, basement membrane develop antibodies reactive with the autologous basement membrane (236). Asherson and Dumonde have reported the production of complement fixing auto-antibodies to rabbit liver in rabbits immunized with rat liver microsomes (237). This antibody, however, lacked organ-specificity and cross-reacted with rabbit heart, kidney, lung, brain, spleen and muscle. The experiments of these workers, particularly those of Kaplan, Steblay and Asherson, suggest that the ability to break tolerance to autologous proteins is a general property of heterologous tissues. Our experiments with rat liver, however, indicate that tolerance can also be broken with homologous tissue.

It would appear from our experience that the use of complete Freunds' adjuvant was of crucial importance to the breakage of tolerance to the hepato-cellular antigens. It has been demonstrated that following the administration of adjuvant there occurs a marked hyperplasia of the reticuloendothelial system with increase in size of both lymph nodes and spleen (38). Within the hyperplastic lymphatic follicles, conspicuous germinal centers are visible (38) and there can be demonstrated an
extensive proliferation of plasma cells (40, 41). It is not surprising, therefore, that animals immunized with antigens emulsified in Freund's adjuvant produce an amount of antibody approximately 5 times the amount produced in control animals immunized with antigen alone (42). Adjuvant is also used to increase the antigenicity of tissue components. Burnet views this particular effect of adjuvant within the context of his clonal selection theory (43). He considers that adjuvant is capable of breaking down normal immunological homeostasis, and that under such unphysiological conditions immunologically competent cells arise within the hyperplastic lymphatic follicle possessing the capacity to react with autologous antigens (43). Therefore, the fundamental defect in experimentally-induced auto-immunization lies in the antibody-forming tissue itself.

In summary, our data suggests that it is possible to induce a selective loss of tolerance in rats to the organ-specific hepatic antigens by repeatedly immunizing them with either rabbit or rat liver emulsified in Freund's adjuvant. No pathological changes were observed in the liver in response to the presence of the auto-antibody. The duration of this break in tolerance is at present unknown and it is conceivable that, if prolonged, it might result in damage to the liver. Certainly, if immune mechanisms are involved in the pathogenesis of chronic liver disease, the defect must surely reside in the antibody-forming tissues, since the liver protein does not normally appear capable of eliciting an antibody response in the studies reported here.
SUMMARY

An investigation of the organ and species distribution of rat hepato-cellular antigens has been undertaken. The tanned red cell haemagglutination technique and fluorescent microscopy were employed. Results obtained with these techniques strongly suggest that liver possesses organ-specific antigens and shares other common antigens with the heart, kidney and lung. As a result of thioacetamide induced hepato-cellular injury, organ specific liver proteins were released into the circulation where they were detected by means of the haemagglutination inhibition technique, employing a specific anti-liver antiserum. During the period of observation the circulating liver proteins were unable to produce an antibody response within the host. Only by repeated immunization of rats with homologous or heterologous liver, emulsified in complete Freund's adjuvant, was it possible to induce a selective loss of tolerance to the organ specific hepatic antigens. The breakdown of immune tolerance resulted in the production of antibody reactive with autologous liver. In the studies reported here no adverse effects could be detected in the rat liver as a result of the presence of such antibodies.
CLAIMS TO ORIGINALITY

1. The relative distribution of those antigens which rat liver shares with rat heart, lung and kidney was established. Lung and heart share a common group of antigens with kidney and liver, while the kidney contains those antigens in common with the liver which are not present in either lung or heart.

2. The presence of organ specific hepato-cellular antigens has been strongly suggested.

3. It has been demonstrated that each species is characterized by the presence of species-specific hepato-cellular antigens, in addition to which there exists a group of antigens which cross species barriers and are present in all species investigated.

4. The use of rabbit anti-rat liver sera to detect the presence of organ specific hepato-cellular proteins, released into the circulation as a result of thioacetamide induced hepato-cellular injury, has been demonstrated for the first time.

