1965

Studies on the Production of yM and yG Antibodies in the Mouse

Richard Charles Blinkoff

STUDIES ON THE PRODUCTION OF γM AND γG ANTIBODIES IN THE MOUSE

A thesis submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy at The Rockefeller Institute

by

Richard C. Blinkoff, A.B.

Approved for publication

Dr. Zanvil A. Cohn

Professor at the Rockefeller University

16 April, 1965

The Rockefeller Institute

New York, New York

PREFACE

Within the last ten years, considerable interest has been aroused within the field of immunology by the discovery of macroglobulins with antibody activity. These molecules were first studied as the peculiar products of neoplastic disease states in man. They gained somewhat more universal importance when it was recognized that they were part of the primary antibody response to a variety of antigens: macroglobulin (γM) antibodies preceded the appearance of 7S (γG) antibodies in the serum of immunized animals. This transition of antibody type in the serum has now been found in so many cases that it appears that a primary response without this event is the exeception rather than the rule.

The studies presented in this thesis began with an attempt to study a primary antibody response in the spleen of the mouse. When it was discovered that γM and γG antibodies were involved, our efforts turned to studies on the relative synthesis of these two antibodies by the spleen and then to experiments attempting to dissociate the two responses so that the cellular basis might be better understood. Finally, experiments in which spleen cells were removed from immunized donors and were injected into X-irradiated recipients were employed in attempts to assess the importance of anatomical continuity and extracellular antigen to the γM-γG transition.

I am grateful to many of the scientists of The Rockefeller Institute for their frequent guidance and discussions. In particular, I am in debt to Dr. Zanvil A. Cohn, my research advisor. His encouragement, critical advice, and warm personal friendship helped to educate me as both a scientist and a human being. This work was done in the laboratories of Dr. James G. Hirsch, and I am very grateful to Dr. Hirsch and Dr. Stephen I. Morse as well as Dr. Cohn for their enthusiastic help during my four years with them.

Thanks are also due to various persons who helped me with technical problems: to Dr. Henry Kunkel, who instructed me in the ultracentrifugal work and allowed me to use his laboratory facilities, to Dr. Merrill Chase who developed the method for separating spleen cells, to Dr. H. W. Burnet of The New York Hospital-Cornell Medical Center for allowing me to use the facilities of the radiotherapy division, and to Miss Lillian Gregg of The Rockefeller Institute Section Cutting Service who prepared all the histological slides.

The tedious job of typing two drafts of this manuscript was ably handled by Mrs. Beatrice Isaacs and Miss Penny Sturrock.

ABSTRACT

The organ localization of intravenously administered C^{14}-labeled intact S. adelaide was studied in mice. In the absence of circulating antibody, the spleen had a higher specific activity than other organs studied. The primary antibody response to this antigen was then studied using a specific flagellar immobilization assay. Spleen homogenate supernatants exhibited two distinct antibody peaks which were well separated in time. The serum titer curve was not detectably bimodal.

Sera and spleen homogenate supernatants were characterized by electrophoresis, ultracentrifugation, and treatment with 2-mercaptoethanol. The primary antibody response was found to consist of the early, transient production of γM antibodies followed by the production of γG antibodies. This response was quantitatively reduced but not qualitatively affected by the removal of spleens from the mice before antigen injection.

Methotrexate and colchicine both exhibited differential effects on the synthesis of γM and γG when they were administered during the antibody response. With certain doses of each, the γG response seemed more susceptible to inhibition. In all cases, however, the γG response recovered to near normal levels after a delay. Histological studies during a methotrexate inhibited response showed that normal levels of γM could occur in the serum at a time when there were no pyroninophilic blast cells or secondary nodules in the spleen. The recovery of γG was well correlated with the appearance of many haemocytoblasts in the red pulp. At that time there were still no secondary nodules in the white pulp. γG antibodies recovered to nearly normal levels despite the fact that maturation of the haemocytoblasts to mature plasma cells was inhibited.

A study was made of the transfer of spleen cells from primed animals to homologous X-irradiated recipients. When spleen cells were taken from immunized donors on day 2 or day 6 after injection of antigen, only γM antibody could be demonstrated in the recipients. Spleen cells taken on day 8 or day 10 after antigen produced mostly γG antibody in the recipients.

TABLE OF CONTENTS

I. INTRODUCTION

A.) γM and γG Antibodies

1.) The unity and heterogeneity of antibodies

The micro-heterogeneity of proteins is a complex problem which cannot properly be considered in this review; it has been well discussed by Colvin, Smith, and Cook (1954). A discussion of the difficulties inherent in relating biological function to chemical and physical structure, however, is of import to any study of different classes of antibodies. Much confusion in antibody classification stems from early studies in the functions of antibodies and from later attempts to postulate a unified theory of these phenomena. We shall therefore begin with the historical background.

At the end of the nineteenth century, the major interest of immunology was the description of phenomena involved in host defense against microorganisms. Very little was known about mechanism or factors involved, and external similarity was the sole criterion for relating one type of defense to another. With each new phenomenon a new word was added to the vocabulary of host defense. Antitoxins were first described by Emil von Behring, who found that animals injected with sublethal doses of tetanus or diphtheria toxins produced serum factors which could neutralize the toxins (von Behring and Kitasato, 1890). These antitoxins were specific, and they would confer specific immunity when transferred to a normal recipient. Other serum factors, the bacteriolysins, were soon discovered (Pfeiffer, 1894;; Pfeiffer and Friedberger, 1903). When bacteria were injected into the peritoneal cavities of previously immunized guinea pigs, the bacteria were lysed, and this lysis was specific for the bacteria used as antigen. The activity could also be transferred to a non-immune recipient with cell-free serum. Although both functions were localized in serum, there was no reason for assuming any relation between the two or means of characterizing either. In 1895, Bordet distinguished the thermostabile lysin activity from heat-labile serum bactericidal factors. This gave one physical means of classifying activity, but the multiplicity and interaction of these factors was still confusing.

Within a few years, many of the other basic phenomena of immunology were described: Denys and Leclef (1895) described phagocytosis-promoting opsonins, Kraus (1897) discovered precipitins, Bordet (1898) observed immune hemolysis, and Bordet and Gengou (1901) discovered complement fixation. Each function was associated with a different hypothetical cause: antitoxin, precipitin, lysin, agglutinin, amboceptor, opsonin, etc.; each term was assumed to represent a different serum factor. There was often a lack of parallelism

on the titration of different activities, and such evidence supported theories of multiple factors.

On the other hand, evidence relating these functions quantitatively was gradually accumulating. Doerr and Russ (1909) showed a quantitative relationship between the precipitin content of an antiserum and its anaphylactic sensitizing properties. Felton and Bailey (1926a) found that the water-insoluble protein from Type I antipneumococcus serum agglutinated the specific cocci, protected mice, gave positive precipitin tests, fixed complement, and stimulated phagocytosis. All the protective antibody was precipitated by the specific polysaccharide (Felton and Bailey, 1926 b) and could be recovered from the precipitate (Felton, 1932). Heidelberger and Kabat (1936) studied the type-specific agglutinins and precipitins in the horse. The amount of each by weight was identical, and the removal of one resulted in the quantitative removal of the other. Similar quantitative studies by Chow and Wu (1937) proved the identity of the precipitating, anaphylactic sensitizing and complement-fixing activities in rabbit antisera. The weight of this and similar evidence led to the view that one antibody could perform all of the above functions. As stated by Zinsser (1931) this "unitarian hypothesis" follows:

By such a conception of antibodies, we do not imply that a complex cell like, for instance, a typhoid bacillus can only give rise to one variety of antibody... we do mean that, were we working with a single antigen in a pure state, one variety of antibody would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen...The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, bactericidal phenomena, opsonization, or sensitizing effect on the anaphylactic sense) would be determined, not by differences in the nature of the antibodies with which the antigen had united, but rather in the co-operating substances (alexin, leucocytes, tissue-cells) and by the environmental conditions under which the observations are made.

We generally accept today the theory that the same antibody can perform more than one function. But we also know that an antigen often, perhaps always, gives rise to different kinds of antibodies. For instance, the Rh antigen gives rise to two different antibodies. Both combine with red blood cells, but only one can agglutinate them. The second is called an "incomplete" antibody. Presumably, both antibodies are produced by the same antigen (Race and Sanger, 1954). As another example, conjugated antigens are known to evoke antibodies with different specificities: some are directed against the haptene, some against the protein carrier, and some against both haptene and protein together (Landsteiner, 1945). Does this disprove the unitarian hypothesis?

Perhaps, as Zinsser points out in the above quotation, what we have been calling an antigen is really a complex body containing more than one antigen - i.e., a conjugated protein is really two antigens, the carrier and the haptene. But among antibodies directed only against the haptenic group there are molecules with different affinities for different parts of the haptene (Landsteiner and van der Scheer, 1938). More recent studies have shown that antibodies directed against a simple dextran molecule are quite heterogeneous with respect to the sizes of their combining sites (Schlossman and Kabat, 1962). In fact, if the concept of "one antigen-one antibody" is to be upheld, we must specify smaller and smaller molecular groupings as antigenic determinants. The concept is then hardly testable and its truth is not an important question here. What is important is that we never work with "pure antigens," and all antibody responses are more or less heterogeneous (Smith and Jager, 1952; Boyd 1956). The amount of heterogeneity ascribed to an antiserum is often a direct function of the number of criteria by which it has been examined. In general, antibody activities are separated by either immunologically specific methods or by physico-chemical, non-specific methods.

Specific methods for separating antibodies depend on the formation of antigen-antibody complexes. For such methods to be useful, one must be able to isolate the complex and remove non-specifically bound contaminants, dissociate the complex, and eventually separate the released antigen from the antibody. Non-specific methods, on the other hand, depend on physicochemical differences among the plasma proteins and among antibodies themselves. These take advantage of such properties as solubility, electrophoretic mobility, and molecular weight. The many variations of specific and non-specific methods have been well reviewed by Cann (1958) and will not concern us here. What might be pointed out, however, is the inconclusiveness of any one separation, specific or non-specific, in terms of the functional or molecular properties of the antibodies isolated. Specific methods may separate antibodies which still differ in combining affinities or physical characteristics. Non-specific isolates may be heterogeneous by other non-specific techniques, and they may or may not separate functions. Later in this review, we shall speak of the classification of antibodies as γG or γM. Perhaps it is important here to illustrate more fully the limitations of such classifications and the possible heterogeneity of the antibodies in each class.

a) <u>That non-specific isolates may be heterogeneous by other non-specific criteria</u>: it is not at all surprising that serum fractions isolated by one

physico-chemical method should still be heterogeneous. For example, one common serum fractionation is the ethanol procedure as defined by Cohn and his associates (Cohn et al., 1940). When human serum is treated with ethanol under carefully controlled conditions, four fractions of gamma globulin can be isolated: γ_1-globulin, II-1,2, II-3, and γ_2-globulin. Each fraction has a different mean isoelectric point and each migrates as a single boundary in an electric field. If examined by reversible boundary spreading, however, each fraction is electrophoretically heterogeneous (Jager et al., 1948). In addition, each fraction has been found to be impure according to sedimentation, solubility, and immunological criteria (Cohn, Deutsch, and Wetter, 1950). Also, γ-globulins have been shown to exhibit almost continuous distributions when examined by various physico-chemical parameters. Thus, Williams et al. (1952) were unable to resolve horse γ-globulin into fractions having discrete sedimentation coefficients. And bovine γ-globulin has been separated into eight fractions of different mean mobilities when examined by reversible boundary-spreading (Cann, Brown, and Kirkwood, 1949). In fact, the designation of fractions isolated by any non-specific method is largely arbitrary and must be employed with reservation. As Cann (1958) has pointed out:

The serum globulins are very heterogeneous with respect to both biological and physico-chemical criteria. Indeed, the globulin fraction contains so many molecules differing only slightly from one to another in electrophoretic mobility that it might well be considered as possessing a continuous, rather than a discrete distribution of mobilities, as often supposed. The various maxima in the distribution can be designated arbitrarily as α, β-, and γ-globulin.

b) That non-specific separation may isolate functional activities: despite the broad heterogeneity of γ-globulins, non-specific methods may, at times, purify certain antibody activities. For instance, one activity may be separated from another by the preferential inactivation of the latter. Thus, Forssman (1922) reported that when anticorpuscle serum was mixed with ether and heated at 56°C the hemolysin activity was destroyed while the agglutinin activity was unaffected. This, of course, depends on the heat-lability of complement. But true separations of activity have also been achieved. Type specific horse anti-pneumococcus antibodies are euglobulins (Felton, 1928), while horse diphtheria antitoxins are pseudoglobulins (Pappenheimer et al., 1940). Deutsch and his associates (Deutsch et al., 1947) found that isohemagglutinins and typhoid O agglutinins were concentrated in the γ_1-globulins while diphtheria antitoxins were concentrated in the γ_2-globulins. Kuhns (1954) found that skin-sensitizing diphtheric antitoxin migrated as a γ_1-globulin,

whereas nonsensitizing, precipitating antitoxin was a γ_2-globulin. Also, complete Rh saline agglutinins, cold agglutinins, heterophile antibodies, and others are associated with 19S γ-globulins separable by centrifugation (Franklin, 1962).

c) That non-specific isolates may be functionally heterogeneous: instead of separating functions non-specifically, it is more usual to find the same function performed by antibodies having a broad range of physico-chemical characteristics. Deutsch et al. (1947) found typhoid H agglutinins and antibodies against influenza virus in both γ_1 and γ_2 fractions. Antibodies with both 19S and 7S sedimentation coefficients have been found in responses to S. typhosa, T_2 phage, diphtheria toxoid, human serum albumin, bovine gamma globulin, and keyhole limpet hemocyanin (Bauer, Mathies, and Stavitsky, 1963). Also, incomplete Rh antibodies have been found broadly distributed throughout the serum globulins or at times having a bimodal distribution (Cann et al., 1952).

d) That specific isolates may be heterogeneous: finally it is worth noting that specifically isolated antibodies may not only have varying physico-chemical properties, but also that they may be functionally heterogeneous. Preparations containing nearly 100% precipitable antibodies have been shown to consist of molecules with a wide range of affinity for the antigen involved (Karush, 1959).

The cause of this variation among antibodies has proven to be a question of great interest in immunology. Some variation certainly results from the multiplicity of antigenic determinants on any one antigen. But this is not the entire answer. For instance, much investigation during the 1930's was concerned with the responses of different species to the same antigen. Heidelberger and Pedersen (1937) found that type specific antipneumococcal antibodies in the horse had sedimentation coefficients of 18.4 while those in the rabbit were mainly 7S. Kabat (1939) extended this work to show that anti-pneumococcal antibodies from cow, horse, and pig had molecular weights of nearly one million; antibodies from human, rabbit and monkey had the same molecular weights as normal γ-globulin (160,000). Goodner and Horsfall (1936) divided antibodies on the basis of their ability to fix complement: thus, rabbit, rat, guinea pig, and sheep antipneumococcal antibodies were complement fixing, while horse, human, canine, mouse, cat, and goat antibodies did not fix complement. Horsfall and his coworkers also claimed that sera from different species could have different therapeutic value (Horsfall et al., 1937).

Another focus of interest was the antibody response to different antigens in the same animal. We have already mentioned that anti-pneumococcal antibodies and diphtheria anti-toxins in the horse had different solubility properties. Van der Scheer, Wyckoff, and Clark (1940) identified a "T component" in horse serum which migrated electrophoretically between and γ and globulins. Antibody responses fell into three groups: 1) elevated γ, no T, 2) elevated T, normal γ, and 3) both classes elevated. In studying sera from 15 horses hyperimmunized against bacterial antigens, these authors found that the responses to each antigen characteristically fell into one of these three groupings.

Experiments on the role of species differences or antigenic differences did not establish any universal principle regarding antibody variation; such experiments often added to pre-existing confusion and sometimes could not be confirmed. Some sense was made out of chaos by Porter and his associates. These authors used partition chromatography on celite to separate the anti-ovalbumin activities in immunized rabbits (Porter, 1955). They found that after repeated intravenous immunizations for three weeks, the antibody produced was located in the slowly moving chromatographic fractions. After a secondary schedule of immunizations two months later, the antibodies were found in faster chromatographic fractions. Antibodies to type III pneumococcus and influenza virus were also separable chromatographically and showed the same pattern of response (Humphrey and Porter, 1956). When two antigens were given simultaneously, the response to each was independent of the other. After a subcutaneous injection of antigen with adjuvant, a granuloma was formed. This granuloma could be shown to be producing at least 80% of the total antibody (Askonas and Humphrey, 1955). Unlike the antibody appearing after intravenous immunization, antibody from granulomas was found in both the fast and slow moving chromatographic fractions within a few weeks and no change was observed over a period of months. This observation led these authors to suggest that perhaps a constant site of antibody formation was associated with the production of a constant type of antibody. The change in type of antibody after an intravenous injection might then be a reflection of a change in site of production. These experiments were important in stressing two things: 1) the possiblity of a bimodal response, and 2) the critical role of route of antigen administration and stage of immunization. We shall review experiments detailing similar responses in the following section.

There can be no doubt that the γ-globulins are a heterogeneous group of proteins. It is unlikely that the heterogeneity is caused by the difference between antibody γ-globulin and γ-globulin with no antibody activity. In fact, many investigators feel that all γ-globulin production is in response to some sort of antigenic stimulus (Gustafsson and Laurell, 1958; Svehag, 1964 b). Also, in view of the differences in size, mobility, and chemical composition among antibodies, it seems unlikely that the heterogeneity is solely the result of differing specificities. Recent results on the structure of antibodies, however, support the idea that within any class or type of antibody the heterogeneity is caused by differences in the number and structure of subunit polypeptide chains. Thus, mammalian γG globulins have been separated by reduction and subsequent starch gel electrophoresis in 8M urea into two kinds of polypeptide chains: light (L) and heavy (H) chains (Edelman and Poulik, 1961). The H chains apparently contain the antigenic differences between the human antibody types γ_2, γ_{1A}, and γ_M (Edelman and Benacerraf, 1962). On the other hand, the heterogeneity of the molecules in any classification according to size or immunoelectrophoretic type appears to depend on differences in their content of L chains. Upon starch gel electrophoresis in urea, "normal" γ-globulin showed a slow band corresponding to the H chains and a faster smear of L chain components. Specifically purified antihaptene antibodies, however, showed sharp bands of distinctive mobility in the L chain region, and the pattern of these bands was characteristic for each antibody specificity (Edelman et al., 1961; Edelman, Benacerraf, and Ovary, 1963). Thus, specific isolation can separate antibodies of remarkable structural similarity. Even antibodies directed against a simple chemical grouping, however, have a range of different structures (Benacerraf, Merryman, and Binaghi, 1964). Also, despite the lack of major differences between pooled and specifically isolated γ-globulins, there is some evidence that small chemical and antigenic differences do exist (Gurevich, Gubernieva, and Miasoedova, 1961; Kunkel, Mannik, and Williams, 1963). So perhaps more than one antibody type is always involved in any antigen-antibody reaction.

Talmage (1959) has suggested that the heterogeneity of γ-globulins enables a multiple reaction theory to account for antibody specificity. According to this theory, antigens combine with pre-existing globulins for which they have some degree of affinity. An antibody response is the selective increase in production of those natural globulins having a high affinity for the injected antigen but overlapping specificities. This theory successfully avoids the

question of whether one antibody is formed in response to a single antigenic determinant. Talmage also says that "in a mixture of a large number of different globulin molecules, the dominant reactivity will be that common to the largest number of molecules present." This conforms to the general opinion that the functional manifestation of an antigen-antibody interaction varies with the environmental test conditions.

The causes of heterogeneity among antibody globulins remain unresolved. Some of the factors affecting the γM and γG responses will be discussed later in this paper. One should remember, however, that γM and γG groupings result from a number of arbitrary criteria for differentiating structures and functions, and that in both cases we are dealing with the average behavior of groups of widely varying protein molecules. There no doubt will be many subdivisions and exceptions to these groups as methods become more sophisticated.

2.) The sequential production of γM and γG and some exceptions to the rule

Porter and his associates were not the first to show that the type of antibody produced might vary with the stage of immunizations. Experiments studying the response in the horse to pneumococci had associated the early antibody produced with an electrophoretic component not present in normal serum: the "T component," migrating between the γ and β peaks. When horses were repeatedly immunized over long periods of time, there were some indications that an antibody of a lower mobility appeared (Tiselius and Kabat, 1939). Sedimentation studies came to similar conclusions: that antipneumococcal antibodies in the horse were of the macroglobulin 19S class, but that after prolonged periods of immunization antibodies with sedimentation constants of 7S, 10S, and 12S appeared (Kabat, 1939). No thorough studies were done on the characteristics of each of these antibodies or of the factors affecting their appearance, and many of the results obtained seemed mutually inconsistent. For instance, Kekwick and Record (1941) found that "the immediate reaction in the horse [to diphtheria toxoid] is the production of the γ-globulin antitoxin. As further injections are given increasing quantities of β-globulin antitoxin are formed..." This sequence differs from that found by Tiselius and Kabat. In support of Tiselius and Kabat, van der Scheer found that early antipneumococcal antibody in the horse migrated between the γ and β peaks and was later replaced by pure γ-globulin, but he in turn could find no evidence for activity in a light 7S component in horses immunized for as long as ten years (van der Scheer, Lagsdin, and Wyckoff, 1941). These different results remained largely unresolved. While there was much evidence that differences in physical properties of antibodies existed, there was no consistent correlation of these properties with species or antigenic differences, stage or route of immunization, or particular manifestations of antibody activity.

At the time of Porter's work, Taliaferro, Talmage, Stelos, and their associates independently studied the antibody response in the rabbit to repeated injections of sheep erythrocytes. They found that the Forssman hemolytic activity of an antiserum declined as immunization proceeded. At the same time, however, the Forssman combining capacity was constant (Talmage, Freter, and Taliaferro, 1956 a). This suggested the possibility of a sequential production of two different antibodies having different hemolytic efficiencies. This assumption was proved correct by the subsequent centrifugal separation of two antibodies with closely related specificities: the heavier of the antibodies was 100 times more effective at hemolysis (Talmage et al., 1956 b). The antibodies could be separated by electrophoresis, with the hemolytic antibody

migrating in the γ_1 region while the combining antibody was found in the γ_2 region (Stelos and Talmage, 1957). Also, the two antibodies differed in rates of metabolic decay after passive transfer (Taliaferro and Talmage, 1956) and in the kinetics of the hemolysis reaction (Weinrach and Talmage, 1958). Whereas Porter had been unable to correlate the chromatographic behavior with other properties of the molecules, these authors convincingly demonstrated a transition from early, short-lived, highly hemolytic, 19S γ_1-globulin antibodies with high anionic binding capacity to late, long-lived, 7S γ_2-globulin antibodies with much less hemolytic activity and low anionic binding capacity. These results were extended to include a number of different antigens, and similar sequences were found with one exception: human type A red blood cells stimulated only the 19S γ_1-antibodies (Stelos, 1958; Stelos and Taliaferro, 1959; Stelos, Taliaferro, and D'Alesandro, 1961).

In the above experiments and the concurrent work of Porter, antibodies were produced in response to multiple immunizations. In fact, Porter believed that the change in antibody distribution was a direct response to the repeated antigenic stimuli and not caused simply by the passage of time. Bauer and Stavitsky (1961), however, soon found identical sequences of antibodies after single injections of a variety of particulate and soluble antigens. These authors also differentiated between the two types of antibody by incubating them with 2-mercaptoethanol: sulfhydryl compounds dissociated macroglobulins into inactive subunits, but had no effect on 7S globulin activity (Deutsch and Morton, 1957; Glenchur, Zinneman, and Briggs, 1958).