5. Repeated immunization of rats with homologous or heterologous livers, emulsified in complete Freund's adjuvant, resulted in the production of antibodies which reacted specifically with autologous liver antigens.

6. The presence of circulating auto-antibodies to rat liver was not found to be associated with demonstrable hepatic pathology.
A typical example of the haemagglutination of rat liver sensitized tanned cells by a rabbit anti-rat liver serum. Reading from left to right, tube 5 was considered as positive, tube 6 as plus/minus and tube 7 as negative.
A typical example of haemagglutination inhibition. From left to right in the bottom row, twofold serial dilutions of rat serum, containing hepato-cellular antigens released into the circulation as a result of thioacetamide induced liver damage, were prepared in 0.5 ml volumes in a series of test tubes. 0.5 ml of rabbit anti-rat liver serum, diluted to a concentration of five haemagglutinating units/ml, was added to each of these tubes. The absence of haemagglutination when liver sensitized cells were added, indicated the presence in the test serum of hepato-cellular antigens identical to those employed to sensitize the tanned cells. The upper row served as the control and depicts the haemagglutination of liver sensitized cells by rabbit anti-rat liver serum.
Rabbit anti-rat liver serum employed as the unconjugated middle layer in indirect fluorescent microscopy. Note that only the cytoplasmic elements of the hepatic parenchymal cells are stained. There is no staining of the nuclear components of the cells or of the connective tissue elements of the rat liver.
Low and high powered photomicrograph of a section of liver obtained from a rat whose serum contained auto-antibodies to liver.
FIGURE V

Photomicrograph of a section of liver obtained from a rat twelve hours after the injection of thioacetamide.
Low and high powered photomicrograph of a section of liver obtained from a rat twenty-four hours after the injection of thioacetamide.
Low and high powered photomicrograph of a section of liver obtained from a rat thirty-six hours after the injection of thioacetamide.
Low and high powered photomicrograph of a section of liver obtained from a rat forty-eight hours after the injection of thioacetamide.
Photomicrograph of a section of liver obtained from a rat ninety-six hours after the injection of thioacetamide.

2. Popper, H., Schaffner, F.
Liver Structure and Function,

3. Popper, H., Zak, F.G.

4. Sherlock, Sheila.


6. Tisdale, W.A.

7. Isselbacher, K.J., Greenberger, N.J.

8. Eaton, M.D., Murphy, W.D., Hanford, V.L.


10. Franklin, E.C.
Progress in Allergy Vol VIII,
Ed. by Kallos, P., Waksman, B.H.

11. Mellors, R.C., Korngold, L.

12. White, R.G.
Ciba Foundation Study Group,
Immunologically Competent Cell,

13. Sainte-Marie, Guy, Coons, A.A.

14. Fahey, J.L., Robinson, A.G.


31. Rose, N.R., Witebsky, E. 
Immunopathology 1st International Symposium, 
Ed. by Grabar, P., Miescher, P. 

32. Rose, N.R., Kite, J.H., Doebbler, T.K. 
Immunopathology 2nd International Symposium, 
Ed. Grabar, P., Miescher, P. 

Cell Bound Antibodies, Ed. Amos, B., Koprowski, H. 

34. Rose, N.R., Kite, J.H., Doebbler, T.K., Witebsky, E. 
Injury, Inflammation & Immunity, 
Williams & Wilkins Co., Baltimore, 1964.

35. Spiegelberg, H.L., Miescher, P.A. 

36. Felix-Davis, D., Waksman, B.H. 

37. Koffler, D., Freedman, A.H. 

38. Ehrlich, Paul. 
Collected Studies on Immunity translated by Charl. Bolduan, 
J. Wiley & Sons, 1906.

39. Raffel, S. 
Immunity 2nd Edition, 

40. Burnet, F.M. 
Clonal Selection Theory of Acquired Immunity, 

41. Fischer, D.S. 

42. Widal, F., Abrami, P., Brule, M. 

43. LeGendre, - Brule, - . 
Fresse Med. 17: 70, 1909.

44. Metalnikoff, S. 

45. Damesheck, W., Schwartz, S.O. 
Medicine, 19: 231, 1940.


Advances in Immunology, Vol. 4, 1964,
Ed. Dixon, F.J., Humphrey, J.H.