The chromatographic, electrophoretic and ultracentrifugal behavior of these types of antibody, as well as the preferential inactivation of the macroglobulins by sulfhydryl compounds, have now been used to study the antibody response in many species and with a variety of different antigens. The two classes of antibody globulins defined by these techniques have been called γM and γG. The general nature of an antibody response has been the same in almost all cases: after an induction period of varying length during which no antibody can be demonstrated, the initial activity detected is associated with γM globulins. The amount of γM increases exponentially to reach a peak in a few days. In most cases, however, production of γM ceases shortly after its highest titers are reached, and the amount of serum γM declines at a characteristic decay rate. At approximately the time γM peak titers are reached, or shortly thereafter, γG appears in the serum and increases exponentially at a somewhat slower rate that γM. Within a month after the initial injection, γG

has usually totally replaced γM in the serum. The amount of serum γG may then remain fairly constant or gradually decline over a period of months.

Much of the work detailing this sequence of events has been summarized in Table I. The range in experimental animals, the variety of antigens, the different routes of antigen administration, and the various kinds of titrations used all give evidence of the importance of this phenomenon.

There are, of course, exceptions to this sequence. In a few cases, γG antibody has arisen without any preceding γM response. Thus, although no kinetic studies were done, Fahey and Humphrey (1962) claimed that the earliest antibodies in mice to spider crab hemocyanin and pneumococcal polysaccharide SS III were 6.5S γ_2-globulins. Also, in studies in guinea pigs with two different viruses, both live and inactivated, complement fixing antibodies were entirely of the γG type whereas virus neutralizing activity followed the expected γM-γG sequence (Graves, Cowan, and Trautman, 1964; Bellanti et al., 1965). The possibility that certain antigens may favor a γG response is raised by the work of Ada et al. (1963). These authors found that soluble flagellin was 100,000 times less effective than particulate flagella in causing a γM primary response. Their results are not clear, however, as to whether this difference is caused by the difference in physical state of the antigens or by the greater comtamination of the particulate flagella with traces of somatic O antigen.

A more common exception to the γM-γG sequence is the exclusive production of γM with no appearance of γG activity. This is often the case with certain kinds of antigens. Thus, agglutinin and hemolysin titers in rabbit anti-mouse erythrocyte sera remained in γ_1-globulin fractions even after eight injections (Lee, Takahashi, and Davidsohn, 1960). Bauer and Stavitsky (1961) found that γG antibodies to Salmonella O antigen in the rabbit were not synthesized even following three injections of intact organisms. Also, no γG activity could be found to typhoid O antigen in sera from 150 different immunized humans (LoSpalluto et al., 1962). The amount of antigen may also be a critical factor. With some antigens known to produce a normal γM-γG sequence, reduction of the antigen dosage below a certain level results in a transitory γM antibody response with no subsequent γG production (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964 a). Perhaps either the type of antigen or the dosage involved causes certain antibodies and related substances in man (Rh saline complete agglutinins, heterophile agglutinins, and cold agglutinins among others) to be found consistently in the γM fraction. In a like manner, the background

Table I. Evidence for the $\gamma M - \gamma G$ Transition

Author	Year	Animal	Antigen	Characterization	Titration Method	Injection Route
Bauer & Stavitsky	1961	rabbit	DPT, BGG, KLH, S-RBC, HSA, Salm.	1,2,3,4	HA, TN	footpad
Bauer, Mathies, & Stavitsky	1963	rabbit	T2 phage	1,2,3,4	VN	IV
Bauer	1963	rabbit	SA-BGG, PABA-BGG (adj.)	1,2,4	HA	IP, IV, SC, IM
LoSpalluto et al.	1962	human - adult	TAB	3,4	AGG	ID, IM
Fink et al.	1962	human - infant	TAB	3,4	AGG	IM
Uhr et al.	1962a	human - infant	ØX-174	1,3,5	VN	IM
Silverstein et al.	1963	fetal lamb	ØX-174	3	VN, HA	IM
Uhr, Finkelstein, & Franklin	1962b	chicken, frog, goldfish	ØX-174	1,2,3	VN	IV, SC, IP
Uhr & Finkelstein	1963	guinea pig, rabbit	ØX-174	1,3	VN	IV, IP
Benedict, Brown, & Ayengar	1962	rabbit	BSA	1,2,4	HA, PPT	IV
Benedict, Brown, & Hersch	1963a	chicken	BSA	1,3,4,6	HA	IV
Benedict, Larson, & Nik-khah	1963	chicken	BSA	2	HA	IV
Dreesman	1963	turkey	BSA	1,2	HA	IV
Smith	1960	human - infant	TAB	2,3,6	AGG	ID
Bellanti et al.	1963	newborn rabbit	Salm.	1,2,3,4,6	AGG	ID, IP
Grey	1963	turtle	KLH, BSA (sol., alum ppt., adj.)	1,3,4,5,6	Farr PPT	IM
Svehag & Mandel	1964a,b	rabbit	poliovirus (live or UV killed)	1,3	VN	IV
Svehag	1964a,b	rabbit	poliovirus (live or UV killed)	2	VN	IV
Nossal et al.	1964	rat	isolated Salm. flagella	1,3	BI	footpad
Kim, Bradley, & Watson	1964a	mouse	MSP8 actinophage	1,2,3,4,5	VN	IP
Bellanti et al.	1965	guinea pig	JE virus (live)	1,2,4,6,7	VN, HA, CF	IP, ID, SC, IC
Brown	1960	cattle, guinea pig	FMD virus (live)	4,5	VN, PPT	ID
Berlin	1963	mouse	influenza virus	1,3	HA	IP

DPT	diphtheria toxoid
BGG	bovine gamma globulin
KLH	keyhole limpet hemocyanin
S-RBC	sheep red blood cells
HSA	human serum albumin
Salm.	intact Salmonellae
SA-BGG	sulfanilazo haptene
PABA-BGG	p-aminobenzoic acid haptene
BSA	bovine serum albumin
TAB	typhoid-paratyphoid vaccine
JE	Japanese encephalitis virus
FMD	foot and mouth disease virus

1	density gradient ultracentrifugation
2	zone electrophoresis
3	reduction by 2-mercaptoethanol
4	DEAE cellulose chromatography
5	immunoelectrophoresis/abs. with antisera
6	analytical ultracentrifugation
7	gel filtration (Sephadex G-200)

HA	tanned cell hemagglutination
CF	complement fixation
VN	viral neutralization
BI	bacterial immobilization
PPT	precipitation
HAI	hemagglutination inhibition
TN	toxin neutralization
AGG	agglutination

IV	intravenous
IP	intraperitoneal
IM	intramuscular
SC	subcutaneous
ID	intradermal
IC	intracerebral

antibody activity found in sera from "normal", unstimulated animals (the so-called "natural antibody") has been found to be γM in a number of cases (Rowley and Turner, 1964; Svehag, 1964 b; Michael and Rosen, 1963).

The genetic constitution of the host may also play a role in the relative production of either γM or γG. For instance, persons of blood group 0 produce relatively more isohemagglutinins of the 7S type than persons of blood groups A or B (Fudenberg, Kunkel, and Franklin, 1959). When rheumatoid factor was present in a patient, however, the 7S type isohemagglutinins were reduced below normal (Rawson, Abelson, and McCarty, 1961). Kim et al. (1964 b) have found that host-associated factors may also alter the type of response: in the primary response to MSP8 actinophage, piglets which were both germ-free and colostrum-deprived produced only γM antibody, whereas conventional colostrum-deprived animals produced both γM and γG. In addition, species differences may be important. In most species, "hyperimmunized" animals manufacture antibody which is totally γG. In one case, however, Franěk has found that antibodies in the pig hyperimmunized with Brucella suis remained mostly in the γM fraction (Franěk, Jouja, and Kostka, 1962). Some differences are correlated with the position of a species on an evolutionary scale. In non-mammalian vertebrates the slowly sedimenting antibody activity characteristically differs from the γG in mammals by being susceptible to inactivation by sulfhydryl reagents. This has been shown to be the case in the frog and goldfish (Uhr et al., 1962 b), in the turtle (Grey, 1963 a), and possibly in the mallard duck (Grey, 1963 b). γG antibody in the chicken may also be more susceptible to sulfhydryl inactivation than mammalian γG (Benedict, Brown, and Hersh, 1963 b). The γM response in these animals seems to be of greater importance than in mammals, or at least of greater duration, and this was particularly true in the turtle. Studies in cold blooded vertebrates indicate that in more primitive animals the γG response does not occur at all. The antibody produced in response to T_2 phage in the elasmobranch guitarfish was sensitive to 2-mercaptoethanol and had a 19S sedimentation coefficient (Good and Papermaster, 1964). Both the primary and secondary responses to Brucella in the California lamprey, paddlefish, and gar consisted of 14-15S antibodies without the subsequent appearance of lighter-weight antibody (Ashbach et al., 1964). Although evidence in this area is very sparce, such findings have led to the suggestion that the γM response is a primitive protective device found early on the evolutionary scale, while the γG response is a later development in the phylogeny of adaptive immunity.

To find a possible selective advantage of the γG over the γM response, one need only look at the most notable exception to the rule of γM-γG transition: the secondary response. The high levels of antibody activity common to the secondary or anamnestic response are almost entirely of the γG type, and there is no comparable preceding γM response. Also, only a primary γG response can stimulate the lasting immunological memory essential for a typical secondary response. Recent work has shown that secondary injections given within a short period after the primary response can stimulate a secondary response of the γM type. Although this response is smaller in absolute titers than the secondary γG, Svehag and Mandel (1964 b) feel that the relative increase in each case is the same. The γM immunological memory is short-lived, however, and it was non-existent within two weeks after primary immunization in all responses examined thus far. There has been a recent report (Nossal et al., 1964) that there may be long term memory in γM production in at least one case, but the details of this work have not yet been published. Long-lasting immunological memory and the rapid production of large amounts of circulating antibody upon second contact is of selective advantage to survival, and the above findings support the theory that the γG response is a relatively recent evolutionary development.

We have seen that the chemical nature of the antigen, the dosage, the genetic nature and species of the host, and the stage in immunization all may alter the transition of γM to γG antibody. There has been a recent suggestion that the route of immunization may affect this sequence (Shulman, Hubler, and Witebsky, 1964). After an intravenous injection of human serum albumin into rabbits, the initial γM response was replaced by γG within 20 days; when antigen was injected intradermally with Freund's adjuvant, however, the γM response was bimodal and was still detectable five months after immunization. On the other hand, Svehag and Mandel (1964 b) could detect no difference in response when rabbits were injected intravenously, intraperitoneally, or in the hind foot pad with poliovirus. The fact that complete Freund's adjuvant was used in the former experiment and live antigen was used in the latter may make both of these experiments somewhat special cases.

A number of findings emphasize the important role of antigen in the γM-γG response: 1) the minimal dose requirement for induction of γM antibody is much less than that for γG, perhaps as much as 50 times less, 2) with low doses of antigen, γM synthesis ceases abruptly at about 4-5 days after immunization, while with higher antigen doses γM synthesis may continue for about two weeks (Svehag and Mandal, 1964 a), 3) the cessation of γM synthesis on days 4-5 does not appear to be due to loss of viability of cells and may be caused by insufficient antigen

(Uhr and Finkelstein, 1963; Uhr, 1964; Finkelstein and Uhr, 1964). With these findings in mind, we might consider the exceptions to the γM-γG transition we have just discussed and reinquire into the role of antigen. One question of interest is why certain antigens evoke activity in the γM fraction for long periods of time which is not followed by γG. As yet, this behavior has not been correlated with any physical state or chemical feature of the antigens. What may be common to these antigens is the manner in which they are removed by the reticuloendothelial system and digested within phagocytic cells. The prolonged γM response to red blood cells and bacterial somatic antigens may be due to the slow degradation of these antigens by phagocytic cells and release to antibody forming cells. "Natural antibody" complexing with these antigens might cause increased sequestration in the liver, an organ which does not form antibody (Benacerraf et al., 1959). In either case, if the antigen for some reason contacted potential antibody forming cells in low doses over a long period of time, one would expect a prolonged γM response but no γG because of its higher threshold. If such was the case, perhaps larger antigen loads might present enough antigen to the producing cells to stimulate a γG response. Recent studies supporting this idea have shown that with repetitive stimuli and/or massive antigen doses good γG responses can be obtained to both red blood cells (Davidsohn et al., 1962) and to bacterial somatic antigens (Pike and Schulze, 1964; Weidanz, Jackson, and Landy, 1964). As another example, the bimodal γM response reported by Shulman et al. (1964) may have been caused by the slow continuous release of antigen from the adjuvant depot. This might stimulate a prolonged γM response without causing any significant secondary γG. Perhaps the reason that antibodies such as heterophile agglutinins and cold agglutinins are associated with the γM fraction is the gradual release of the antigens involved in amounts insufficient to stimulate γG. The lower threshold of the γM response might also be the cause of the association of "natural antibodies" with γM and not γG: perhaps these antibodies are formed in response to minute quantities of antigen derived from foods or minor infections. There have been suggestions that the particulate nature of an antigen favors the γM response. Though this remains to be proven, the only common characteristic of particulate antigens would seem to be the way they are localised, phagocytized, or otherwise handled by the reticuloendothelial system.

Before finishing this section, a few words of caution might be said about evaluating studies dealing with the γM-γG transition. Firstly, one should be attentive to the methods for determining antibody titers. We have already noted

that γM molecules have a much greater hemolytic efficiency than γG (Weinrach and Talmage, 1958). Other studies have shown that the tanned cell hemagglutination method detects γM antibodies out of proportion to its ability to detect γG (as compared to spontaneous precipitation of labelled antigen or ammonium sulfate precipitation of bound antigen). This conclusion has been reached after thorough work in both the chicken (Benedict, Hersh, and Larson, 1963; Dreesman et al., 1965) and in the rabbit (Freter, 1957; Greenbury, Moore, and Nunn, 1963; Shulman et al., 1964; Grey, 1964). Such findings question the desirability of using hemagglutination or hemolysis titrations to evaluate γM and γG responses. To mention still another possible functional difference of importance in determining titers of γM and γG, there has been a recent report that γM in contrast to γG diphtheria antitoxin fails to neutralize diphtheria toxin in vivo (Robbins, 1965).

Secondly, quantitative studies of both types of antibodies should be carried out over long periods of time to get a true picture of the response. This is especially true in studies of the selective repression of either γM or γG. Comparisons of titers at the same time interval after the injection of antigen seldom give a true picture of the magnitude or course of a response. Various experimental manipulations may lengthen the induction period of a particular antibody type and displace the titer curve. It would be interesting to know, for instance, whether the different responses to flagella and flagellin found by Ada et al. (1963) were as specific as they seemed or whether the induction time for the flagellin response was simply lengthened until that antigen was removed from the circulation. One cannot conclude that an antibody response has been destroyed simply because no antibody of that type is found on the day of its normal peak titer. There is a meaningful difference between permanent inhibition and delay, and experimental conclusions should be critically evaluated in this light.

In this discussion, we have concentrated on the transition during the primary antibody response of γM to γG type antibodies. There are many areas less germane to this thesis which are associated with studies on γM and γG. Such topics include the chemical and physical properties of both immunoglobulins, studies on their digestion or dissociation and their sub-chain structure, the respective functions of γM and γG and how they differ, antibody activity in the γA class of immunoglobulins, and proteins in disease states related to either type. A review of any one of these could cover as many pages as are already written here. A number of excellent reviews covering these topics have appeared recently, and the reader is referred to them (Kunkel, 1960; Franklin, 1964; Cohen and Porter, 1964; Tomasi, 1965; Franklin and Lowenstein, 1964).

B.) The Role of the Spleen in Antibody Formation

It is generally accepted that following an intravenous injection of antigen, especially a particulate antigen, the spleen is a major source of antibodies. This has been tacitly assumed in much of the work to be presented in this thesis: antibody found in spleen homogenates is assumed to have been produced in the spleen, and histological events in the spleen are taken to reflect antibody formation. A consideration of the historical evidence implicating the spleen in the production of antibodies is therefore important to the results presented later. This evidence is derived from various areas of research, and the discussion of the following section has been divided according to these areas.

1.) Antibody activity in splenic extracts: The work of Paul Ehrlich toward the end of the nineteenth century stimulated great interest in antibodies and the organs producing them. At the same time that the basic phenomena of immunology were being detailed in blood serum, the other body "humors" were being investigated. For instance, the soluble portions of organs were examined by grinding the organs with sand and examining the centrifugal supernatants. Pfeiffer and Marx (1898), Wasserman (1898), and Deutsch (1899) had all been able to find antibody in soluble extracts of spleens. These experiments suggested that the spleen might be involved in antibody formation. There was the possibility, however, that antibody formed elsewhere might accumulate in an organ by leakage from blood vessels. Knowledge of the role of the spleen in antibody formation awaited more direct evidence.

2.) The effect of splenectomy: Because the removal of the spleen was not lethal to the animal involved, this device was used to study the importance of the spleen more directly. In 1892, Tizzoni and Cattani showed that splenectomized rabbits failed to develop immunity to tetanus. With the advent of *in vitro* functional tests to measure amount of antibody, other investigators restudied this effect of splenectomy on antibody production. Rath (1899) found a decrease in agglutinin production after splenectomy, Luckhardt and Becht (1911) observed a similar reduction of hemolysin production in dogs, and Hektoen (1920) extended these observations to the rat and rabbit. Hektoen also found that injections of human blood intraperitoneally into splenectomized rabbits yielded good titers of agglutinins but no precipitins. This suggested that the spleen might play a special role in the formation of certain types of antibody. Despite such findings many persons were unable to show consistently that removal of the spleen decreased subsequent antibody formation. Motohashi (1922) found that one source of confusion was the amount of antigen injected. With large doses of antigen given

intravenously, the effect of the spleen removal was masked and titers were as high as normal. With smaller doses there was no doubt that removal of the spleen decreased subsequent antibody titers. Much of this early work on the function of the spleen in antibody formation has been reviewed by Krumbhaar (1926), Topley (1930), and Jaffé (1931).

The effect of splenectomy has been more recently studied by Rowley (1950 a,b). He confirmed Motohashi's finding that the decrease in serum antibody titers was only dramatic when small doses of antigen were used. In fact, when antigen doses were small enough perhaps all antibody production occurred in the spleen - i.e., splenectomy after the primary response abolished the capacity for any subsequent secondary response. Also, the route of immunization was very important. If the injection was intraperitoneal or intraportal the antibody response was the same in splenectomized rats as in intact rats. Despite reports in the early literature that hypertrophy of other reticuloendothelial tissue would rapidly compensate for the removal of the spleen, Rowley found that the antibody response was the same whether antigen was given one hour or six months post-operatively. Wolfe et al. (1950) extended this work to study precipitin formation in chickens, and they found results similar to studies in mammals.

In a series of lengthy papers, Taliaferro and Taliaferro (1950; 1951; 1952) investigated the kinetics of the hemolysin response in intact and splenctomized rabbits. Comparing the antibody curves in both animals they concluded that the spleen forms most of the antibody during the initial rise in titer and then abruptly stops forming antibody at or about peak titer. Splenectomy at the time of peak titer did not affect the rate of decrease in serum titer afterwards. Splenic antibody apparently disappeared due to normal metabolic degradation until the continuing level of antibody from non-splenic sources was reached. After that time, splenectomized and intact rabbits had identical titers and identical rates of antibody decay. With multiple antigen injections, not only was the spleen relatively less important, but also hemolysin titers rose much higher in splenectomized than in intact animals. This might have been caused by hypertrophy of other reticuloendothelial tissue in the absence of the spleen, or by greater contact of other tissues with antigen in the absence of a spleen which could capture most of the antigen but produce only limited amounts of antibody. These authors also concluded that the spleen played the same role after a second injection of antigen (i.e., the production of the initial antibody titers) as it did after a first injection.

3.) Shielding the spleen from X-rays: Removing the spleen provides an indirect estimate of the amount of antibody it produces. A better experiment could be done by removing all antibody-forming tissues except the spleen and then following an antibody response. By utilizing severe X-irradiation to destroy reticuloendothelial tissue and by shielding the spleen from this destruction, the above experiment can be approximated. Jacobson and his associates were the first to show that lead shielding of the spleen during irradiation protected the immune response (Jacobson, Robson, and Marks, 1950). They found that 70-90% of the peak hemolysin titer was protected by shielding, although the time of peak titer was delayed. The capacity to respond was protected even if the spleen was removed as early as 24 hours after irradiation (Jacobson and Robson, 1952). These authors felt that their results proved the role of the spleen in forming antibody. In later, more extensive experiments, Taliaferro and Taliaferro (1956) and Süssdorf and Draper (1956) found that shielding the appendix provided even greater protection of the antibody response than shielding the spleen, this despite the fact that removal of the spleen showed that it, and not the appendix, played the major role in hemolysin production following intravenous immunization. Also with thymidine labeling of cells, they were able to show that cells originating in a protected appendix were responsible for the repopulation of an irradiated spleen. These authors therefore concluded that the important factor in these experiments was the mass of lymphatic tissue shielded rather than the antibody-forming capacity of the shielded organ. The shielding experiments only proved that the protected spleen possesses some factor able to induce the regeneration of antibody-forming capacity. These results were recently reviewed by Taliaferro, Taliaferro, and Jaroslaw (1964).

4.) Cellular changes associated with antibody formation: It is well known that following an injection of antigen there occurs, in those organs which are beginning to produce antibody, a characteristic change in cell types and histological relationships. The evidence describing this cellular response will be reviewed in the following section. In discussing the lines of evidence which have implicated the spleen in antibody production, it is important to note here that this cellular response does occur in the spleen and that its occurrence is related to the amount of antibody produced by the spleen - i.e., after an intraperitoneal or intradermal injection, when splenic antibody is known to be less, the cellular changes in the spleen are fewer.

5.) Localization of antigen and antibody in the spleen: Within the last decade there has been much concern about the organ and cellular localization of antigens

and antibodies. Studies in both of these areas have provided indirect evidence of antibody formation by the spleen. Implying the organ site of antibody production from the cellular localization of antibody assumes, of course, that neither the cells nor the antibody have been translocated from somewhere else. On the other hand, the relationship of antigen localization to antibody production is based on the more questionable assumption that where there's fire, there's eventually going to be smoke.

Whatever its relationship to the site of antibody production, the organ and cellular localization of antigen has been intensively studied. Radioactive labeling of antigen has provided one of the best means of tracing antigen deposition. With this method, both particulate antigens (Benacerraf, Sebestyen, and Schlossman, 1959) and soluble antigens (Hawkins and Haurowitz, 1961; Roberts and Haurowitz, 1962) can be found in the spleen after intravenous injection. When there is less antibody produced by the spleen, as after intradermal injection, there is less antigen found in the spleen. Radioactive labeling may denature the antigen slightly, and some investigators feel that even small modifications could change the organ and cellular localization. Such objections may be avoided by staining tissue sections with fluorescein-conjugated antibody produced against the antigen. This technique requires no modification of the antigen prior to its injection. Kaplan, Coons, and Deane (1950) used this method to follow the localization of injected pneumococcal polysaccharide in the spleen. They found most of the antigen in the red pulp of the spleen, although smaller amounts could be found in the white pulp.

The immunogenicity of an injected antigen is perhaps more pertinent to antibody formation than its localization. Franzl (1962; 1964) has used the anamnestic response as an experimental test of the ability of antigen recovered from different organs still to induce an antibody response. He found that spleen homogenates injected intraperitoneally into primed mice provoked antibody synthesis in these recipients even though donor spleens were taken many days after antigen injection. In the liver, however, the immunogenicity of the antigen was destroyed within 12 hours. Haurowitz and his associates have conducted many studies on the lifetime of antigenic label in the liver. These results of Franzl along with other experiments showing that the liver rarely produces any antibody (Thorbecke and Keuning, 1953; Askonas and White, 1956; Askonas and Humphrey, 1958) would seem to question further the relationship of antigen deposition to antibody formation. Much of this work on the localization and fate of antigen has been reviewed by Campbell and Garvey (1963).

Antibody localization is perhaps better related to antibody formation, and fluorescent techniques have been used to great advantage in this area. Both specific antibody (Coons, Leduc, and Connolly, 1955) and "normal gamma globulin" (Ortega and Mellors, 1957) have been localized in the spleen in cells around the periphery of follicles, in cells of the red pulp, and in cellular cuffs around small arteries. Staining in the follicle centers was generally diffuse and cell outlines were indistinct there. In more recent work, different kinds of antibodies and also subunits of antibody globulins have been localized in the spleen using fluorescent techniques. Chiappino and Pernis (1964) have demonstrated the presence of both γM and γG globulins in the spleen. Also, Bernier and Cebra (1964) found both H- and L- chains of 7S γ-globulin in individual lymphoid cells of spleens.

6.) Homologous transfer and in vitro culture of spleen cells: Perhaps the most convincing proof that the spleen is involved in antibody formation comes from the ability of spleen cells to produce antibody in homologous recipients or in in vitro cultures. As early as 1899, Deutsch transferred spleen cells from animals infected with typhoid organisms and found agglutinins in the sera of recipients. Since he presumably transferred antigen along with the cells, the experiment does not prove that the donor cells were capable of antibody production. More recently, Chase (1953) transferred spleen cells from guinea pigs having anaphylactic antibodies and high skin sensitivity to simple chemical compounds. After transfer of cells to normal recipients, he found a rise in both types of antibody in the recipient sera. In addition, the amount of antibody produced in heavily irradiated, isologous recipients has been quantitated and found to be a direct function of the number of spleen cells transferred (Makinodan, Kastenbaum, and Peterson, 1962). The relative capacity of various tissues to form antibody was studied by Stoner and Bond (1963) who transferred bone marrow, spleen, lymph node, and thymus cells into irradiated recipients. The donor animals received subcutaneous primary and secondary immunizations with tetanus toxoid. After transfer of equal numbers of each cell type to recipients, tertiary responses were elicited in the recipients by intravenous injection. The spleen cells produced neutralizing titers which were 36 times higher than the titers produced by any of the other three tissues. Although the results convincingly demonstrate the capacity of the spleen to produce antibodies, they are somewhat surprising in view of the fact that the immunization of donors was subcutaneous, and this route is known not to involve the spleen maximally.

Experiments involving similar transfer of cells have been summarized in a review by Cochrane and Dixon (1962).

If animals are immunized and antibody producing tissue is removed after an appropriate interval, this tissue will continue to produce antibodies *in vitro*. Such an experiment avoids the possiblity that recipient tissue might also be involved in the formation of antibodies and allows the conditions for the antibody response to be more carefully defined and controlled. Many such experiments have employed splenic tissue. Fagraeus (1948a) showed that red pulp removed during a secondary response to *S. typhi*. produced more antibody *in vitro* than the corresponding white pulp. Many of the factors affecting such an *in vitro* response have been detailed by LaVia, Uriu, and Ferguson (1960) and Vaughan, et al. (1960) using splenic tissue, but they are too numerous to be reviewed here. In studying the primary response, Kong and Johnson (1963) found that in order to get antibody production they had to wait at least three days after antigen injection of donors before transferring spleen fragments to *in vitro* cultures. For transfer to homologous recipients, tissue could be removed one day after antigen. Apparently, certain conditions of the induction period are not satisfied in the cultures but are satisfied in irradiated recipients. One of the most interesting questions to arise from this work is the nature of these conditions. Stavitsky, who did much of the early work proving the possibility of the antibody response *in vitro*, has reviewed his own experiments and those of many others in this field (Stavitsky, 1961).

Considering the lines of evidence reviewed here, there can be no doubt that the spleen produces antibody, and that under certain circumstances, particularly after intravenous injection of antigen, the spleen is the major and sometimes the sole source of antibody. The cellular changes accompanying antigen uptake and antibody production in the spleen will be reviewed in the next section.

C.) The Cellular Basis of Antibody Production

1.) The histological response in the spleen

An antigenic stimulus evokes a characteristic differentiation and proliferation of cells in the reticular tissues. These cellular changes have been associated with the production of antibody by fluorescent techniques and other methods. It is clear that the antibody is formed by a special population of cells which is almost completely non-existent before the antigen is administered. A complete review of the many studies of this response is beyond the scope of this discussion. Rather we shall briefly outline the essential changes in spleen architecture and cell type which occur after an injection of antigen. The following description is based primarily on the work of Thorbecke and Keuning (1956) and Langevoort et al. (1963).

On the first day after antigen injection, haemocytoblasts begin to appear in the periarteriolar lymphocyte sheaths of the white pulp. There have been various suggestions that these cells are derived from the germinal center reticulum cells (Fagraeus, 1948 b; Marshall and White, 1950), from the lymphocytes surrounding these arteries (Langevoort et al., 1963), or from small (circulating?) lymphocytes (Gowans, Gesner, and McGregor, 1961; Porter and Cooper, 1962). The haemocytoblasts are large rounded cells with large, central pale nuclei. They have prominent nucleoli and a narrow zone of pyroninophilic cytoplasm. During the second and third post-antigen days, these cells increase in number and apparently migrate to the periphery of the white pulp. The haemocytoblasts are collected there around the penicilli arterioles so that they are characteristically seen in groups of "aggregates." Sometime these aggregates are within the red pulp, but usually they are located on the border of the red and white pulp.

By the fourth and fifth days, germinal centers (secondary nodules) begin to appear in the white pulp. These often contain mitotic figures, blast-like cells, and medium lymphocytes in increased numbers. At the same time, the haemocytoblasts begin to differentiate into immature plasma cells. They become smaller, more basophilic, with darker muclei caused by some condensation of the chromatin. The nuclei begin to be more eccentrically located. The nucleoli are still clearly visible. Within another two days, these cells become typical mature plasma cells: they have eccentric nuclei with cartwheel patterns of densely clumped chromatin, no visible nucleoli, and strongly pyroninophilic cytoplasm with a characteristic juxtanuclear clear zone. Mature plasma cells are the smallest of the three types. With most antigens, the plasma cells are seen in

large numbers bordering the white pulp lymphoid tissue for only a week or so; by the end of the second week after antigen administration, the plasma cells disappear rather rapidly and can be found only occasionally in animals given no further stimuli. Throughout the time that the plasma cells become predominant at the borders of the red pulp, the germinal centers show increasing signs of cell disintegration and phagocytosis (tingible-body macrophages), and they exhibit a typical "starry sky" appearance.

The white and red pulp of the spleen can be separated with the aid of a dissecting microscope and cultured apart in vitro. Fagraeus (1948 a), Thorbecke and Keuning (1953), and Langevoort et al. (1963) have studied the relative antibody production of these two parts of the spleen in this manner and have concluded that most of the antibody is formed in the red pulp rather than in the germinal centers of the white pulp. Presumably, the plasma cell aggregates along the border between the two are removed with the red pulp in this technique. These conclusions have been substantially supported by studies of antibody localization with fluorescence. What then is the role of the secondary nodules? It seems curious that the secondary nodules reach their peak of development after the production of antibodies has subsided, and that these sites of greatest morphological change and cellular proliferation are topographically removed from those parts of the spleen which produce antibody. There have been recent suggestions that these nodules do not play a role in the primary response, but that they form those cells which rapidly respond to a booster injection - that they are the sites of the immunological memory. The exact function of these nodules, however, is still somewhat controversial (Thorbecke et al., 1962; White, 1963).

As a final note to the discussion of histology: in recent years, many experiments have stressed the possible importance of intimate cell associations in the immune response, particularly the association of macrophages with cells of the lymphocytic and plasma cell families. The lack of phagocytic activity in cells producing antibody has always been problematical to theories of antibody formation. Is antigen necessary in these cells, and if so, how does it get in? What happens to the antigen taken up by phagocytic cells? Recent studies of the primary response in vitro (Fishman, 1961; Fishman, Hammerstrom, and Bond, 1963) have suggested that the macrophage may be involved in the transfer of some activating principle to immunologically competent cells. These experiments have stimulated other investigators to look for histological evidence of cell

interactions which may play a role in the antibody response (White, 1963; Foy and Kondi, 1964; Schoenberg et al., 1964 a; Miller and Nossal, 1964). A good evaluation of the role of such cell interactions, however, will have to wait for more convincing in vitro experiments.

2.) Cell types producing γM and γG antibodies

Immunology as a subject of inquiry has often been plagued with redundant and imprecise terminology. Within this subject, a major area of debate has been the cellular basis of antibody production. Since the work of Fagraeus (1948 b), antibody production has been associated with the plasma cell and its immature form, the transitional cell of Fagraeus (haemocytoblast). Experiments detailing the role of these cells almost always involved hyperimmunized animals so as to insure high levels of γG antibodies. After the discovery of γM production in such disease as Waldenström's macroglobulinemia and the importance of γM in the primary antibody response, questions arose about the cellular source of this antibody - are γM and γG produced by different cells? If so, does a change in cell morphology accompany the transition from γM to γG? Despite much interest and experimental work, this one cell - two cell controversy continues. It would perhaps be easiest to review the evidence for each side of this debate in a tabular form, much as we presented the experiments showing γM-γG transition, but this is not possible because the confusion in terminology is such that the work of a single author has at times been claimed by both sides, and a definitive statement of opinion is rare indeed. We shall be forced to make as good an assessment as possible by reviewing the individual papers.

Most studies on the cellular basis of γM production have taken advantage of situations in which γM is the dominant antibody type. Macroglobulinemia and rheumatoid arthritis have both been well studied. The technique most commonly used has been fluorescent staining of cells for the presence of antibody. Yet despite the use of similar materials and methods, the results and conclusions have seemed contradictory. Thus, Curtain and O'Dea (1959) and Curtain (1961) studied cellular localization of antibody in macroglobulinemia and found specific fluorescence in plasmablasts but not mature plasma cells; and Mellors et al. (1959), Mellors, Nowoslawski, and Korngold (1961), and McCormick (1963) all studied the cellular origin of rheumatoid factor and found that both γM and γG globulins were located in both immature and mature types of plasma cells. Mature lymphocytes were not stained. All these authors felt that the same cell therefore produced both γM and γG. On the other hand, Braunsteiner, Fellinger, and Pakesch (1957) found organized ergastoplasm in lymphoid cells of macroglobulinemia suggesting protein synthesis of a large order, Waldenström (1958) found that "lymphocyte reticulum cells" were preponderant in spleen, nodes, and bone marrow in macroglobulinemia, and Dutcher and Fahey (1959; 1960) described specific fluorescent staining of "lymphoid

plasma cells" in similar patients. In a study which included both fluorescent localization and production of macroglobulins in vitro, Zucker-Franklin, Franklin, and Cooper (1962) found that patients with macroglobulinemia had no increase in typical plasma cells. Cells staining for γM antibody were either large or medium lymphocytes of "lymphoid reticulum cells." Thus, one might conclude that there are two different cells involved.

Such studies have not been confined to disease situations; the primary antibody response has also been a source of conflict. For instance, Asofsky and Thorbecke (1962) studied the production of γM and γG in vitro and found that the "ability of tissues to form both 7S and 19S γ-globulin was closely correlated with the appearance of immature plasma cells." Langevoort et al. (1963) concluded that "immature and mature plasma cells" in the red pulp were responsible for γM production in the primary response. Yet Schoenberg, Rupp, and Moore (1964 b) report the production of γM antibodies by "a large mononuclear cell with cytoplasmic basophilia." They feel that this cell is unrelated in origin to the plasma cell which produces γG and that there is no morphological transition from one to the other.

What then is the source of confusion in this work and what can be concluded from the experiments reviewed here? For one thing, a major source of difficulty is the similarity of the cells central to the debate - the large lymphocyte and immature plasma cell are very similar in appearance. Oftentimes, no light microscopy accompanies the fluorescent observations and the determination of cell type is done without reference to nucleoli, chromatin density and arrangement, or cytoplasmic basophilia. When rather poor, low power pictures are presented, the reader is at the mercy of the investigator's skill at differentiating the two cell types. Secondly, some of these conclusions are based merely on the "preponderance" of one cell type in a disease state or the presence or absence of a cell type at times of γM production. Even studies employing immunofluorescence are by no means conclusive. The demonstrated immunologic cross-reactivity of γM and γG globulins (Thorbecke and Franklin, 1961) makes the application of immunofluorescent techniques at best problematical. Careful absorption controls and elimination of background staining have not always been done.

Another source of difficulity has been the multiplicity of names used to describe the same cell type and the surprising number of cell types often included under the same name. For instance, do "lymphocytoid plasma cells" and "plasmacytoid lymphocytes" belong to the same or different families? Or consider a study of γ and β_2A myelomas by Burtin and Buffe (1963) in which they stated that "fluorescent cells were always plasma cells"; yet, the size of the cells described was

variable, the nuclei showed a broad range of size and location, some cells had budding or irregular nuclei, and even bi- or multinucleated cells were seen. Also, Zucker-Franklin et al. (1962) show a "large lymphoblast" with a distinctly eccentric nucleus, a property which many investigators find ipso facto definitive for a plasma cell.

The controversy in this field has largely been directed to the question of whether two different cells produce the two globulins. Perhaps much of the confusion stems from what we mean by "two different cells." For some persons, production by two different cells implies different cell families, different cell morphology, and no possible transitions between the two. For others, differences in morphology alone constitute different cells. As an example, if a haemocytoblast produces γM antibody and then matures to a plasma cell which produces γG, is this the production of both types of antibody by one cell or two different cells? Though the distinction may seem insignificant, the failure to make it has caused much confusion throughout the literature. Thus, in a study of dysgammaglobulinemia, Cruchard et al. (1962) found γM in transitional cells of Fagraeus. Because of a failure to distinguish basic definitions, this one paper has recently been cited both in support of a one cell theory (Nossal et al., 1964) and also in support of "two major cell lines" (Robbins and Smith, 1964). To show that this is not an isolated example: Curtain and O'Dea (1959) are cited as support for the role of the plasma cell by both Nossal et al. (1964) and Franklin (1964), while the same paper is cited by Schoenberg et al. (1964 b) as supporting the production of γM by the lymphocyte. Perhaps the criterion for differentiating between one cell production and the production by two cells should not be a morphological difference but rather the ability of the γM produceing cell to change over or transform into a cell producing γG antibody. Histological studies are largely inconclusive on this point.

Therefore, it may be that different cell types produce γM and γG but that one cell line or family is involved. Perhaps the transition from a large blast-like cell to a mature plasma cell is accompanied by a transition from γM to γG production. Macroglobulinemias or myelomas might then represent a maturation arrest at one or another stage in this globulin-producing cell line. The globulin type may not always be exactly correlated with the cell morphology. Thus, while most macroglobulinemias exhibit a preponderant lymphocytic reticulum cell, there have been reports of macroglobulinemias where plasma cells were dominant in the bone marrow (Engle, 1959).

In this regard, three recent papers are most interesting (Solomon, Fahey, and Malmgre, 1963; Mellors and Korngold, 1963; Chiappino and Pernis, 1964). These papers contain observations on the cellular localization of γM and γG by the use of immunofluorescence. They include studies of large numbers of patients with multiple myeloma, macroglobulinemia, rheumatoid arthritis, and also patients with various unrelated disorders. All three studies find no correlation between cell type and type of antibody globulin. Both γM and γG were observed at different times in large, blast-like, immature forms, in germinal center reticular cells, in cells resembling intermediate stages between lymphocytes and plasma cells, and in immature and mature plasma cells. Thus, in large samplings in the absence of specific antigen stimulation there may be little correlation of either γM or γG with any one cell type.

If γM and γG can be associated with a variety of cell types, what of their occurence together in a single cell? Despite the failure of some authors to find cells that produce two antibodies (Burtin and Buffe, 1963; Solomon et al., 1963; Chiappino and Pernis, 1964), there have been other reports with double fluorescence of both γM and γG in the same plasma cell (Mellors et al., 1959; Mellors et al., 1961; Mellors and Korngold, 1963; McCormick, 1963). The low incidence of cells producing both types agrees with previous work on single cell producing antibodies of two specificities (Attardi et al., 1959; Makela and Nossal, 1961).

Perhaps the most direct and convincing evidence that one cell line can be involved in both γM and γG production comes from recent single cell studies of the primary response (Nossal et al., 1964). Of 123 cells containing antibody examined at various times during a primary response, 21% were blast cells, 31% were immature plasma cells, and 47% were mature plasma cells. No morphological differences were observed between γM and γG containing cells. At a time when γG was first beginning to replace γM in the serum, 7 cells were found which contained both types of antibody. Though there is no proof that the same cell produced first γM and then goes on to produce γG, such a theory would seem to have the most support from evidence presently available.

The work reviewed thus far has dealt exclusively with various kinds of morphological evidence. Before ending this section, we might mention some other authors who favor the involvement of two cell types on the basis of kinetic or functional studies. Reasoning from such facts as a) the concurrent synthesis of γM and γG in certain cases, b) the differences in kinetics of formation of the two antibodies, c) differences in antigen dosage threshold, d) the presence of

long-term memory of only one kind, and e) the different susceptibility of each to depression by X-ray, these investigators have concluded that two major cell lines are necessarily involved (Bauer, Mathies, and Stavitsky, 1963; Svehag and Mandel, 1964 b; Robbins and Smith, 1964). Such logic is on rather tenuous ground at present, since there seems to be no reason to exclude major physiological differences between two members of the same cell line.

II. MATERIALS AND METHODS

A.) ANIMALS: mice used in all experiments were of the Nelson-Collins-Swiss strain developed at the Rockefeller Institute. The mice are specific pathogen free and are randomly bred in a closed population. Mature male mice weighing 23-28 grams (5-7 weeks old) were used throughout. Animals were fed ad libitum a diet of pathogen free commercial mouse pellets (Dietrich and Gambrill, Frederick, Maryland) and tap water. None of these mice were found to have detectable antibody titers against any of the antigens used in these studies.

B.) ANTIGEN: the flagellated salmonellae used in these studies were obtained from Dr. P.R. Edwards of the Communicable Disease Center, Atlanta, Georgia. S. adelaide (35:f,g) was used for immunization and subsequent titration. Control titrations were done using S. umhlatazana (35:a) and S. derby (1,4,12:f,g).

Overnight cultures of S. adelaide were grown in penassay broth (Difco Laboratories, Detroit) on a reciprocal shaker at 37° C. Formalin was added to a concentration of 0.25% and the flasks were further shaken at 37°C for 15 minutes and allowed to stand at 4°C overnight. The bacteria were washed three times in saline, collected by centrifugation at 9,000 X G, and resuspended in sterile, physiological saline. Optical density was measured at 650 mμ in the small tubes (0.9 cm. I.D.) of the Coleman Junior Spectrophotometer. The O.D. was adjusted to 0.4 which corresponded to a viable count of 5×10^9 organisms per ml. and a dry weight of 0.95 mg./ml. This antigen was stored at 4°C without preservatives. The stock was often checked for contamination and loss of immunogenicity, and it was never kept longer than three months. Intact bacteria were used for immunization in all experiments.

C.) IMMUNIZATION: maximal primary antibody responses were elicited by injection of 0.2 ml. of stock antigen into the tail vein. When the secondary antibody response was studied, stock antigen was diluted 1 to 4 in saline, and mice were injected with 0.2 ml. of this dilution 35-40 days after the primary injection.

D.) COLLECTION OF SERA: blood was collected from mice under ether anesthesia by direct heart puncture and allowed to clot at room temperature. The clot was rimmed and allowed to retract overnight at 4°C, then centrifuged, and the serum was removed and stored at 4°C until assay.

E.) HOMOGENIZATION OF SPLEENS: spleens were removed under ether anesthesia, trimmed of connective tissue, and homogenized in 1 ml. of cold saline with a motor driven teflon pestle (Tri-R Instruments Jamaica, N.Y., 2 ml. capacity). Twenty passes of the pestle over a period of 2 minutes were performed while the

tube was kept in an ice bath. The resulting homogenate showed very few unbroken cells when examined under phase contrast. The homogenate was centrifuged at 4°C using 5 ml. lusteroid tubes (Lourdes, 9RA rotor). After centrifugation for 15 minutes at 11,000 rpm (15,000 X G), the supernatants were collected and were stored at 4°C for titration the same day. The pellets were discarded.

F.) ANTIBODY TITRATIONS: anti-flagellar antibody was detected by a modification of the technique originally described by Nossal (1958) utilizing the specific immobilization of motile bacteria. Bacteria were maintained at maximal motility by daily passage through a semi-solid nutrient medium (0.4% agar, 8% gelatin in penassay broth) in a petri dish (Lederberg, 1956). Organisms from the leading edge of the culture were taken up with a 4mm. loop and innoculated into 5 ml. of fresh penassay broth. The culture was incubated for 2-3 hours at 37°C before use in the titrations. Liquid cultures grown in this way routinely showed 95-100% motile organisms. If heavier growth was allowed, the percentage of motile bacteria decreased.

Sera and spleen homogenate supernatants were diluted in saline in 2 ml. cups of disposable plastic trays. Serial two-fold dilutions were done with an automatic pipet (Arthur H. Thomas Co., Philadelphia, No. 8212-E) of 0.2 ml. capacity. The final volume of all dilutions was 0.1 ml. An equal volume of a 2-3 hour culture in penassy broth was mixed with each dilution and allowed to stand for 20 minutes before counting. All operations were performed at room temperature.

Counting chambers were similar to those of de Fonbrune (1949). A thin layer of mineral oil (Nujol, Plough Inc., N.Y.) was spread over the surface of a clean glass microscope coverslip (No. 1, 22x50mm.). Kimax capillary tubing (0.7 - 1.0 mm.) was drawn into micropipets and a separate pipet was used to deposit a droplet of each dilution beneath the layer of oil. On a 3"xl" glass microscope slide, a rectangular area was enclosed with liquid wax on three sides. The coverslip was inverted on the hardened wax and the space beneath it was filled with mineral oil. This method gave chambers of varying depths depending on the thickness of the wax, however, this did not affect the results of the experiments. The resolution in phase contrast was better if the microdroplets contacted both the top and the bottom of the chamber.

Microdroplets were observed with high dry phase optics (Zeiss Ultraphot II; 400-640 magnification). Bacteria showing any movement other than Brownian motion were judged motile. Fifty to one hundred bacteria were counted. Care was taken

always to count throughout the height of the drop, as there were sometimes more immotile bacteria towards the bottom. The specificity of the immobilization of bacteria by antibody, a representative titration, and the establishment of end-points will all be discussed in the section on "Results."

G.) ZONE ELCTROPHORESIS OF SERA: a modification of previous techniques was used (Kunkel and Slater, 1952; Müller-Eberhard, 1960). One ml. of serum was placed in a trough 4" from the end of a block of polyvinyl chloride (Pevikon, Mercer Chemical Co., New York distributor) 18x7x0.6 inches. Electrophoresis was carried out in 0.1 μ barbital buffer, pH 8.6, for 18 hours at 250 volts, 50 ma., and a temperature of 4°C. One-half inch strips were cut from the block, and the protein was eluted with 3 ml. saline under negative pressure through a coarse glass filter. The protein content of the eluates was determined by the method of Lowry et al. (1951).

H.) ULTRACENTRIFUGATION OF SERA AND HOMOGENATES: density gradient centrifugation was carried out according to methods described by Edelman, Kunkel, and Franklin (1958) and Kunkel (1960). A Spinco model L centrifuge at a constant temperature of 4°C was used with the SW-39 swinging bucket rotor. A continuous gradient ranging from 10-40% sucrose from the top to the bottom of the tube was made by dropwise addition of 40% sucrose to 10% sucrose in a mixing chamber. The gradient was cooled to 4°C, and 0.2 ml. serum or homogenate supernatant was carefully layered over the sucrose. The lower portion of the protein solution was stirred slightly into the sucrose in order to minimize droplet formation, and the ultracentrifugation was begun immediately. All gradients were centrifuged for 18 hours at 35,000 rpm. Sera were not concentrated before application to gradients; spleen homogenate supernatants were concentrated two-fold by means of ultrafiltration through collodion bags under negative pressure (Carl Schleicher and Schuell Co., Keene, N.H.; porosity less than 5 millimicrons).

Fractions of the centrifuged density gradient were obtained by piercing the bottom of the plastic centrifuge tube with a standard needle and collecting a given number of drops in each tube of a fraction collector. The speed of efflux was controlled by negative pressure above the solution. The fractions were titered for antibody without prior dialysis.