63. Brent, L., Medawar, P.B.
Tolerance & Auto-Immune Phenomena
C.C. Thomas, Springfield, Ill.

64. Medawar, P.B.
The Immunologically Competent Cell,
Ciba Foundation Symposium,

65. Yoffey, J.M.
Annual Review Medicine, Vol. 15, 1964,

66. Dameshek, W.,
Conceptual Advances in Immunology and Oncology,

67. Gell, P.G.H., Benacerraf, B.
Advances in Immunology, Vol. 1, 1961,

68. Venters, H.D., Good, R.A.

69. Amos, B.
Cell Bound Antibody P. 127,
Ed. Amos, B., Koprowski, H.

70. Arnason, B.G., Waksman, B.H.

71. Waksman, B.H.
Immunopathology II International Symposium,
Ed. by Grabar, P., Miescher, P.


73. Gell, P.G.H.
Cellular & Humoral Aspects of the Hypersensitive States,
Ed. H.S. Lawrence,

74. Turk, J.L., Stone, S.H.
Cell Bound Antibody, Ed. by Amos, B., Koprowski, H.
75. Waksman, B.H., Arbouys, S., Arnason, B.G.

76. McGregor, D.D., Gowans, J.L.

77. Waldenström, J.
Progress in Allergy Vol. VI,
Ed. by Kallos, P., Waksman, B.H.

78. Franklin, E.C., Fudenberg, H., Martensson, L., Meltzer, M.,
Stanworth, D.R.
Immunopathology III International Symposium,

79. Harboe, M., Osterland, C.K.
Immunopathology III International Symposium,

80. Fudenberg, H.
Ed. Kallos, P., Waksman, B.H.
Prog. in Allergy Vol. VII: 1, 1963.
S. Karger, Basel.

81. Fudenberg, H., Franklin, E.C.

82. Osserman, E.F., Takatsuki, K.

83. Goldberg, B.
Immunopathology III International Symposium,

84. Bitensky, L.

85. Pitts, R.F.
Physiological Basis of Diuretic Therapy,

86. Stetson, C.A., Jensen, E.

87. Shulman, M.R.
Immunopathology III International Symposium,
and
88. Ackroyd, J.F., Rook, A.J.
   Clinical Aspects of Immunology,
   Ed. by Gell, P.G.H., Coombs, R.R.A.

89. von Pirquet, C.F.,
   Arch. Int. Med. 7: 259, 1911. 7: 383, 1911.