The sedimentation coefficients of the antibodies were not precisely determined in the analytical ultracentrifuge. The activities sedimented, however, in the same gradient fractions as 19S and 7S antibodies in the rabbit and in man

(Kunkel, Rockey, and Tomasi, 1961). For convenience, therefore, antibodies sedimenting in the central portion of the gradient will be called γG (for 7S γ-globulin) and antibodies sedimenting to the bottom of the gradient will be called γM (for 19S γ-globulin or γ-macroglobulin). This is in accord with the changes in Nomenclature for Human Immunoglobulins (Bull. Wld. Hlth. Org., 30;447, 1964).

I.) MERCAPTOETHANOL TREATMENT OF SERA: 2-mercaptoethanol (2-ME) was added to serum to a final concentration of 0.1M (1/128 by volume), mixed, and allowed to stand at room temperature for two hours in a tightly sealed tube. Titrations were carried out in the presence of 2-ME without any decrease in control motility.

J.) SPLENECTOMIES: spleens were removed from normal mice under light ether anesthesia. A single silk ligature was placed around the pedicle to control bleeding. Care was taken to remove any accessory splenic tissue. Wounds were closed with 11 mm. Michel clips and collodion. The mice were allowed 11 days of post-operative rest before antigen injection. At the time they were bled for antibody determination, all mice were re-examined for regrowth of splenic tissue. Laparotomy was performed on all control animals.

K.) LOCALIZATION OF ANTIGEN: uniformly labelled D-glucose-C-14 was obtained as a lyophilized syrup (Volk Radiochemical, Skokie, Illinois; No. CC-24, 4.66 mc/mM). The following basic medium was used for growth of bacteria: 0.2% ammonium sulfate, 1.4% dipotassium phosphate, 0.6% monopotassium phosphate, 0.1% sodium citrate, 0.02% magnesium sulfate · $7H_2O$, 0.1% yeast extract dialysate, and 0.01% casamino acids (Cohn, 1963). The final medium contained 10μc of C^{14} - glucose per ml. A small inoculum of S. adelaide was added to 10 ml. of this medium in a tightly stoppered 50 ml. flask and incubated at 37°C on a reciprocal shaker for 12 hours. Unlabelled glucose was then added, and the culture was killed with formalin after one additional hour. The bacteria were washed three times with saline, collected by centrifugation at 9,000 X G, and resuspended to an O.D. of 0.40 as described above for unlabelled antigen. Normal mice were injected with 0.20 ml. (162,000 cpm) of this stock antigen to elicit a primary response. To study the localization of the antigen in the secondary response, 0.20 ml. of a 1/4 dilution of C^{14} stock antigen was injected 37 days after the primary injection of unlabelled antigen.

Heparinized cardiac blood was obtained at varying times after the injection of antigen. The spleen, lungs, and liver were digested in appropriate volumes of

1N NaOH. The digestion proceeded at 37°C for 15 hours.

Known volumes of each of the four fractions were placed on metal planchets and dried slowly under an infra-red lamp. A small drop of Roccal (Winthrop Laboratories, New York) was added to each planchet to give even spreading. Radioactivity was then assayed in a micromil end-window gas flow counter (Nuclear-Chicago) At least 2,000 counts above background were recorded for each sample. Self-absorption curves were prepared for each fraction, and samples were corrected and expressed to infinite thinness.

L.) INHIBITION OF THE PRIMARY IMMUNE RESPONSE: methotrexate sodium (4-amino-N^{10}-methyl pteroylglutamic acid sodium) was obtained from Lederle Laboratories, Pearl River, and was dissolved in distilled water; colchicine, USP, was obtained from Amend Drug and Chemical Co., New York, and was dissolved in physiological saline. Both drugs were administered intraperitoneally in volumes of 0.5 ml. Dosage and frequency is indicated in each experiment.

M.) TRANSFER OF PRIMED SPLEEN CELLS: the suspending medium in all parts of the transfer experiments was Medium No. 199 with sodium bicarbonate (Microbiological Associates, Bethesda) and 10% fresh normal mouse serum. Donor mice were anesthetized with ether and their spleens were removed sterilely and placed in a small petri dish. The spleens were minced using a sharp, single-edge razor blade. The resulting pulp was then wrapped in a square of hem from a woman's nylon stocking which had previously been boiled free of dye and autoclaved. This stocking bag was immersed in 1 ml. of transfer medium per spleen and very gently squeezed to free the cells from within. Very little manipulation was necessary to free most cells. Any attempt to free all of the cells in the bag required rougher treatment and resulted in a greater percentage of dead cells. The connective tissue was left in the stocking bag and was discarded.

The cells were centrifuged at 800 rpm for 6 minutes and resuspended to a concentration of 2×10^8 cells per ml. All cell counts were done in a standard hemocytometer using Turk's solution as a diluent. A differential count of these wet preparations showed an average of 13% large mononuclear cells, 85% small and medium lymphocytes, and 2% other types. One-half ml. (1×10^8 cells) was injected into the recipient's tail vein using a 26 gauge needle. The number of cells injected was approximately 30-50% of the cells which could easily be freed from one donor spleen. All media were kept at room temperature during an experiment, and the transfers were routinely completed in less than two hours.

The uptake of trypan blue was used to determine the percentage of cells living at the end of an experiment. Equal volumes of 1% trypan blue in saline, normal mouse serum, and cell suspension were mixed and incubated under a sealed coverslip for 15 minutes at 37° C. Only cells whose nuclei did not stain blue were considered alive. The viability of cells as determined by this method was routinely 85-90% at the end of an experiment.

N.) IRRADIATION OF RECIPIENT MICE: the facilities of the radio-therapy division of New York Hospital were used for all X-irradiation of recipient mice. Mice were separated from each other by dividers in a cardboard box, and six mice at a time were given 650r surface dose using a GE Maxitron according to the following specifications:

250 KV 30 ma	TSA: 50 cm.
filters: ½mm. Cu, 1mm.Al	HVL: 1.5 mm. Cu
100 r/min. (air)	135 r/min. (skin)
backscatter: 1.35	area: 300 cm^2

The mice were irradiated 24 hours prior to receiving cells. At this dosage, better than 90% of the mice survived more than 40 days whether or not they received cells.

O.) HISTOLOGICAL STUDIES: all sections were prepared from whole spleens fixed in formalin:distilled water (10:90). The spleens were paraffin embedded and stained with methyl green - pyronin according to the method of Kurnick (1955) with the exception that ethyl alcohol was substituted for butyl alcohol.

All photographs were taken with a Zeiss Ultraphot II microscope using 4" x 5" Anscochrome 3200 film plates.

III. RESULTS

A.) GENERAL CONSIDERATIONS: the method of antibody titration used in the following experiments was adapted from earlier studies on the production of antibodies by single cells (Nossal, 1958). Nossal had taken the immobilization of more than 90% of test bacteria as a positive indication of antibody production. In the present work, it was felt that a more precise and possibly more sensitive determination of endpoint was needed to achieve accurate kinetic studies. A technique was developed for growing cultures which were routinely 99+% motile and endpoints of titrations were determined by counting the percentage of test bacteria immobilized at each dilution. A typical titration curve is shown in Fig. 1. There were usually four to five doubling dilutions between complete immobilization and control values. The range of values at the same dilution was less than 15%. The spontaneous immobilization of controls never exceeded the indicated range in any of the following studies. The endpoint of a titration was chosen as the highest dilution containing more than 25% immotile test bacteria. On repeated titration of the same serum, the standard deviation of endpoints was less than one doubling dilution.

The specificity of the immobilization was tested using salmonellae of known serological types. Mice were immunized with S. adelaide of flagellar type: f,g (H antigen). The resulting antisera were titered with the original immunogen, with a strain having a common H antigen, and with a strain having a common O antigen (Table II). Only those test bacteria having an H antigen in common with the immunogen showed significant immobilization. Other controls in which mice were immunized with S. derby or S. umhlatazana gave similar results.

B.) THE ORGAN LOCALIZATION OF INJECTED ANTIGEN: the clearance and organ localization of injected particulate antigens have been studied by many investigators (Bull, 1915; Sullivan, Nekerman, and Cannon, 1934; Kerby, Holland, and Martin, 1950; Benacerraf, Sebestyen, and Schlossman, 1959). Because antigen localization is known to vary dramatically with the type of organism, the animal used, and the natural opsonins present, it was decided to restudy the distribution of injected antigen under the present circumstances. Carbon-14 label was used because it was more stable than the P-32 used in previous work (Benacerraf et al., 1959).

Fig. 2 presents the summation of data collected during the first week of the primary response. During the first six hours after immunization, counts in the blood decreased exponentially to less than 2% of the initial injection.

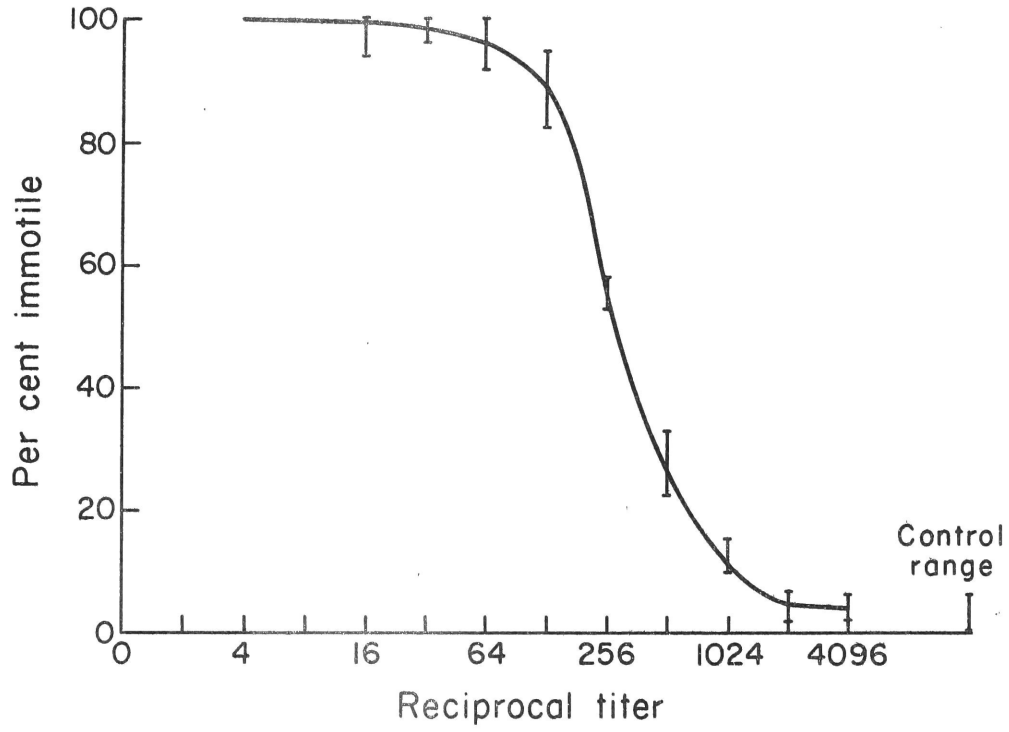


Fig. 1 The immobilization of motile test bacteria by a specific antiserum.

Test Bacteria	Reciprocal Titer	
	5 day	15 day
S. adelaide (35 : f,g)	256	512
S. derby (1,4,12 : f,g)	256	512
S. umhlatazana (35 : a)	0	4

Table II **The specificity of immobilization by an antiserum produced against S. adelaide (35 : f,g).**

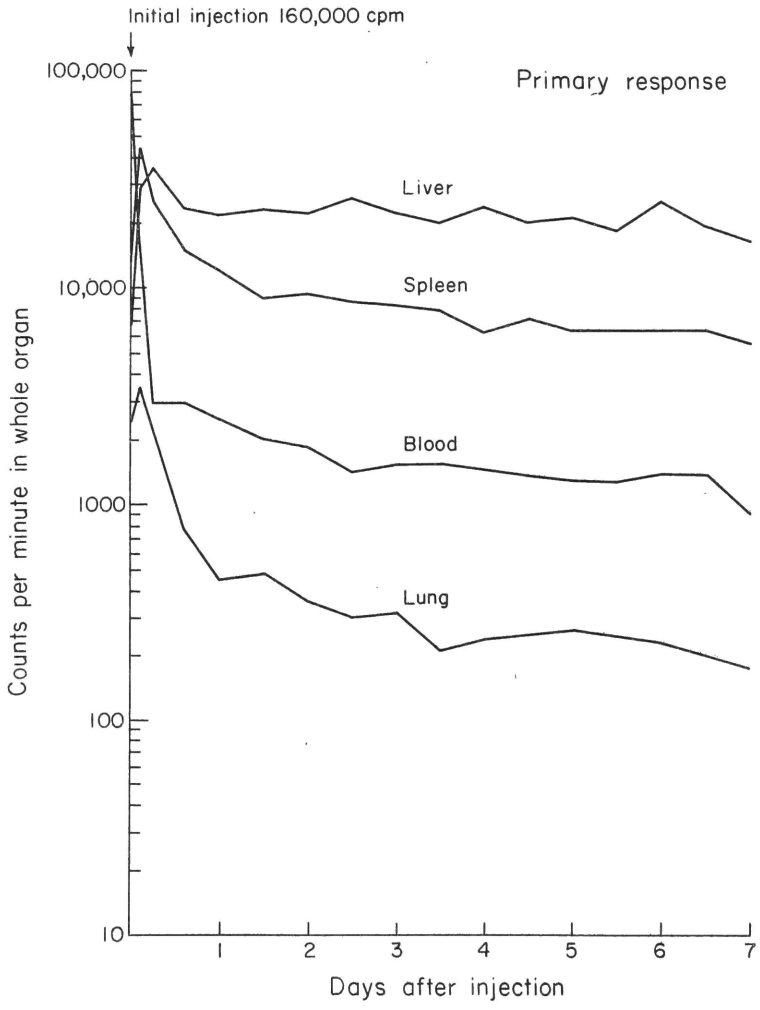


Fig. 2 **The localization of C^{14} labeled S. adelaide during the first week after primary injection.**

The counts in the three organs studied rose rapidly during this time, but they sharply decreased in the lung and the spleen during the next 18 hours. After the first 24 hours of the response, the levels of radioactivity declined at a steady rate in the three organs studied and in the blood. These rates remained the same when followed for as long as 15 more days.

Fig. 3 and Fig. 4 show the recovery of injected antigen in each of the organs and the radioactivity per wet weight of tissue. The spleen had a higher specific activity than either lung or liver and showed relatively greater phagocytic capacity than was expected from the results of studies in other animals. This might be caused by the relatively poor phagocytic efficiency of the mouse liver which has been described by Dobson and Jones (1951). They attributed their results to the greater speed of blood passage through the liver in the mouse as compared to other mammals. In the present studies, the spleen captured relatively larger amounts of antigen than the liver when there was no circulating antibody detectable. In the presence of specific opsonins, however, the liver removed almost all of the injected antigen and also had a higher specific activity than the spleen. These latter results will be presented in Appendix I. This dependence of organ localization on level of circulating antibody is in agreement with the results of Benacerraf et al. (1959).

C.) THE PRIMARY IMMUNE RESPONSE: In view of the relatively large uptake of S. adelaide by the spleen after a primary injection, it was of interest to assess the role of the spleen in antibody production as well as antigen localization. The primary response was therefore studied in both spleen homogenates and in sera of mice.

A preliminary study was made of the change in weight of the spleen after antigen injection. It is well known that during the inductive phase of antibody formation, there are cellular changes in lymphoid tissue which are accompanied by enlargement of the tissue and an increase in its weight (Bjorneboe and Gormsen, 1943). In the spleen, this is caused by hyperplasia of the white pulp and not by edema or erythrocyte infiltration (Norton, Wolfe, and Crow, 1950.) This weight increase has been correlated with increases in the organ content of DNA and RNA (Ehrich, Drabkin, and Forman, 1949; Harris and Harris, 1949; Makinodan, Ruth, and Wolfe, 1954) and with increased levels of protein synthesis (Wust and Novelli, 1962). In the present study (Fig. 5), spleen weights increased sharply from the time of antigen injection to peak at nearly three times control values on day 4.

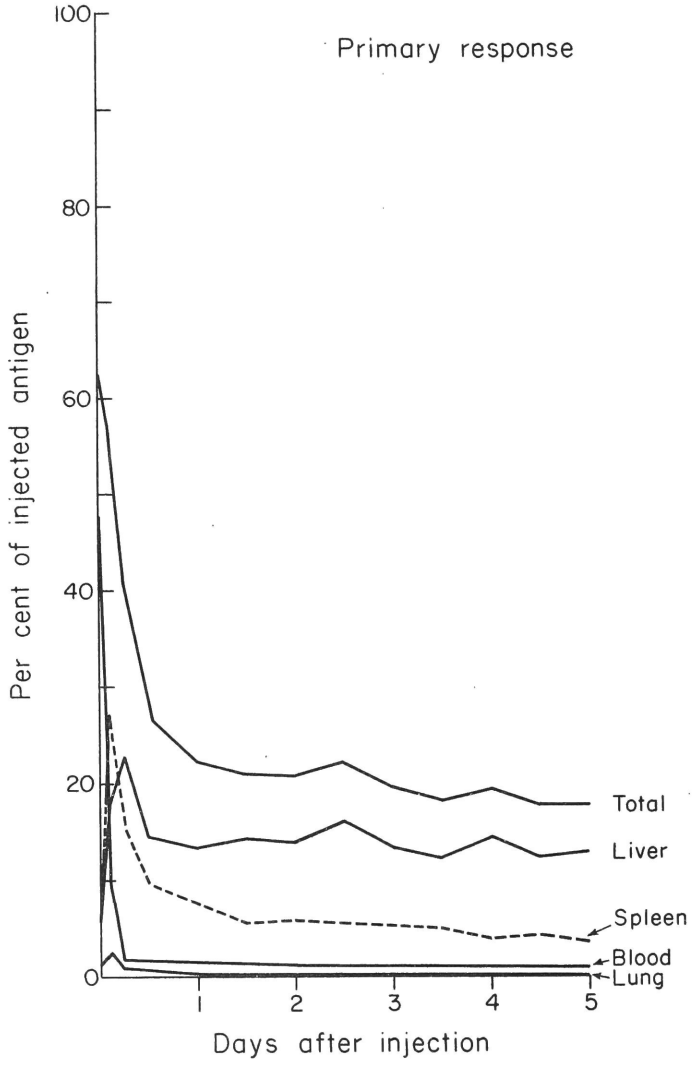


Fig. 3 The relative percentage of injected antigen recovered in each of the organs and in the blood.

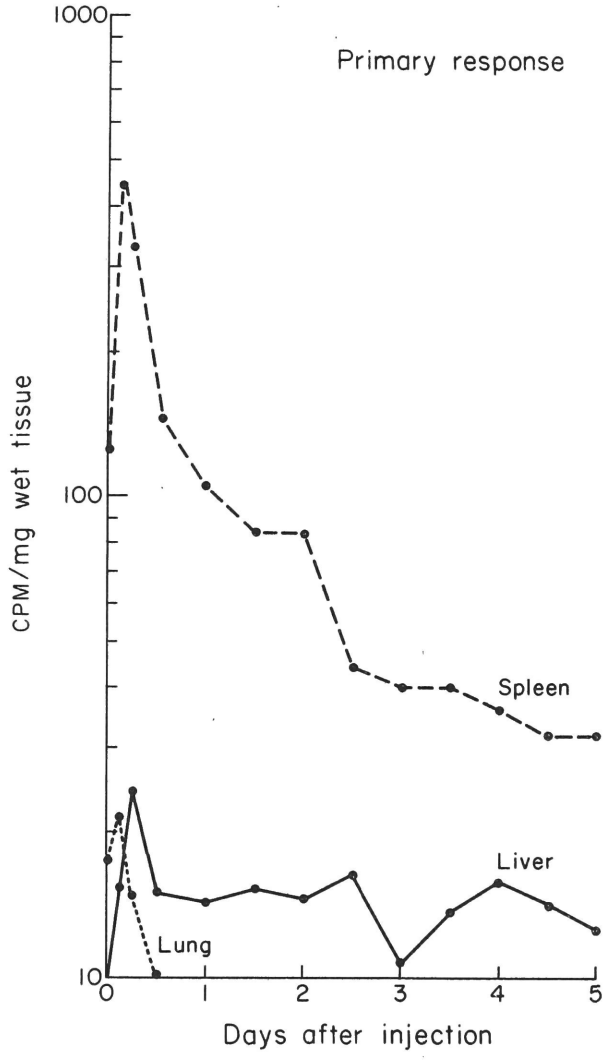


Fig. 4 The specific radioactivity of organs during the primary response.

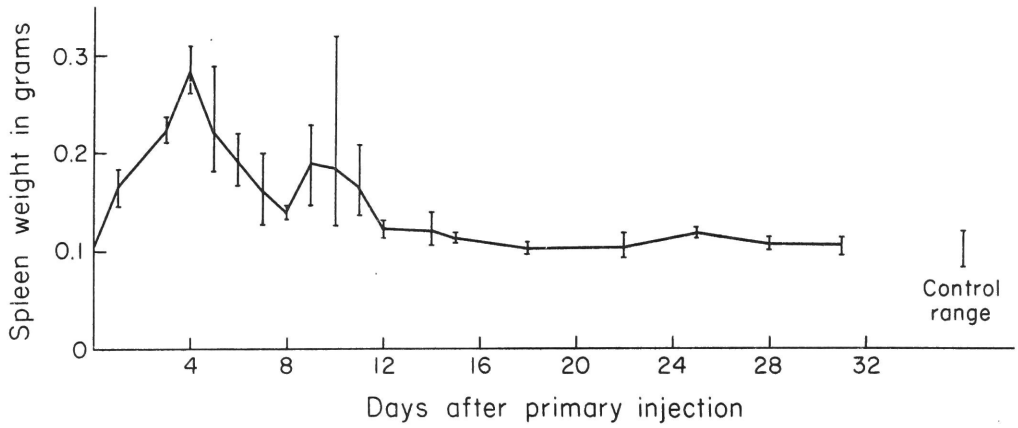


Fig. 5 **The change in the wet weight of the spleen during the primary response.**

This increase occurred despite the fact that during the first 24 hours of the response the mice underwent a characteristic endotoxin reaction: a loss in total body weight, drop in peripheral leucocyte count, decrease in body temperature, and diarrhea. The spleen weights then declined until day 9 when a smaller second increase in weight was seen. The second peak rapidly declined to control values by day 12. The bimodal nature of the spleen weight curve with this antigen was confirmed in a number of experiments. Similar bimodal curves were not noted in any of the studies cited above.

Antibody titers found in the serum and in spleen homogenate supernatants are shown in Fig. 6. Specific antiflagellar antibodies first appeared in the serum on day 4 and could be found in the spleen one day earlier. The serum titer increased exponentially until day 7 and then slowly declined during the remainder of the first month. The antibody content of the spleen was represented by a bimodal curve with a small peak on day 5 and a second, larger peak on day 10. Although the spleens were not perfused to remove blood before homogenization, the change in splenic titer was not correlated with any change in the serum titer. Also, controls done with equivalent weights of non-perfused liver tissue did not show detectable antibody titers. Thus, it seemed a fair assumption that homogenate titers were a measure of the antibody produced by the spleen itself.

It was of interest that the curves representing the change in weight of the spleen and its antibody content were each bimodal and corresponded to each other in time. Bjorneboe and Gormsen (1943) associated sharp increases in spleen weight during a primary response in rabbits with increases in the percent of antibody protein in the serum. In the present study, there was no relationship between the weight of a spleen and its antibody content (Fig. 7). It is not clear whether the fact that the two curves are both bimodal is significant in some other way.

The previous experiments of Stelos and Talmage (1957) and Bauer and Stavitsky (1961) suggested that the bimodal curve of antibody content of the spleen, as observed in these experiments, might be caused by the asynchronous production of two different kinds of antibody globulin. Attempts were made, therefore, to characterize the antibody activity both early and late in the primary response. In the results presented below, spleen homogenate supernatants were generally harder to work with because of the extremely low titers, and experiments with sera will be emphasized.

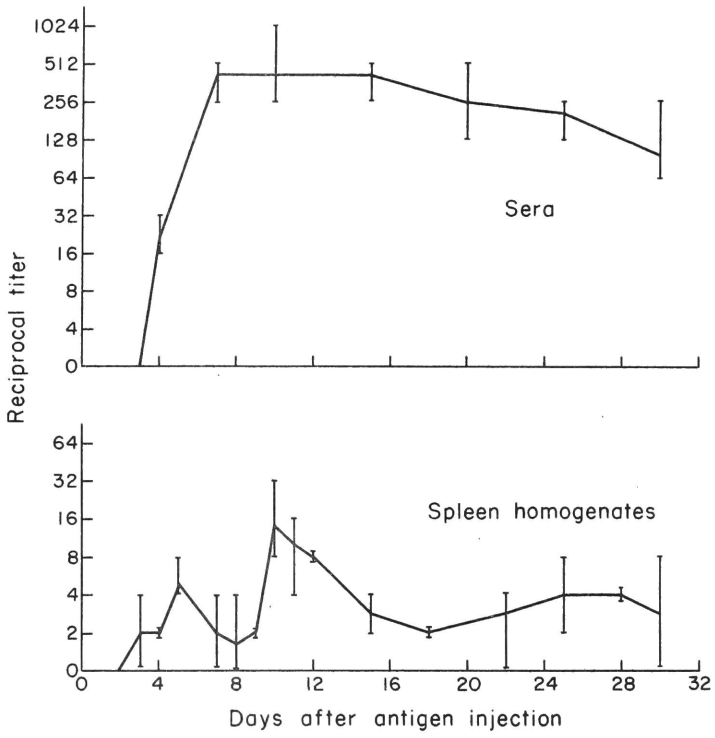


Fig. 6 The primary immune response to S. adelaide.

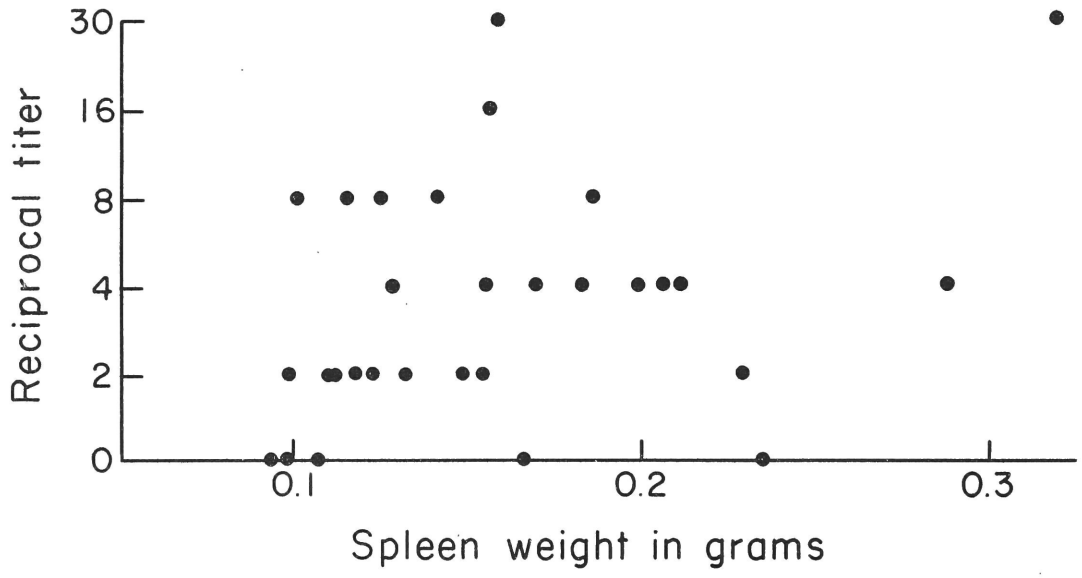


Fig. 7 The relationship between the wet weight of the spleen and its antibody content.

D.) PHYSICOCHEMICAL PROPERTIES OF PRIMARY RESPONSE ANTIBODIES:

1.) ELECTROPHORETIC SEPARATION: both 5-day and 10-day antisera were examined by zone electrophoresis and antibody activities were determined in consecutive segments (Fig. 8). Some increase in antibody activity migrating in the γ-2 region could be found at 10 days with respect to day 15. Although the separation of activities by this method was not optimal, these results were consistent with earlier suggestions cited above that the primary response consisted of the initial synthesis of γ-1 globulins followed by the later synthesis of antibodies with γ-2 mobility. With repeated attempts, it was difficult to sharpen the distinction between days 5 and 10 using this technique, and the electrophoretic characterization was not pursued.

2.) ULTRACENTRIFUGATION: a better distinction of the early and late antisera was achieved by ultracentrifugation in sucrose density gradients. Antibody titers were determined in consecutive fractions of each gradient, and the results at different times during the primary response are shown in Fig. 9. At day 5, all the antibody activity was associated with the fastest sedimenting fractions, indicating that these antibodies were macroglobulins. On day 7, more slowly sedimenting antibody activity began to appear. By 10 days, this lighter antibody accounted for approximately half of the antibody content of the serum. The macroglobulin activity continued to decrease, until by 20 days after antigen injection, the transition from heavy to light antibody activity had been completed in the serum.

Centrifugation of spleen homogenate supernatants in density gradients showed that the first peak detected in the spleen consisted entirely of macroglobulin activity. The second spleen peak, at 10 days, was composed solely of lighter antibody. Both antibodies found in the spleen sedimented in exactly the same gradient positions shown in serum studies.

3.) MERCAPTOETHANOL TREATMENT: the depolymerization and loss of activity of macroglobulin antibodies in the presence of sulfhdryl reagents has been reviewed by Franklin (1964). This specific inactivation has become a convenient means for differentiating macroglobulin from ligher weight (7S) antibody activity. When the density gradient fractions isolated above were incubated for two hours at room temperature in the presence of 0.1 M 2-ME, the fast sedimenting fractions lost all antibody activity. More slowly sedimenting antibody was not affected.

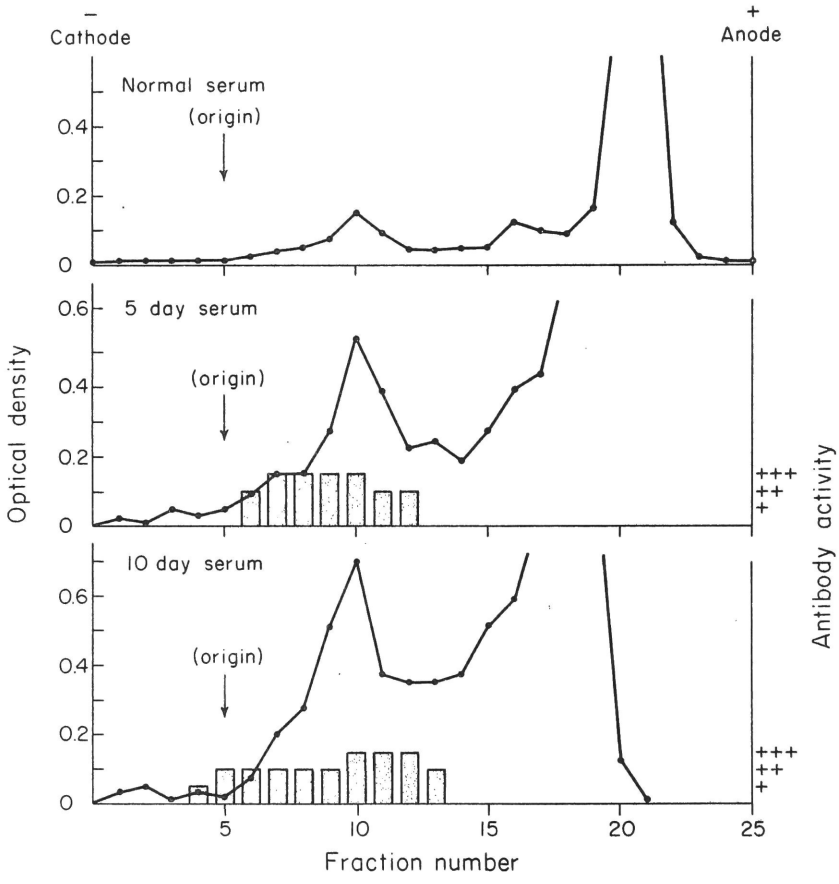


Fig. 8 The electrophoretic separation of immune sera.

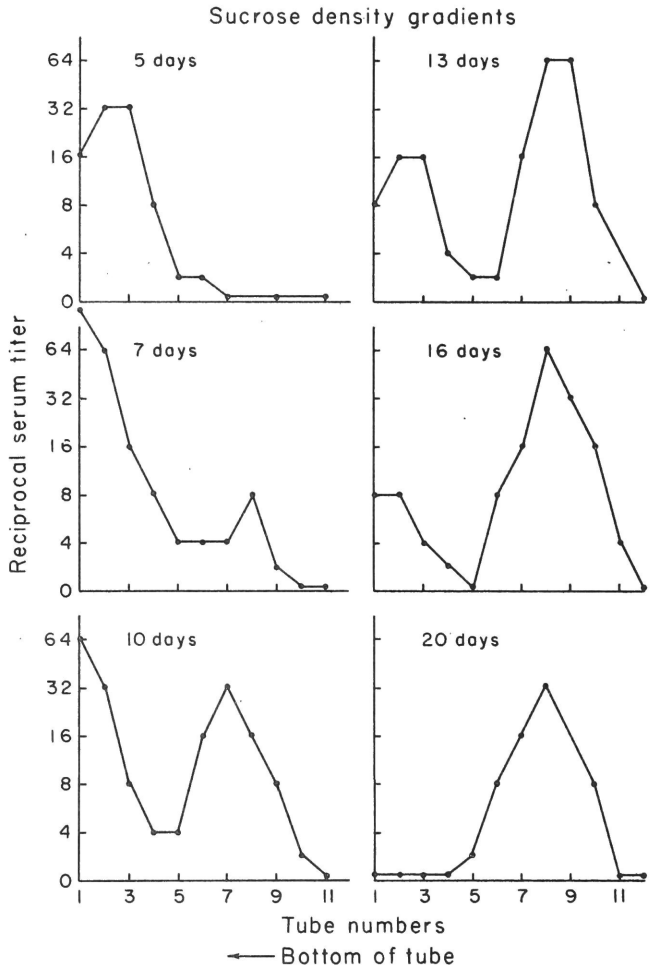


Fig. 9 **The ultracentrifugation of primary response antisera in sucrose density gradients.**

The correlation of sensitivity to sulfhydryl inactivation with ultracentrifugal and electrophoretic behavior was consistent with the idea that the two antibodies described here were identical with γM and γG antibodies found in other studies. This terminology will be employed below. Also, throughout the following experiments, only the sensitivity to 2-ME was used to differentiate γM from γG activity.

In almost every system studied thus far, γM antibodies lose all antibody activity upon reduction with 2-ME while γG antibodies are not affected. There are, however, at least two unconfirmed reports of residual γM activity after 2-ME treatment (Smith, 1960; Chan and Deutsch, 1961). Also, subunits of 2-ME treated macroglobulins may retain some binding capacity (Onoue et al., 1964; Schrohenloher, Kunkel, and Tomasi, 1964; Hill and Cebra, 1965). In certain cases, 2-ME may inactivate more than γM alone. The activity of antibodies with intermediate sedimentation coefficients in man is sensitive to reduction (Rockey and Kunkel, 1962) as is the activity of 7S antibodies in non-mammalian vertebrates (Uhr et al., 1962b). 7S antibodies in man, rabbit, and sheep may lose complement-fixing or sensitizing activities after treatment with 2-ME (Ishizaka, Ishizaka, and Sugahara, 1961; Wiedermann, Miescher, and Franklin, 1963; Schur and Christian, 1964). Depending upon the occurrence of similar effects of 2-ME on mouse serum proteins, the two classes of antibodies as defined by sulfhydryl inactivation and described in this paper may include types of gamma globulin other than 19S and 7S antibodies.

4.) OTHER PROPERTIES: various other properties of γM and γG anti-flagellar antibodies are summarized in Table III. These experiments show that neither early nor late antisera were dependent on complement for their activity. Also, neither of the antibodies lost activity during the storage times before titration.

E.) THE KINETICS OF PRIMARY RESPONSE γM and γG: the results of 2-ME characterization of antibody type during the primary response are shown in Fig. 10. The unbroken line represents titers in untreated sera; the dotted line shows the residual titer after the sera were incubated for two hours at room temperature with 2-ME. Each point represents the average of at least three mice, and the range of individual titers is shown.

The response was quite similar to primary responses already described in other species with a variety of other antigens. Beginning at four days after antigen injection, there was a sharp increase in antibody susceptible to reduction

Treatment of Immune Sera	Reciprocal Titer 5 day	15 day
dilution in saline	256	512
dilution in saline - 10% guinea pig serum	256	1024
heated 56°C : 30 minutes	128	256
0.01M sodium-EDTA	128	512
untreated: 2 hrs. room temperature	256	512
0.1M mercaptoethanol: 2 hrs. room temperature	8	256
frozen-thawed: 6 times	---	512
-15°C : two weeks	256	---
4°C : five days	256	1024
4°C : two weeks	128	512

Table III Various treatments of early and late primary antisera.

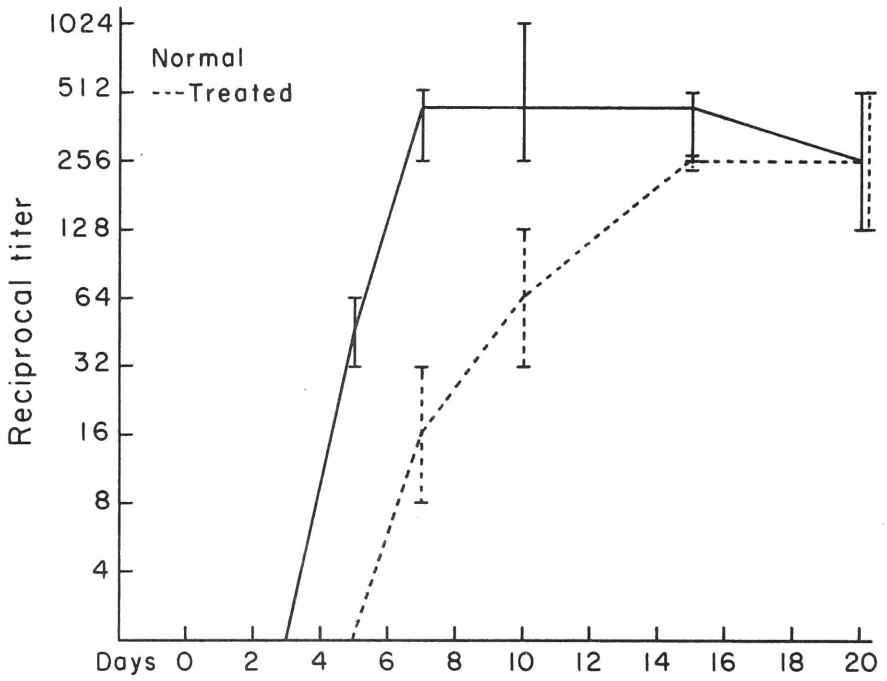


Fig. 10 The transition from γM to γG in the primary response as determined by incubation of antisera with 2-ME. The dotted line represents residual titer after 2-ME incubation.

by 2-ME. The production of this γM antibody was very short-lived and apparently ceased between day 7 and day 10. By subtracting titer values of untreated and 2-ME-treated antisera, γM could be estimated to decline after day 10 with a half-life of 24-48 hours. This is in fair agreement with the mean biological half-life of passively administered γM anti-ØX (Uhr and Finkelstein, 1963).

Three days after the first appearance of γM in the serum, antibody that was resistant to 2-ME reduction first appeared. At the antigen dose used, the rate of increase of this γG antibody was relatively slower than the rate of increase of γM. No attempt was made to study the effect of dosage on the rates of formation of either antibody. By day 20, γG accounted for all the antibody activity present and was declining with an approximate half-life of 6-7 days.

F.) THE EFFECT OF SPLENECTOMY: there have been many studies suggesting that the early antibody in a primary response is derived from the spleen and that later antibody is produced in the lymph nodes and bone marrow (Taliaferro and Taliaferro, 1950; Benedict et al., 1962; Svehag and Mandel, 1964a). Such evidence, as well as studies on the cessation of macroglobulin antibody synthesis after splenectomy in dysgammaglobulinemic patients, led Rosen (1962) to suggest that "19S gamma-globulin production occurs principally in the spleen." On the other hand, Askonas, Humphrey, and Porter (1956) suggested that the spleen produces antibody late in the response, not early, and Davidsohn, Lee, and Zandrew (1964) reported that splenectomy removes the ability to produce γG, not γM From the studies with spleen homogenates above and from other reports (Asofsky and Thorbecke, 1962; Chiappino and Pernis, 1964), there seemed little doubt that the spleen could produce both γM and γG antibodies; however, the relative production of these types by the spleen as compared with the rest of the body had not been established. This was studied indirectly by utilizing splenectomized mice.

Mice were splenectomized and allowed to recover fully from the effects of the operation before antigen injection. Sham-operated animals consistently gave the same response as normal animals. The response in the splenectomized animals is seen in the lower half of Fig. 11. The serum titers in these mice were much reduced below the normal response levels. Early samplings were not frequent enough to determine whether there were minor differences in the time of antibody appearance or in the rate of increase in the serum between the operated and intact mice.

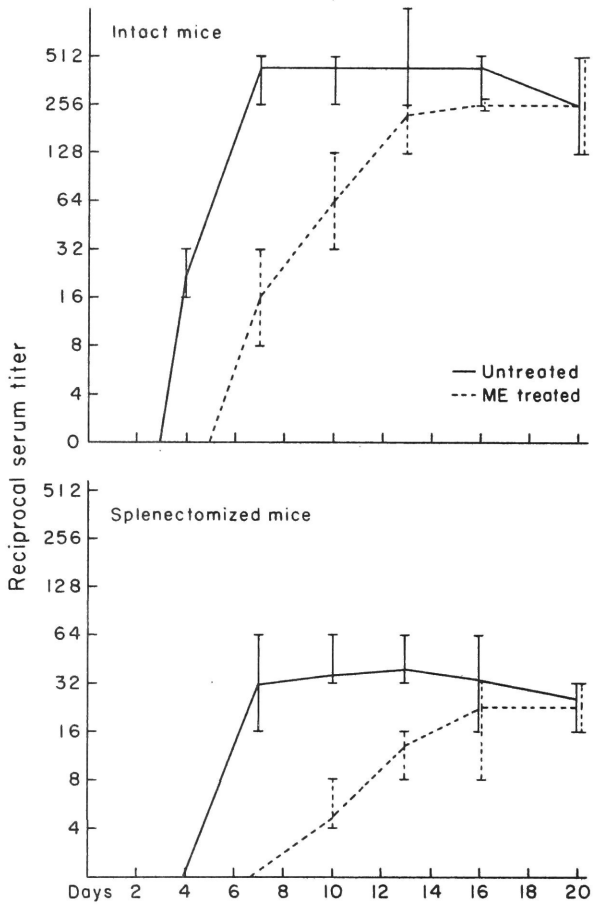


Fig. 11 The effect of splenectomy on the relative production of γM and γG antibodies.

It seemed clear, however, that splenectomy had equally depressed both types of antibody without altering the amount of one relative to the other in the serum during the first 20 days. At any time point, the ratio of γM to γG was the same in splenectomized as in intact mice. The experiment did not answer the question of whether the spleen produced antibody relatively early or late in the response. It did show that the spleen not only produced both types of antibody, but also produced them in the same proportion as extra-splenic sources.

G.) INHIBITION OF THE PRIMARY RESPONSE:

1.) USE OF METHOTREXATE: in the effort to determine the nature of the γM-γG transition and possibly to investigate the cellular basis of production of each, some attempts were made to dissociate the two kinds of antibody in a primary response. Turk and Stone (1963) reported that methotrexate administered I.P. to guinea pigs failed to inhibit the production of large pyroninophilic cells in the lymph node draining a sensitized ear; the methotrexate did, however, block the development of plasma cells. It thus seemed possible to use this drug to investigate the relationship of haemocytoblasts and plasma cells to γM and γG antibody production.

Various doses of methotrexate were given to mice to determine the inhibition of the immune response (Fig. 12). In this figure and those to follow, the arrows below the abscissa indicate the days on which methotrexate was administered. The amounts indicated were the daily doses given to each mouse and all injections were intraperitoneal. At least three mice were sacrificed at each time point.

When methotrexate was given every 48 hours throughout the response, doses of 2 mg/kg depressed serum titers while doses of 20 mg/kg totally inhibited the immune response. The weight gain or loss was determined as an indication of the general health of the animals (Fig. 13). With 2 mg/kg all the mice looked normal and gained weight as fast as normal or antigen injected controls. This dose could be used throughout the response. On the other hand, 20 mg/kg given every 48 hours caused mice to lose weight rapidly. The mice were outwardly sick and 50% usually died within 10 days. This dose was therefore used only for short periods.

The primary γM and γG responses obtained when low doses of methotrexate were given every other day are shown at the top of Fig. 14. The mice varied in sensitivity to the drug causing a wide spread in titers at some points. Also,

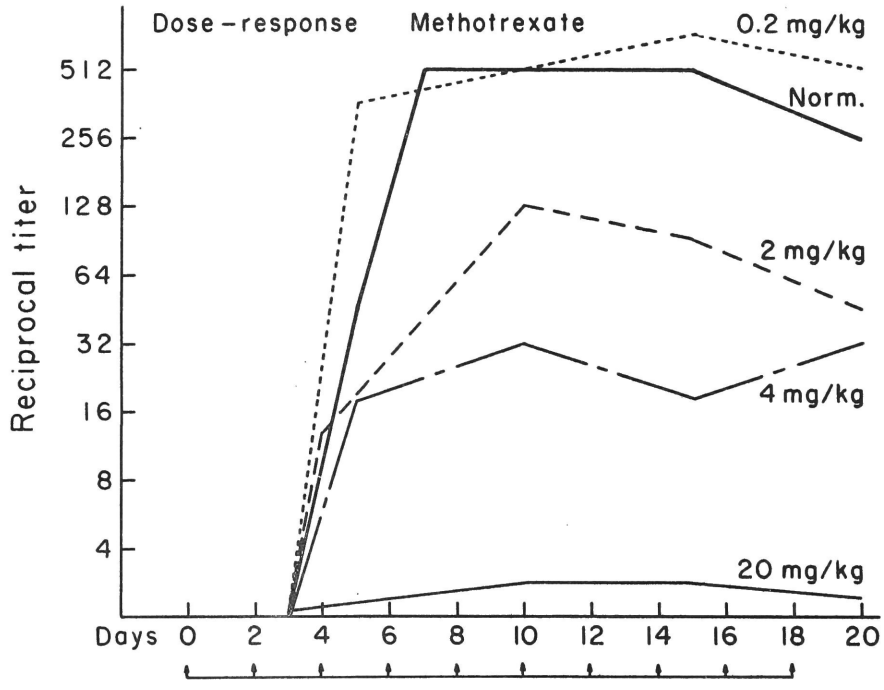


Fig. 12 Inhibition of the primary response by different doses of methotrexate.