90. Hawn, C.V.Z., Janeway, C.A.

91. Germuth, F.G.,


94. Dixon, F.J.
   Harvey Lectures Series 58, 1962-63.

95. Dixon, F.J.
   Immunopathology II International Symposium,
   Ed. by Grabar, P., Miescher, P.,

96. Dixon, F.J.

97. Germuth, F.G., McKinnon, G.E.

98. Weigle, W.O.
   Advances in Immunology Vol. 1, 1961,

99. Ishizaka, K.
   Prog. Allergy Vol. VII,
   Ed. by Kallos, P., Waksman, B.H.,

100. Christian, C.L.

101. Mota, I.

102. Miescher, P., Straessle, R.
    Vox Sanguinis (N.S.) 1: 83, 1956.

103. Straessle, R., Miescher, P.
104. Cohn, Z.H., Hirsch, J.G.

105. Thomas, L.
The Streptococcus, Rheumatic Fever & Glomerulonephritis,

106. Barbaro, J.F.

107. Barbaro, J.F.

108. Feldman, J.O.

109. Boyden, S.

110. Sabesin, S.M., Banfield, W.G.

111. Dixon, F.J.
Advances in Immunology & Oncology,

112. Movat, H.Z., Fernández, N.V.P.

113. Benoceriof, B., McCluskey, R.T.
Annual Reviews Inc. Palo Alto, California.

114. McCluskey, R.T., Benoceriof, B., Potter, J.L., Miller, F.


116. Gowans, J.L.
The Immunologically Competent Cell,
Ciba Foundation Study Group #16

117. Gowans, J. L.

118. Wiener, J., Sprin, D., Russel, P.S.

119. Billingham, R.E., Silvers, W.K., Wilson, D.B.
120. Johanovsky, J.
   Immunology. 3: 179, 1960.

121. Voisin, G.A., Toullet, F.
   Ciba Foundation Symposium.
   Cellular Aspects of Immunity,
   Ed. Wolstenhome, G.E.W., O'Connor, P.

122. Inderbitzin, T.
   Mechanism of Hypersensitivity,

123. Algire, G.H., Weaver, J.M., Prehn, R.T.

124. Woodruff, M.F.A.

125. Rosenau, W.
   Cell Bound Antibody,
   Ed. by Amos, B., Koprowski, H.

126. Benacerraf, B., McCluskey, R.T.

127. Turk, J.I.

128. Kay, K., Rieke, W.D.

129. Najarian, J., Feldman, J.D.

130. Lawrence, H.S.
   Quotes David & Al-Askari in Cell Bound Antibody P.5.
   Ed. by Amos, B., Koprowski, H.

131. David, J.R., Al-Askari, S., Lawrence, H.S., Thomas, L.

132. Lawrence, H.S.
   Mechanisms of Hypersensitivity,

133. Lawrence, H.S., Rapaport, F.T., Converse, J.M., Tillett, W.S.
   Ciba Foundation Symposium on Transplantation,
134. Boyden, S.V.
    Cell Bound Antibody,
    Ed. Amos, B., Koprowski, H.

135. Rosenau, W., Moon, H.D.

136. Koprowski, H., Fernandes, M.V.

137. Stastny, P., Sternbridge, V.A., Ziff, M.

138. Stastny, P., Sternbridge, V.A., Ziff, M.
    Arth. & Rheumat. 6: 64, 1963.

139. Stastny, P.

140. Helyer, B.J., Howie, J.B.

141. Sutherland, D.E.R., Archer, O., Peterson, R.D.A.,
    Eckert, E., Good, R.A.

142. Waksman, B.H.

143. Waksman, B.H.

144. Rumke, P., Hellinge, G.

145. Witebsky, E., Rose, M.R., Nadel, H.

146. Guyer, M.F.

147. McCartney, J.L.

148. Kennedy, W.P.
    Quart. J. Exper. Physiol. 14: 279, 1924.

149. Waksman, B.H.
    Henry Ford Symposium,
    Mechanisms of Hypersensitivity,
150. Steiner, J.W., Volpe, R. 

151. Steiner, J.W., Langer, B., Schatz, D.L. 

152. Freund, J. 
Annual Reviews Inc., Palo Alto, California.

153. Pasteur, L., Jonbert, G. 

154. Freund, J., Lipton, M.M., Thompson, G.E. 

155. Freund, J., Lipton, M.M., Thompson, G.E. 

156. White, R.G. 
Henry Ford International Symposium, 
Mechanisms of Hypersensitivity, 

157. Smith, R.T. 
Tolerance Acquise et Tolerance Naturelle a L'egard de 
Substances Antigeniques Definies. P. 117. 
Centre National De La Recherche Scientifique, 