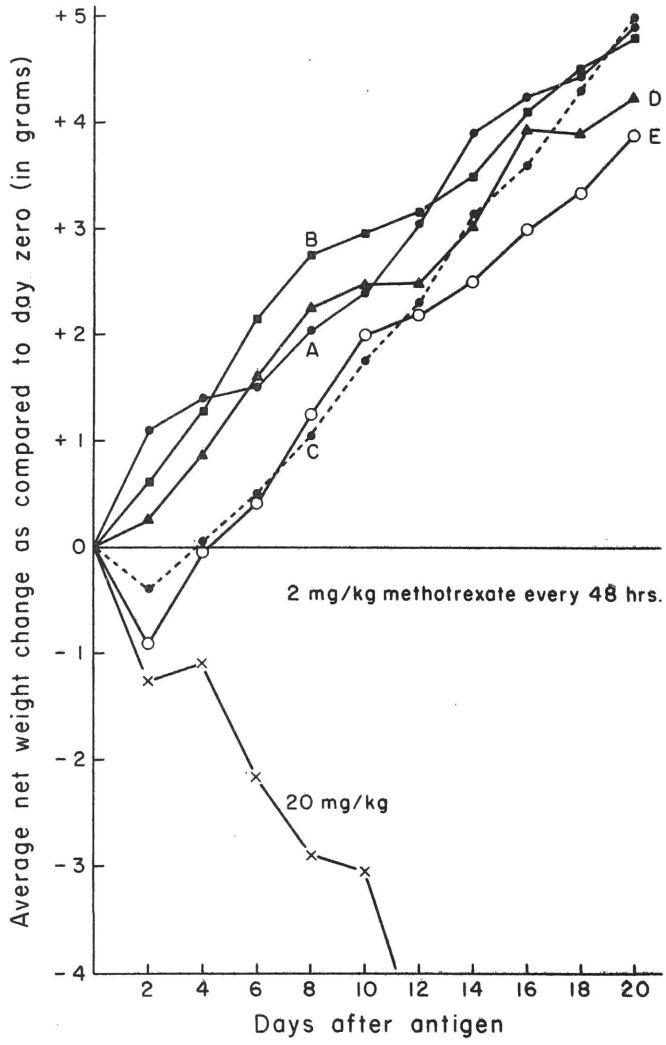


Fig. 13 Weight changes in mice receiving methotrexate.

A) normal control mice

B) mice receiving methotrexate only

C) antigen control - no methotrexate

D) methotrexate started at the time of antigen injection

E) methotrexate started 18 hours after antigen injection

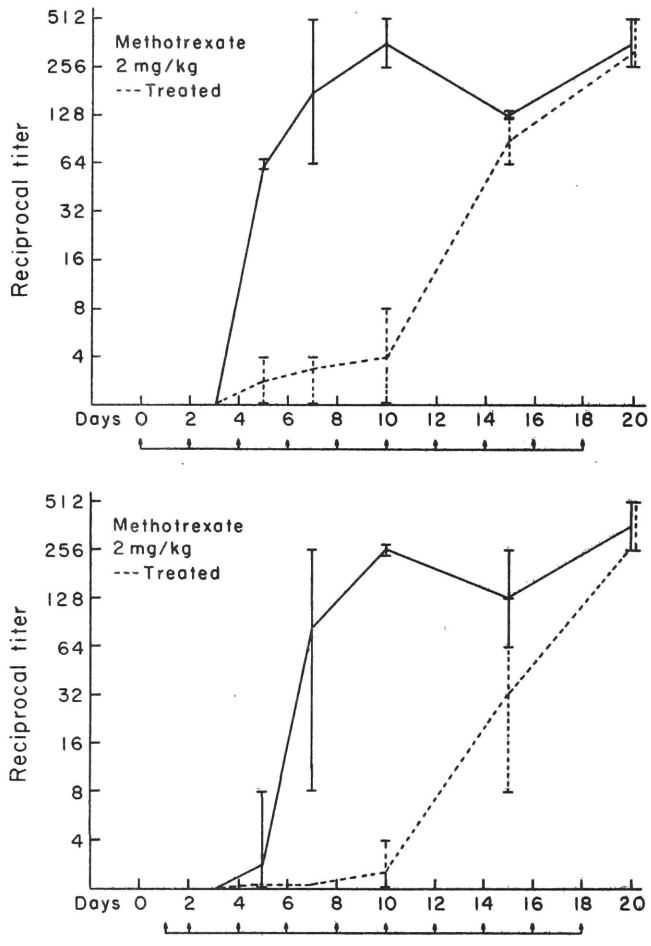


Fig. 14 The differential inhibition of γM and γG by methotrexate. The line labeled "treated" represents the residual titer after 2-ME incubation.

the mice used in these experiments were unaccountably somewhat less sensitive to methotrexate than the mice used in the dose-response curve. Nevertheless, the features described below were confirmed in repeated experiments and were also true of a number of different doses and administration schedules which will not be discussed.

Methotrexate given in sub-inhibitory doses throughout the response did not affect the onset or amount of γM production: there was a substantial titer at five days, reaching a maximum at 7-10 days, and γM disappeared by 20 days. The onset of γG production, however, was delayed 3-5 days. Yet despite continued administration of the drug, the γG did appear in the serum, increased at its normal rate, and reached its normal peak titer which it maintained when followed for two additional weeks. The two features of this response, the apparent greater sensitivity of γG synthesis to methotrexate and the invariable total recover of γG synthesis, were typical of a number of different experiments.

The bottom graph in Fig. 14 shows that the timing of dose administration was as important as the amount given. The γM response was delayed 2 days, in this case, by simply giving the first two injections of methotrexate closer together. The following γG response seemed to be little different than the γG in the top half of the figure.

When doses of 2 mg/kg were given every 48 hours for 12 days preceding antigen injection and were stopped before antigen was given, or when doses were not begun until day 8 of the primary response there was no effect on the onset or peak titer of either γM or γG. Apparently, the events in antibody formation which were sensitive to methotrexate at this dosage occurred between the time of administration of antigen and day 7.

It should be noted that the delay in γG antibody did not prolong the γM antibody response or seem to affect the rate of γM decline. The mechanism for terminating γM production seemed to be independent of the presence or absence of normal levels of γG.

The effect of a ten-fold increase in methotrexate dosage on the immune response was investigated. Because a single injection failed to noticeably inhibit either γM or γG, it was necessary to give at least 3 separate doses in the space of four days. A comparison of the two experiments in Fig. 15 shows the extremely different results obtainable with different injection schedules. The top graph

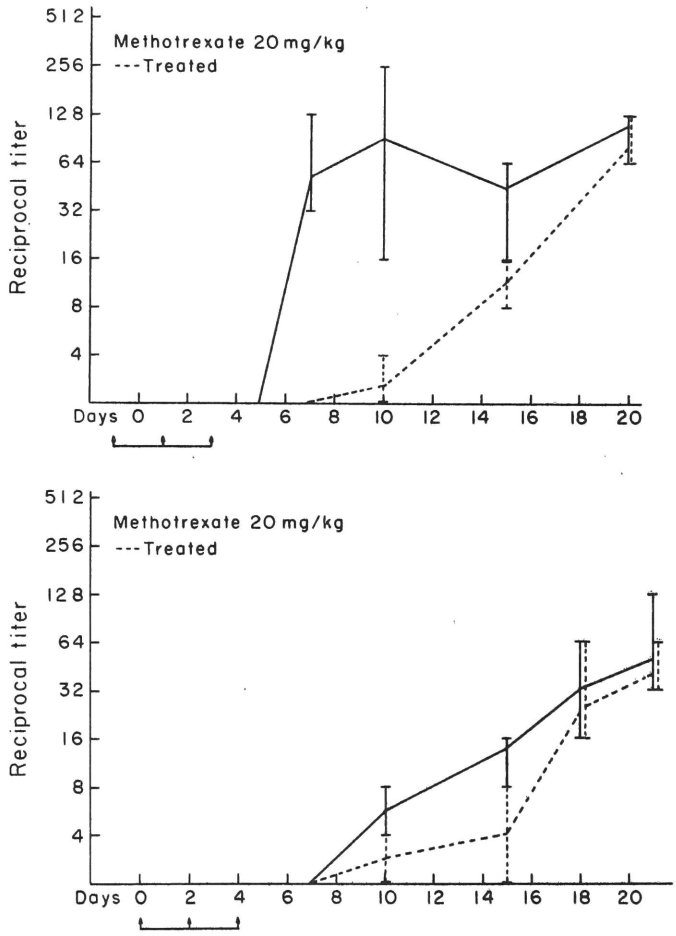


Fig. 15 **Differential inhibition of γM and γG by large doses of methotrexate given early in the response. Residual titers after 2-ME incubation are shown by the dotted line.**

is similar to experiments with 2 mg/kg except that both responses are more depressed. In the bottom graph, a shift of one day in each injection has caused the γM response to be almost totally inhibited. It was most interesting that in the latter case the γG response was little different from the γG response in the upper graph. Apparently, the near absence of a detectable γM response did not alter the recovery of γG production.

The last experiment in this series (Fig. 16) is included to emphasize that inhibition of either γM or γG involves interruption of some sensitive step in the inductive period and is not the result of general inhibition of protein synthesis. Large doses of methotrexate given every other day starting at day 8 failed to accelerate the decline of γM or to inhibit γG production and the eventual recovery of normal γG titers.

2.) USE OF COLCHICINE: colchicine was found to depress the immune response in animals given only one dose intraperitoneally. It was decided to use colchicine in an effort to pinpoint the inhibition-sensitive periods in the γM and γG response. Although known primarily for causing metaphase arrest, colchicine has too complex an effect in vivo to conclude that its inhibition of either γM or γG results from inhibition of cell division alone. The biochemical sites of inhibition are probably different in the case of colchicine from that of methotrexate.

Despite the differences in the two drugs, the effect of colchicine on γM and γG production was strikingly similar to results found with methotrexate (Fig. 17). A single injection of colchicine given on day 4 was able to delay the γG response to roughly the same degree as seen with methotrexate. γG synthesis again seemed able to recover substantially from this inhibition and to regain near-normal titers. After colchicine was given on day 4, the titers of γM on the following day were higher than normal. This possibly was the result of immediate cell damage caused by the colchicine and the consequent release of antibody. Perhaps because of such cell damage, the γM in the serum was lower than usual on day 10.

When colchicine was given on day 1, γM was sharply reduced in almost all subsequent samples. The large range of titers shows that a few mice were not as sensitive as most others to this dose. Although the γG response was more delayed than in the upper graph, the experiment tended to reinforce the evidence from the methotrexate studies that the fate of the γG response was independent

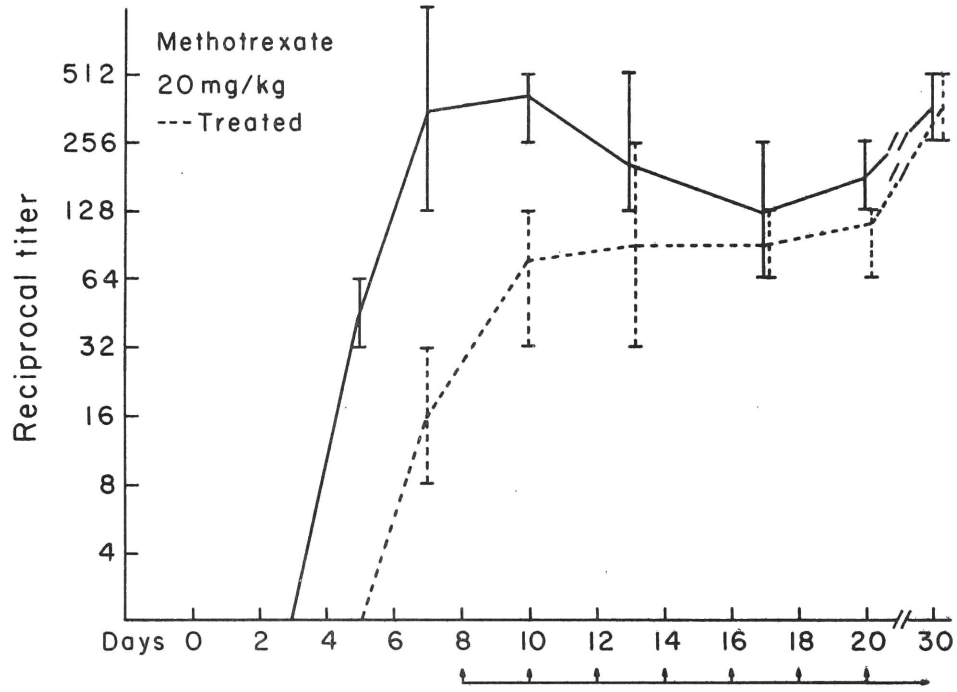


Fig. 16 The failure of large doses of methotrexate late in the immune response to alter the γM and γG transition. The dotted line represents 2-ME treated sera.

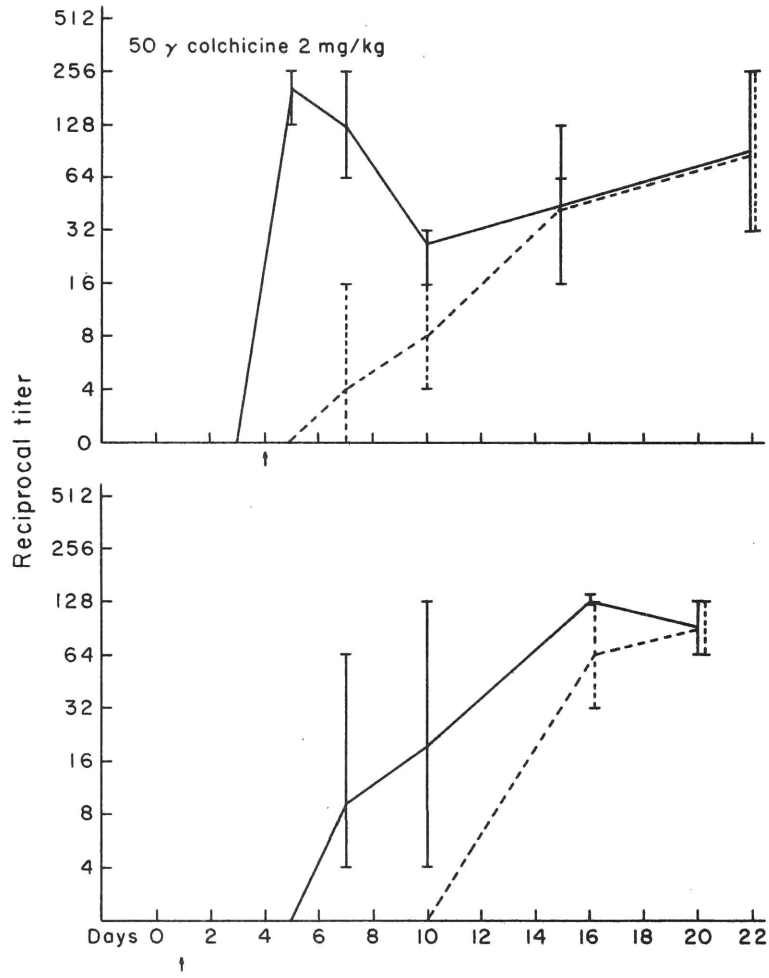


Fig. 17 The differential inhibition of γM and γG by single injections of colchicine. The dotted line show residual titers after 2-ME incubation.

of the presence of a preceding γM response. Since the γG response recovered to near normal levels, cell destruction was probably not involved in this colchicine effect.

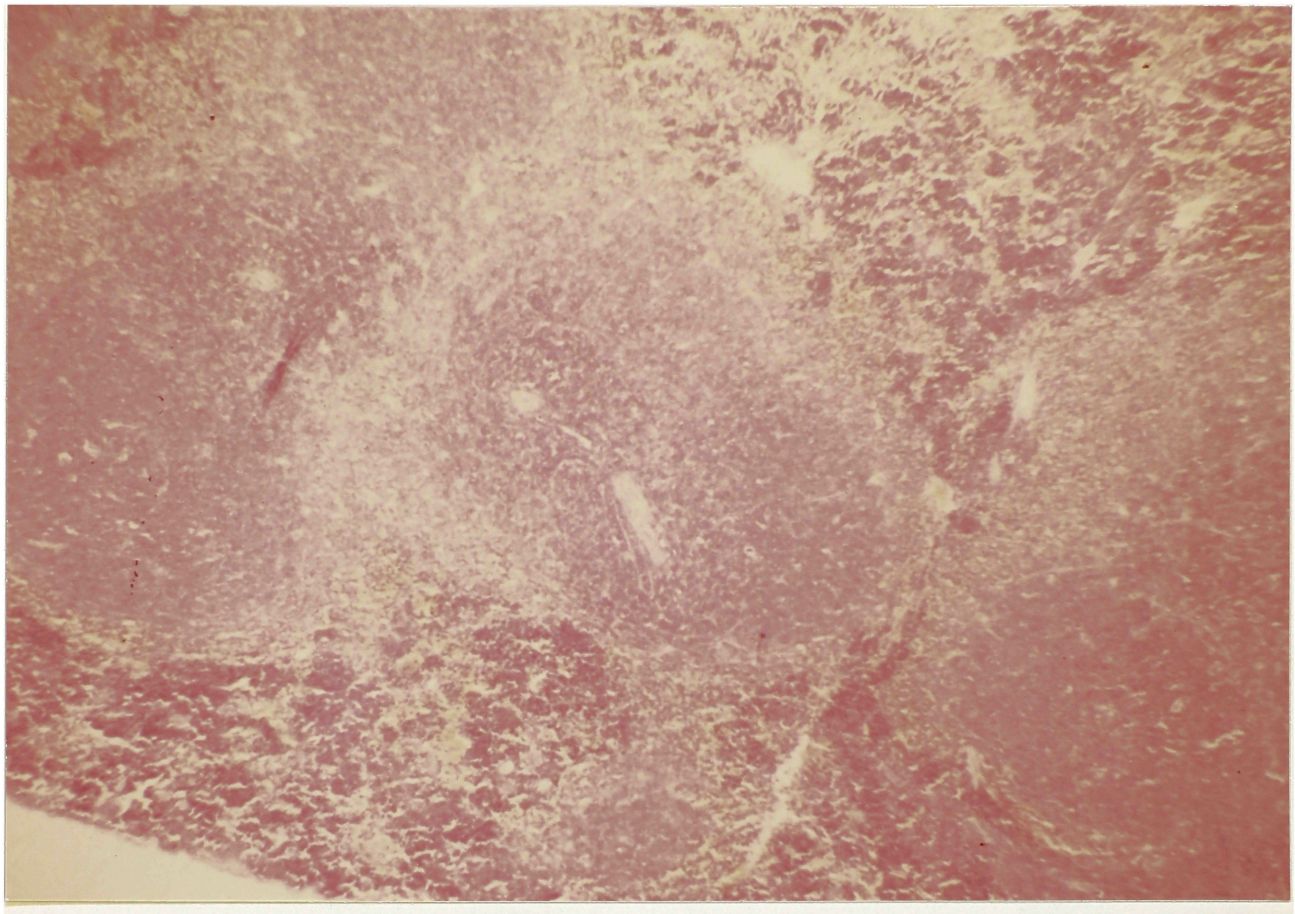
H.) HISTOLOGICAL STUDIES: the experiments on inhibition were prompted by Turk's contention that methotrexate stopped the maturation of haemocytoblasts to mature plasma cells in guinea pigs. It was therefore of interest to examine the delay and eventual recovery of the γG response in the light of simultaneous cellular events. In the discussion below, the histological response in antigen-injected controls will be discussed only briefly since it has already been described in the "Introduction." The methotrexate-treated animals are represented by the graph in the top half of Fig. 14. Spleens were removed at the times of sacrifice for serum antibody determinations. Representative sections from antigen controls and methotrexate-treated mice are shown in Figs. 18-26.

1.) THE CELLULAR RESPONSE TO S. ADELAIDE: all spleens showed sharp distinctions between red and white pulp with both hematoxylin-eosin and methyl green-pyronin staining. By day 6 of the primary response there were numerous secondary nodules in the follicles. These nodules were distinct from the rest of the white pulp and consisted of many large mononuclear cells with large, pale, central nuclei and thin, bright rims of pyroninophilic cytoplasm. A few nodules already had a "starry sky" appearance. These same large, pyroninophilic cells were found along arterioles in the white pulp, at the border, and leading into the red pulp. There was some aggregation of cells in the red pulp. By day 10, the secondary nodules had enlarged and still consisted of large, haemocytoblast-like cells. Tingible-body macrophages were evident in almost all nodules. In the red pulp, there were distinct aggregates of pyroninophilic cells, many large and medium sized, along with many typical, mature plasma cells. On days 16 and 21, the nodules remained prominent in the white pulp. Aggregates of mature plasma cells were present throughout the red pulp. The response in these antigen controls was quite typical and somewhat longer-lived than responses to soluble antigens in the absence of endotoxin

2.) THE CELLULAR RESPONSE DURING METHOTREXATE ADMINISTRATION: spleens taken on day 6 after antigen injection had a light-stained appearance in sections due to the depletion of lymphocytes in the red pulp. In many mice the boundary between the red and white pulp was very indistinct. There were no secondary nodules in most follicles. In a very few follicles a cluster of some pyroninophilic cells could be found. These consisted of only a very few cells having the appearance of

Fig. 18 Day 6. Antigen control (X 100)
Many secondary nodules can be seen in the white pulp. Cell aggregates are evident in the red pulp.

Fig. 19 Day 6. Antigen control (X 310)
Haemocytoblasts are clustered throughout the white pulp and along the edge of the arteriole. They are characterized by large, pale blue, central nuclei and thin rims of brightly pyroninophilic cytoplasm. The secondary nodule is well defined from the surrounding white pulp. A few "tingible body" macrophages are seen as white areas with dark inclusions. The aggregated cells in the red pulp are mostly immature plasma cells.



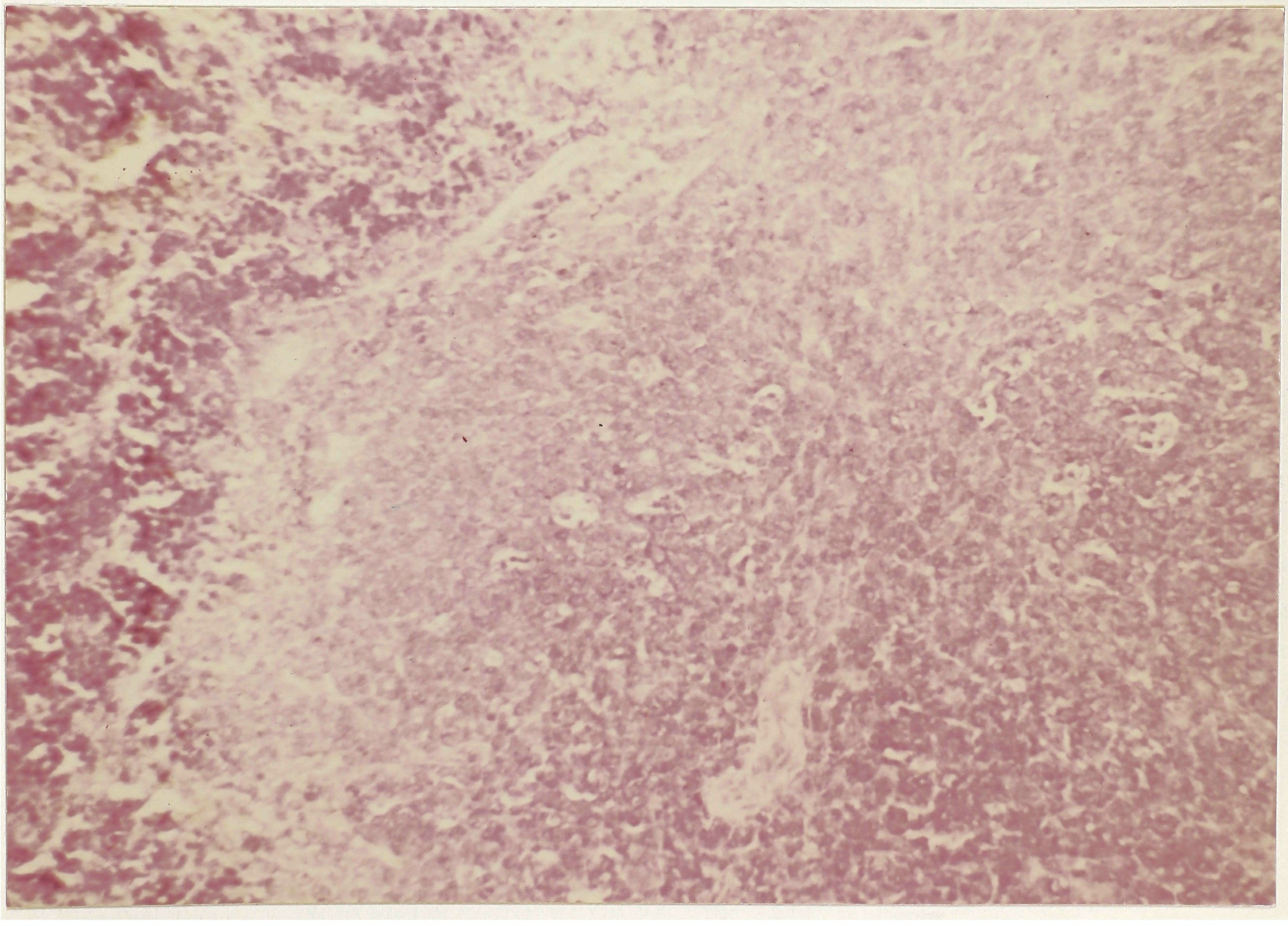
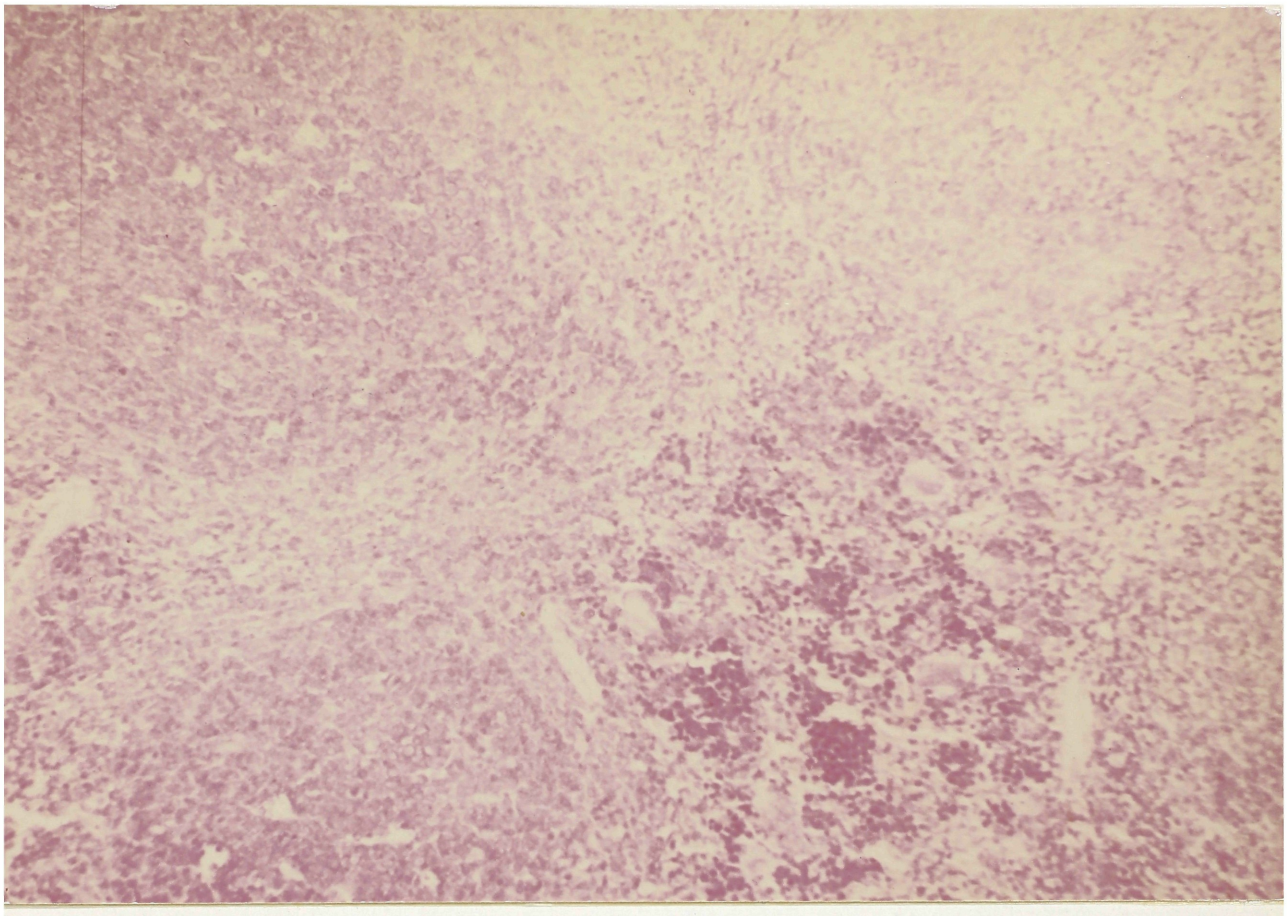


Fig. 20 Day 10. Antigen control (X 255)
Secondary nodules in the white pulp have enlarged and are surrounded by distinct cuffs. Large aggregates of plasma cells are seen in the red pulp.

Fig. 21 Day 16. Antigen control (X 100)
Nodules are well defined with the typical starry sky appearance. They are found in almost all white pulp follicles. Aggregates in the red pulp are distinctly separated and consits of typical mature plasma cells.



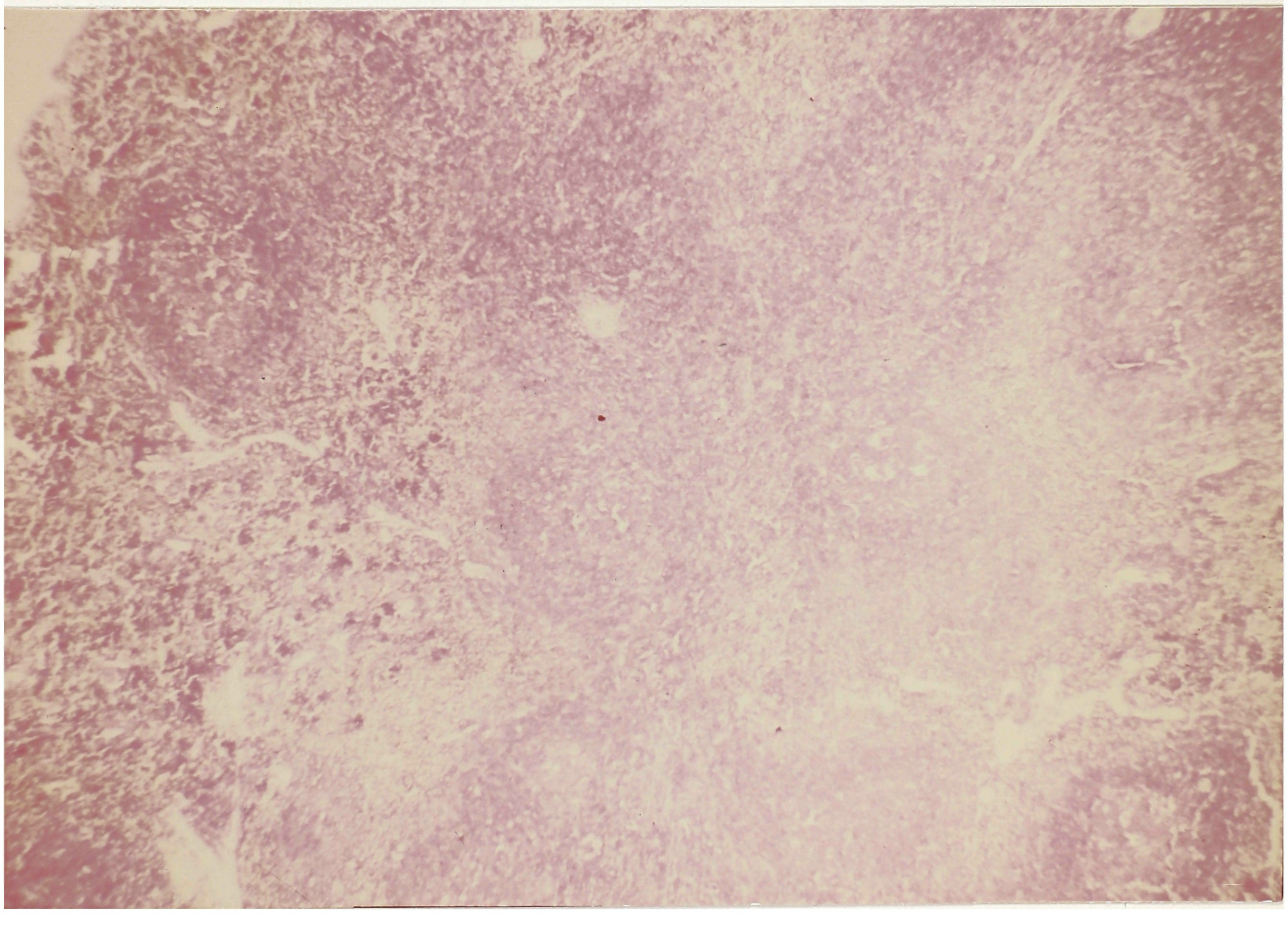
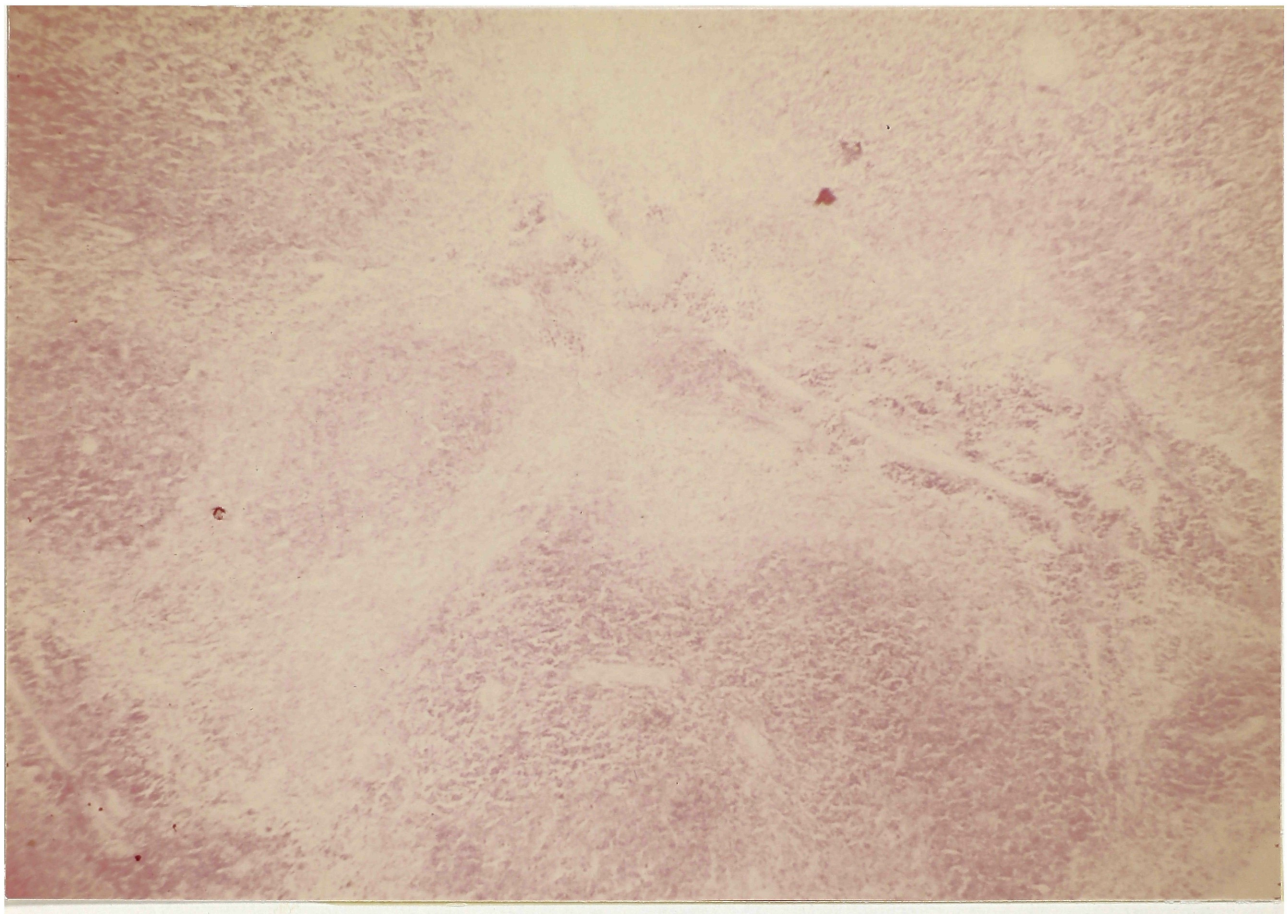


Fig. 22 Day 6. Methotrexate treated (X 100)
Red pulp is severly depleted of cells and white pulp only stains lightly. There are no secondary nodules, haemocytoblasts, or cell aggregates evident. A number of cells with pyknotic nuclei are present along the edges of the arteriole.

Fig. 23 Day 10. Methotrexate treated (X 255)
Many haemocytoblasts are seen in the red pulp. The cells are not aggregated. None of these cells are found in the white pulp, and no secondary nodules are evident.



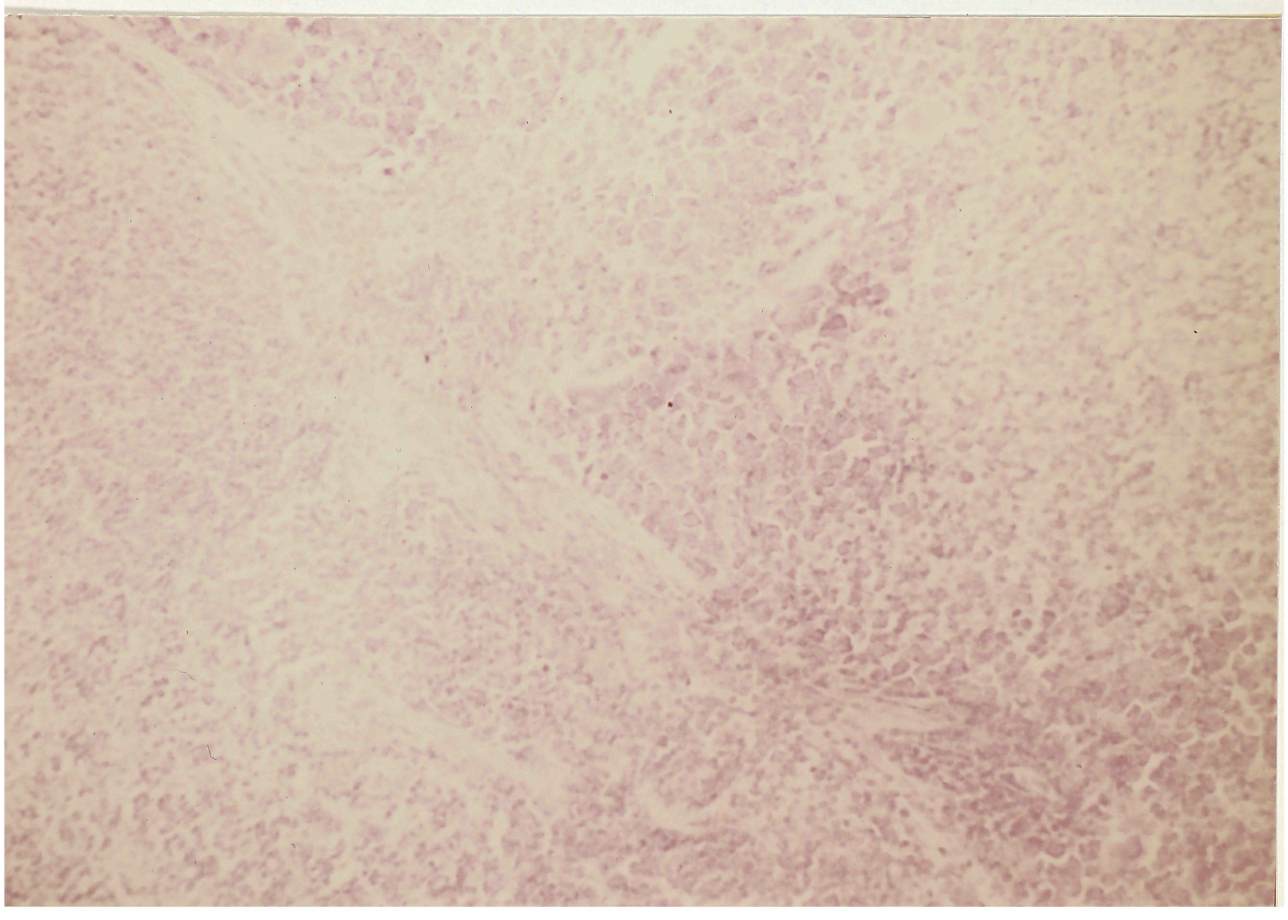


Fig. 24 Day 16. Methotrexate treated (X 255)
The red pulp pyroninophilia has diminished but is still present. There are still no haemocytoblasts or secondary nodules in the white pulp.

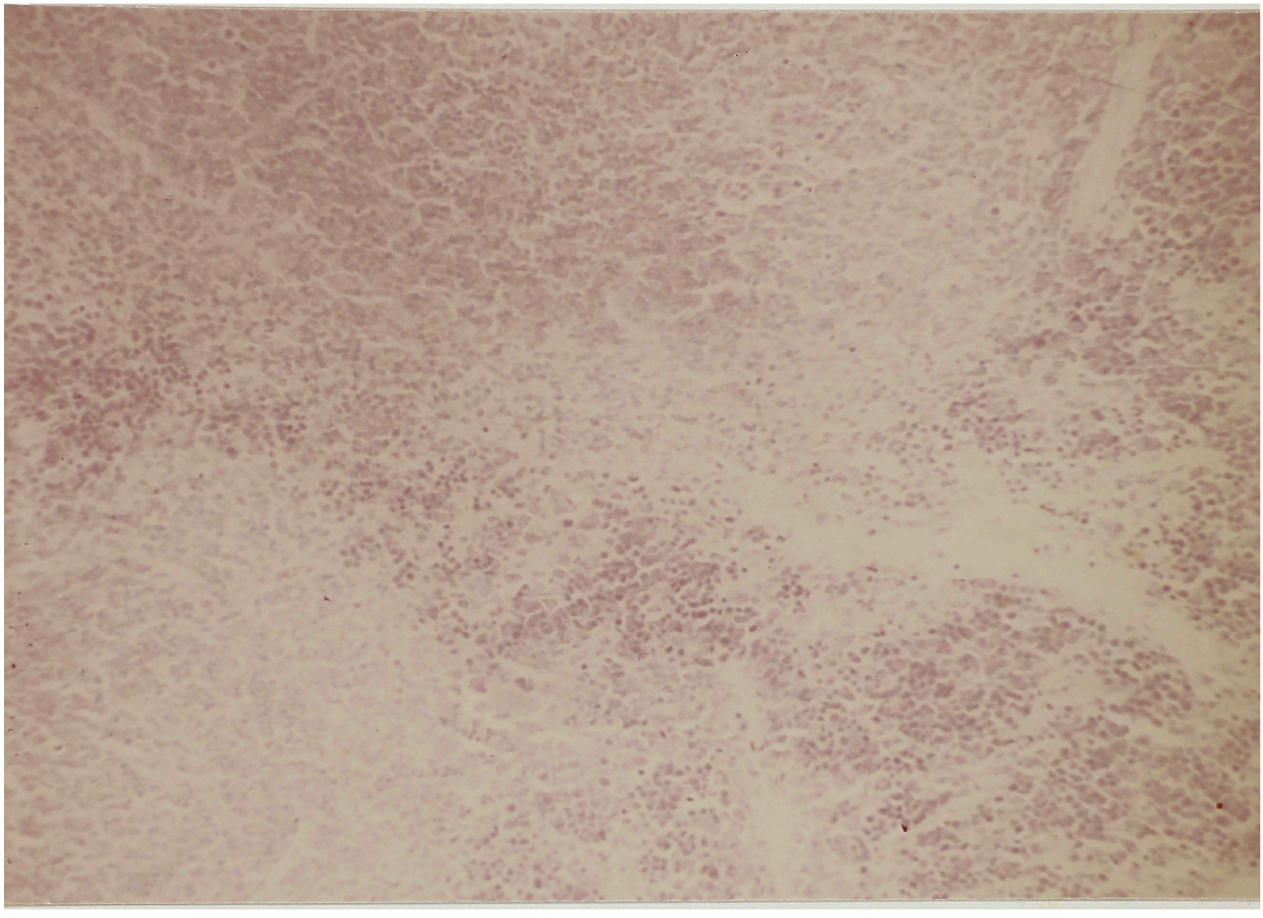
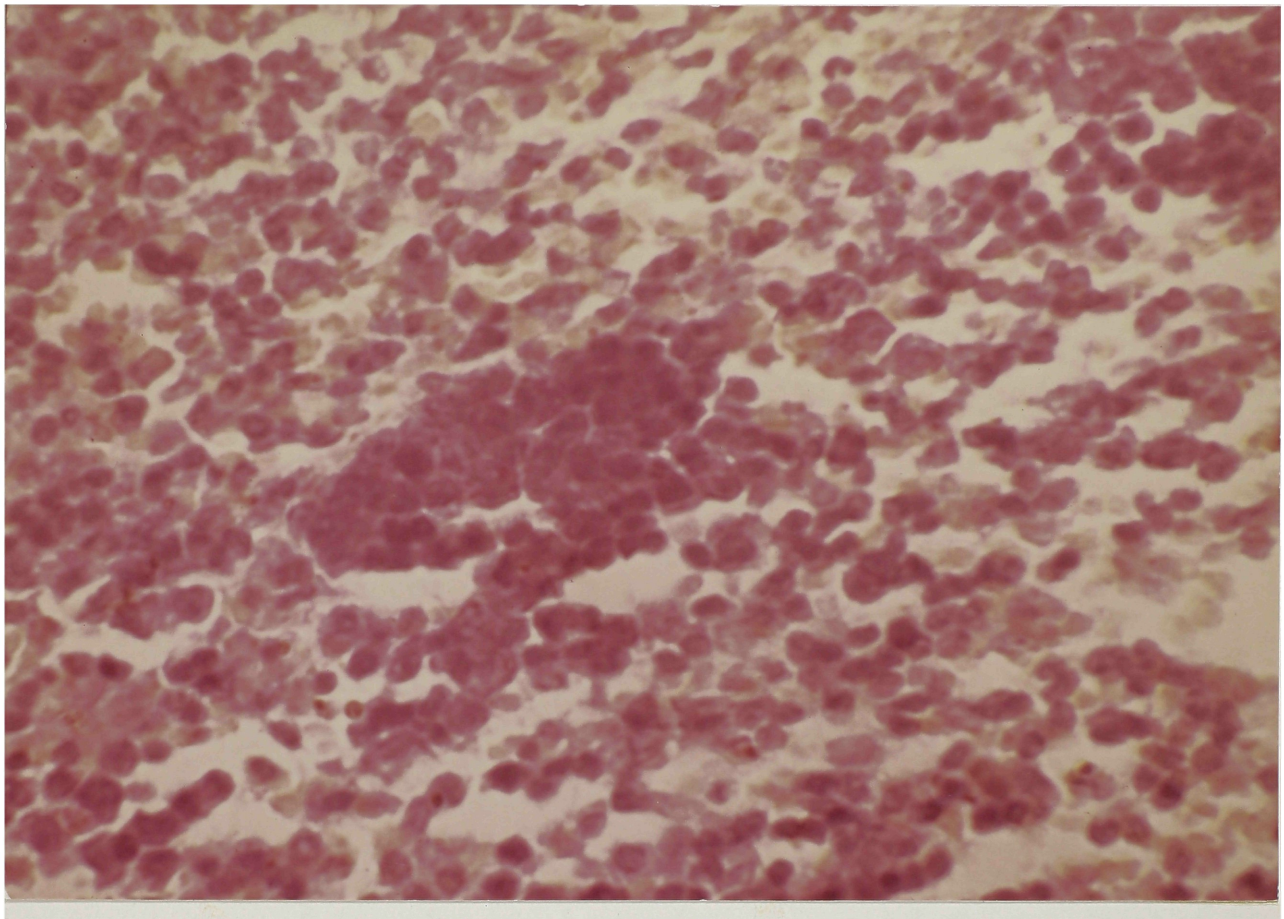
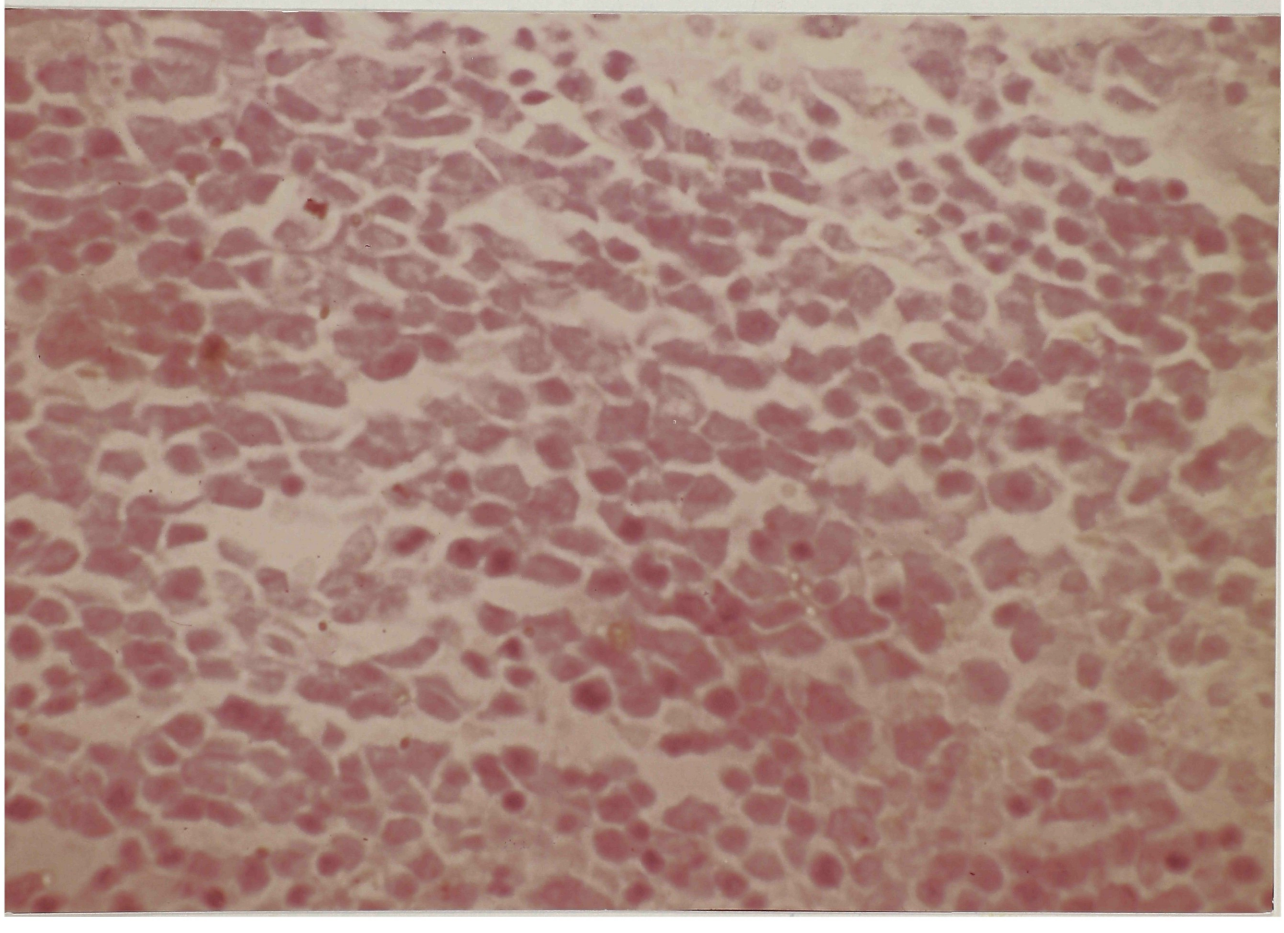