158. Freund, J., Lipton, M.M. 

159. Boyd, W.C. 
Fundamentals of Immunology, 3rd Ed. 

160. Ehrich, W.E., Halbert, S.P., Mertens, E., Mudd, S. 

161. Holt, L.B. 

162. White, R.G., Coons, A.A. 


165. Bishop, F. 
Conference on Auto-Immunity, 

167. Paterson, P.Y.
Cell Bound Antibody,
Ed. Amos, E., Koprowski, H.


169. Schwartz, R., Andre, J.

170. Aisenberg, A.C., Wilkes, E.

171. Hoyer, L.W., Good, R.A., Condie, R.M.

172. Swanson, Beck, J.

173. Kaplan, M.H., Bolande, R., Rakita, L., Blair, J.

174. Kaplan, M.H., Meyeserian, M.

175. Kaplan, M.H., Dallenbach, F.D.

176. Kaplan, M.H., Meyeserian, M., Kushner, I.

177. Kaplan, M.H., Meyeserian, M.

178. Kaplan, M.H., Craig, J.M.

179. Kaplan, M.H.

180. Kaplan, M.H., Suchy, M.L.

181. Kaplan, M.H., Svec, K.H.

182. Lawrence, H.S.
183. White, R.G.
The Immunologically Competent Cell,
Ciba Foundation Study Group,

184. Editorial

185. Dacie, J.V.
The Haemolytic Anaemias Part II


187. Green, H.N.

188. Rosenthal, M.C., Fisciotta, A.V., Komninos, Z.D.,
Goldenberg, H., Dameshek, W.

189. Billingham, R.E., Brent, L., Medawar, P.B.

190. Simonsen, M.

191. Simonsen, M.
Progress in Allergy Vol. VI
Ed. by Kallos P., Waksman, B.H.

192. Nisbet, N.W., Heslop, B.F.

193. Aisenberg, A.C., Waksman, B.H.

194. Venters, H.O., Good, R.A.

195. Frick, P.G.


Ciba Foundation Symposium on Cellular Aspects of Immunity,

199. Holman, H.R., Deicher, H., Robbins, W.C. 
Henry Ford Hospital Symposium, 
Mechanisms of Hypersensitivity, 


201. Levin, M.B., Pincus, H. 

202. Ziff, M. 

203. Peterson, R.D.A., Good, R.A. 
Annual Reviews, Inc. Palo Alto, California.

Tanner, M. 

205. Holman, H.R. 

206. Epstein, W.V. 

207. Holmes, M.C., Gorrie, J., Burnet, F.M. 

208. Helyer, B.J., Howie, J.B. 

209. Tyler, A. 

210. Miescher, P. 
Recent Progress in Microbiology Vol. 7 1958, P. 205 
C.C. Thomas, Springfield, Ill.

211. Thomas, L. 
Quotes P. Miescher 

212. Weissmann, G., Thomas, L. 

213. Paterson, P.Y., Harwin, S.M. 
214. Paterson, P.Y.
Conference on Auto-Immunity,

215. Paterson, P.Y.
Cell Bound Antibody,
Ed. by Amos, B., Koprowski, H.

The Serology of Conglutination,

217. Kunkel, H.G., Williams, R.C.
Annual Reviews, Inc. Palo Alto, California.

218. Williams, R.C., Kunkel, H.G.,

219. Boyden, S.

220. Kabat, E.A., Mayer, M.M.
Experimental Immunochemistry 2nd Ed.
C.C. Thomas, Springfield, Ill. 1962.

221. Nairn, R.C.
Fluorescent Protein Tracing,

222. Sainte-Marie, G.
J. Histochem. & Cytochem. 10: 250, 1962.

223. Gell, P.G.H., Coombs, R.R.A.
Clinical Aspects of Immunology,

224. Witebsky, E.
London, 1936.

225. Milgrom, F., Centeno, S., Shulman, S., Witebsky, E.

226. Richter, M.
Personal communication.


228. Perlmann, P., D'Amelio, V.
229. Vogt, P.

230. Vogt, P.

231. Beall, G.H.

232. Witebsky, E., Rose, N.R.

233. Milgrom, F., Witebsky, E.

234. Weir, D.M.,

235. Weigle, W.O.
Immunopathology,
IIIrd International Symposium,

236. Steblay, R.W.
Immunopathology,
IIIrd International Symposium,

237. Asherson, G.L., Dumonde, D.C.

238. Richter, M.
Personal communication.