Fig. 25 Day 10. Antigen control (X 950)
The aggregated cells in the red pulp are typical mature plasma cells.

Fig. 26 Day 16. Methotrexate treated (X 950)
Red pulp. The cells are typical haemocytoblasts.





haemocytoblasts. Cells with pyknotic nuclei were clustered in the white pulp and were often found around arterioles in the red pulp. These were not pyroninophilic. On the whole, pyroninophilic cells were quite scarce. Some could be found in the red pulp. These were large, mononuclear cells.

Day 10 sections from treated mice showed the red pulp filled with pyroninophilic cells. Many were clustered around arterioles and under the capsule, but they were not arranged in striking aggregates as they were in control animals. These cells appeared to be haemocytoblasts. Only an occasional follicle contained anything resembling a secondary nodule. When seen, these consisted of only a few pyroninophilic cells. No mature plasma cells were evident.

By day 16, the pyroninophilia was much less marked in the red pulp. Some pyronin-stained cells under the capsule seemed to have no structure and looked dead. There were areas of the red pulp which contained large, pyroninophili cells, but these were still not distinctly aggregated. Mature plasma cells and secondary nodules could neither be found at this time nor at day 21.

The distinctive differences between the methotrexate-treated and control animals were the absence of secondary nodules in the white pulp, the lack of distinct aggregates in the red pulp, and the failure to develop mature plasma cells in all animals receiving the drug. The possible importance of these differences will be discussed below.

I.) THE TRANSFER OF γM AND γG PRODUCTION: there have been many theories that the γM-γG transition in some way depends on intimate cell relationships in situ or on the presence of antigen late in the primary response. Many of these theories have been discussed in the Introduction". The transfer of primed spleen cells to X-irradiated recipients seemed a possible means of exploring these questions. In effect, the separation of the cells at the time of transfer would disrupt and rearrange any existing cellular relationships. The washing of the cells would probably remove any extracellular antigen.

Donors were immunized with S. adelaide and their spleens were removed 2, 6, 8 and 10 days after antigen. The cells were separated, washed, resuspended, and injected into recipients as described in "Materials and Methods." With the technique used, it was impossible to obtain all the cells in any spleen. The number of cells injected into each recipient was approximately one-half the number of cells usually extracted from a spleen, and this number corresponded to one-half or one-third the number of cells obtained from a comparable mouse spleen by

Jerne (Jerne, Nordin, and Henry, 1963).

The results of the transfer of cells taken early in the primary response are shown in Fig. 27. When cells were transferred two days after antigen only γM was produced in the recipients; cells transferred on day 6 produced convincing titers of γM but barely detectable levels of γG. When cells were transferred on day 8 or day 10, however, there was very little γM in the recipients, but quite high titers of γG were detected (Fig. 28).

It was necessary to control these experiments in a variety of ways. In the first place, it was possible that the recipients had recovered from the X-irradiation and were producing antibody in response to antigen transferred with the cells. When antigen was given to mice 24 hours after X-irradiation, however, these mice never produced detectable serum antibody titers within 12-14 days after antigen. Additional controls employed frozen and thawed cells taken on the second day after antigen. When these cells were injected into either X-irradiated recipients or normal mice no subsequent antibody titers could be detected. Apparently there was not enough antigen transferred with the cells to stimulate antibody production even in normal mice.

Secondly, the experiments were open to the objection that the cells transferred on days 2 or 6 had simply died after a short time in the recipient and released the antibody which they contained. It is unlikely, however, that no γG was produced in these responses because the transferred cells were dead. In the first place, X-irradiated recipients are known to provide good cultural conditions for transferred cells. Also, in these two cases, no antibody could be detected in the recipients until at least four days after transfer, so the cells did not die immediately. The transferred cells were 85-90% alive as measured by trypan blue staining at the time of transfer. In addition, the previous studies in this paper on homogenization of spleens showed that at the times of transfer the spleens did not contain enough antibody to passively immunize the recipients to the extent shown. Finally, the half-life of passively administered γM antibody is known to be relatively short, and it is unlikely that small amounts of passively transferred γM antibody would continue to be detectable in the recipient serum for as long as 15 days after the day of transfer.

From these studies, it would seem possible to conclude that 1) the events necessary in the immunized donor for γG synthesis had not yet been completed by day 6 after antigen, that 2) the cells producing γM were no longer dependent on

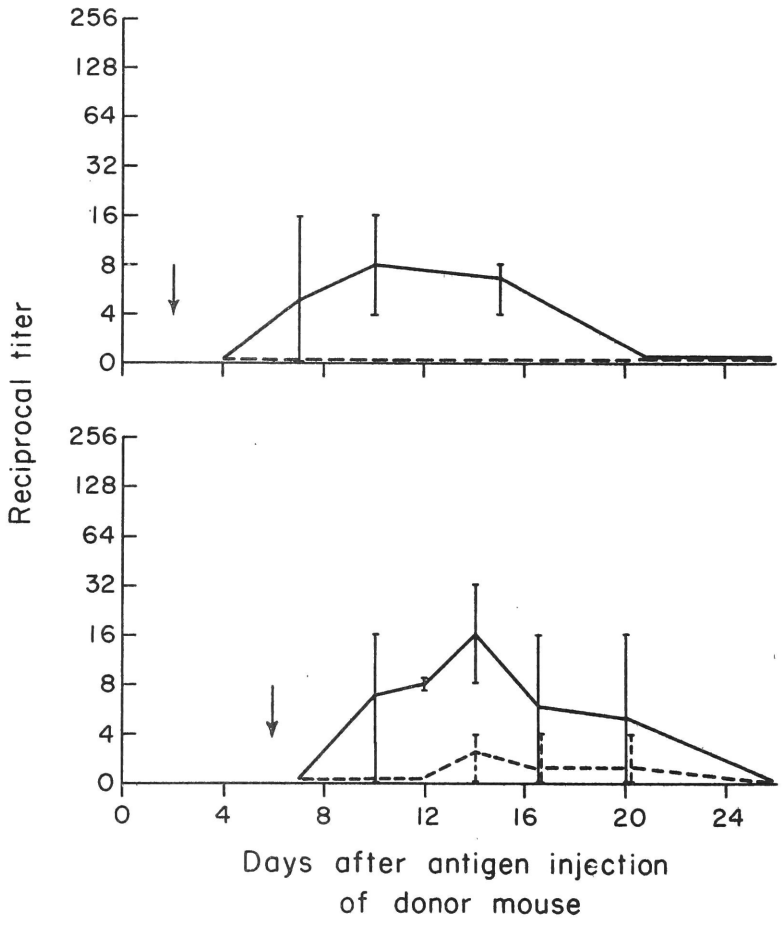


Fig. 27 The transfer of γM production by primed cells taken early in a response. The arrows indicate the day of transfer.

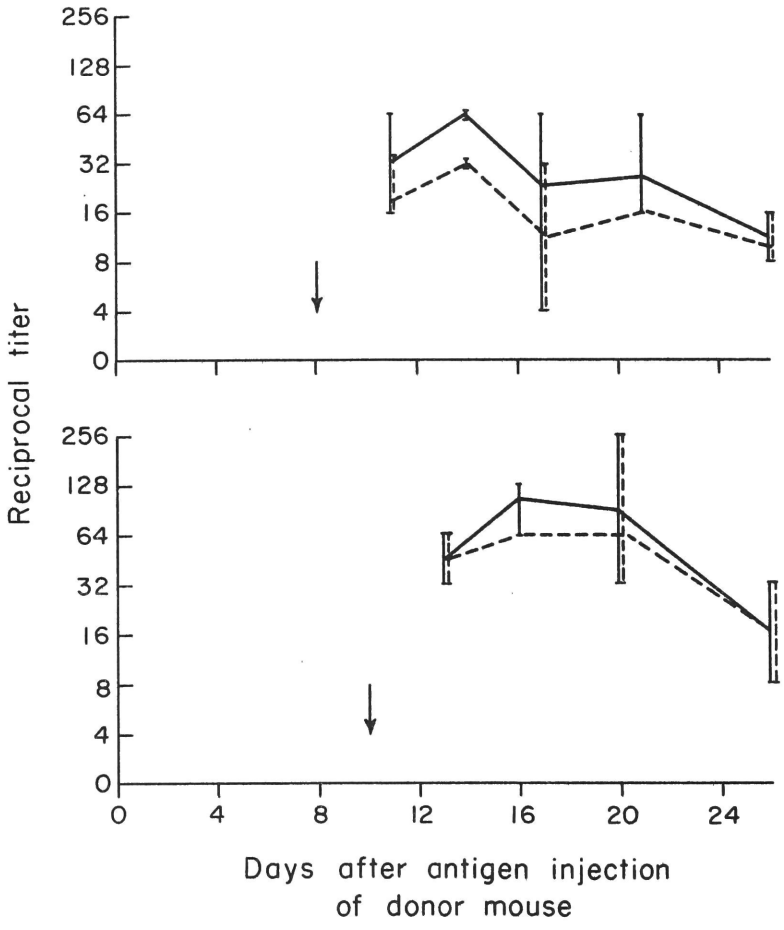


Fig. 28 The transfer of γG production by cells taken late in a response. The arrows indicate the day of transfer.

extracellular antigen or nearest-neighbor cell relationships after day 1, that 3) γM production had ceased in the donor by day 8 but not before day 6, and that 4) γM production was prolonged past its normal shut-off time by transfer to recipients on day 6.

IV. DISCUSSION

The findings that two types of antibodies were involved in the primary response raised the possibility that these antibodies were produced either by different organs, by different cell lines, or sequentially by the same cell. The experiments in this thesis were aimed indirectly at answering which of these possibilities was more probable. Attempts were made during the course of this study to assay directly the production of either γM or γG by different types of cells (employing bacterial adherence to cells in vitro), but the cellular types proved difficult to identify consistently or with certainty, and no conclusions were reached. Although the present experiments have not solved the one cell-two cell controversy, they have clarified in some ways the relationship of the γM to the γG response, they have described some of the factors which can alter the γM-γG transition, and they have raised many questions which should be open to further experimentation.

The early experiments in this study dealt with the role of the spleen in the formation of γM and γG antibody. The results of spleen homogenization were consistent with the conclusions of Asofsky and Thorbecke (1962) and Chiappino and Pernis (1964) that the spleen produced both γM and γG.

The distinct separation of the two antibody peaks gave some evidence that γM production ceased relatively early in the response. There was probably an overlap of these peaks which was not detected because of the low levels of activity. Nevertheless, the peak titer of γM in the spleen occurred on day 5 and none was found by day 8.

These studies did not, however, provide any evidence about the relative quantities of each type of antibody produced by the spleen. It was still possible that one type of antibody was primarily associated with the spleen and another with lymph nodes or with bone marrow. In vitro culture of a primed organ might have established the quantitative relations desired, but one could never be sure that differences in the quantities of γM and γG produced were not reflections of preferential cell survival under the conditions of culture or were not caused by the removal of the organ from various circulatory factors. Indirect experiments were therefore pursued. There seemd to be little doubt that under these conditions removal of the spleen prior to antigen administration did not influence the relative proportions of γM to γG. What appeared to be a lack of γG at day 7 probably resulted from the inability to detect the generally lower titers at that time. It would seem that antibody producing organs are equally capable of producing γM and

γG antibodies. This conclusion appeared reasonable, since studies by other investigators had employed foot pad injections to evoke typical γM-γG responses. A foot pad injection would involve primarily the draining node while an intravenous injection would involve primarily the spleen. Yet the responses summarized in Table I are generally all the same.

This conclusion is contradicted by the report of Davidsohn et al. (1964); however, the use of erythrocytes as antigens might make that report a specialized case. The antigen used by these authors as well as their method of titration is known to favor the production and detection of γM antibodies. With red blood cells, many injections must be given in order to stimulate γG production. This may be due to the inability of this antigen to reach antibody forming cells in doses above the γG threshold (see p. 14 of this thesis). Splenectomy would probably accentuate the antigen distribution problem. In the absence of the spleen, more antigen would be trapped by the liver which apparently destroys the immunogenicity of erythrocytes (Franzl, 1962).

One word might be added about the bimodal spleen weight curve and its similarity to the curve of antibody content. This result was surprising since there was no correlation between the weight of a spleen and its antibody content. Very little is known about the rate of secretion of antibody from the spleen, so that assays of homogenates may give only a rough index of splenic antibody synthesis. It was of interest, therefore, to find that Wust and Novelli (1962) had studied the incorporation of C^{14}-glycine into spleen and liver protein after antigen injection. These authors found a distinctly bimodal curve of incorporation into spleen protein with the first peak on day 5 and the second peak on days 10 and 11. These authors felt that the increase in spleen weight was caused by increased protein synthesis. No reason was offered by them as to why the incorporation curve was bimodal or what its relationship was to antibody production or cell division.

The experiments with methotrexate suggested that the γG response was more sensitive to inhibition than γM. It was difficult, however, to visualize what this meant in terms of the action of the drug or what it implied about the cells involved. Methotrexate is known to inhibit the conversion of folic acid to tetra-hydrofolic acid; a derivative of this latter compound is needed as a co-factor for DNA synthesis. The principal action of the drug is therefore to inhibit

DNA metabolism and consequent cell division. Less is known about the in vivo action of colchicine, but it is likely that metaphase arrest occurred and division was inhibited. Nevertheless, little could be said about the action of these drugs in terms of the site of inhibition of the immune response. Arrest of cell division or inhibition of cell differentiation and maturation may have been equally responsible for the results obtained here. It was difficult to determine the most sensitive times in the response to methotrexate. At best, it was possible to show that pretreatment with the drug or treatment after day 8 did not inhibit serum levels of antibody. The experiment shown in the bottom half of Fig. 14 suggested that on the first day after antigen administration antibody producing cells might be especially liable to inhibition by methotrexate. This possibility was consistent with the results of colchicine inhibition: a single dose of colchicine given on day 1 was more effective at inhibiting both γM and γG antibodies than a single dose given on day 4. So it may be that the most sensitive periods of both responses occur early in the response. The 24-48 hour period after administration of antigen is known to be the time of maximum cellular proliferation. That γG synthesis is still sensitive to colchicine injection on day 4 while γM is apparently not inhibited may be interpreted to mean that cell division and/or differentiation must occur late in the response for γG production to proceed.

No firm evidence was obtained about whether one cell or two major cell lines were involved in producing γM and γG. The two responses appeared to be independent: delaying the γG response did not affect the rise or subsequent decline of γM, while inhibition of γM did not affect the eventual recovery of γG. Such evidence encouraged but did not prove a conclusion that two cell types were involved.

The histological studies reinforced this hypothesis. At early times in the response of methotrexate-treated animals, γM levels were near normal yet there were no collections of haemocytoblasts in the spleen, and there were only a very few pyroninophilic cells in both the red and white pulp. No conclusion could be made about the cell responsible for the γM production in these experiments. On the other hand, the dramatic increase of haemocytoblasts in treated animals on day 10 was clearly correlated with the concurrent rise of γG in the serum. This evidence might suggest that γM can be produced by a cell not commonly

associated with γG production (other than the haemocytoblast-immature plasma cell - mature plasma cell line). It does not suggest what that cell might be nor does it claim that γM is not produced by these latter cells as well as γG. The γM cell might, of course, transform into one of these pyroninophilic cells, in which case two cell lines would not be involved.

The histological studies were also of interest during the recovery of γG synthesis. They showed that the formation of mature plasma cells, the anatomical grouping of these cells into the classical "aggregates," and the establishment of secondary nodules were not essential to the recovery of the γG response. In the light of suggestions that the secondary nodules are the sites of immunological memory, it would be of interest to see whether these methotrexate treated animals, which recover γG production but do not produce nodules, could be stimulated to produce a typical secondary response.

It has been suggested (Finkelstein and Uhr, 1964) that γM synthesis is transitory during a primary response because of a "feedback" mechanism exerted by the presence of γG antibody. These authors assume that the mechanism of this feedback is the capture of antigen by the γG molecules. Previous studies by these same authors (Uhr and Finkelstein, 1963) and by others (Svehag and Mandel, 1964b) suggested that the duration of γM synthesis was dependent on a persistent supply of antigen. There has been no evidence provided, however, concerning the antigen source or the localization of the hypothetical γG-antigen interaction.

With regard to this feedback theory, two observations seemed pertinent. In the first place, the γM response followed its normal course in those methotrexate experiments where the γG response was delayed. This was not consistent with a theory in which the increase in γG is responsible for inhibiting γM. Secondly, when cells were transferred to X-irradiated recipients on day 6 after injection of antigen the γM synthesis apparently continued for at least 15 more days. The continuation of a γM response in the absence of γG synthesis seems to support a feedback mechanism. The key to a feedback theory, however, is the availability of antigen to the γM-forming cell. Where does this antigen come from in the recipient to stimulate γM for 15 days? The transfer experiment would indicate that the source of the antigen is neither extracellular nor dependent on the maintenance of the original "anatomical continuity between reticuloendothelial cells and lymphoid cells" as suggested by Finkelstein and Uhr (1964). If antigen

is required for continued γM synthesis, it is either in the γM-forming cell itself or one must postulate the re-establishment of the previous intimate cell relationships so that, let us say, macrophages can "feed" antigen to surrounding lymphoid cells. The latter event seems unlikely. Let us assume for a moment that the antigen source is within the γM cell. Where then is the γG-antigen interaction? Is it within the cell or at its surface? Is the interaction toxic to the cell? Why is γM ineffective at inhibition?

The present experiments have suggested that the site of γG-antigen interaction is the γM producing cell and not the reticuloendothelial phagocyte. This suggestion is open to experimental investigation.

During the course of the experiments reported here, a number of studies were made of the secondary response. They have not been reported in this thesis because we could not demonstrate a γM response preceding the large γG response. The studies included, however, experiments on the localization of antigen similar to those experiments conducted during the primary response. The results of these localization experiments are included here so that the effect of circulating antibody on the fate of these bacteria can be seen.

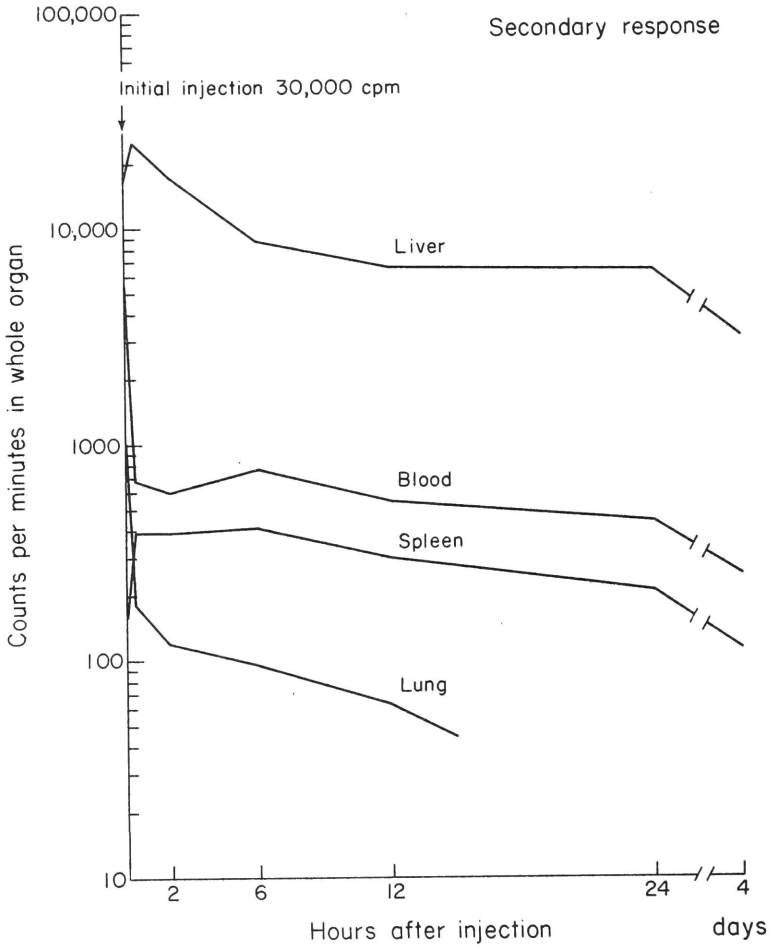


Fig. 29 The localization of C^{14} labeled S. adelaide during the first day after secondary injection.

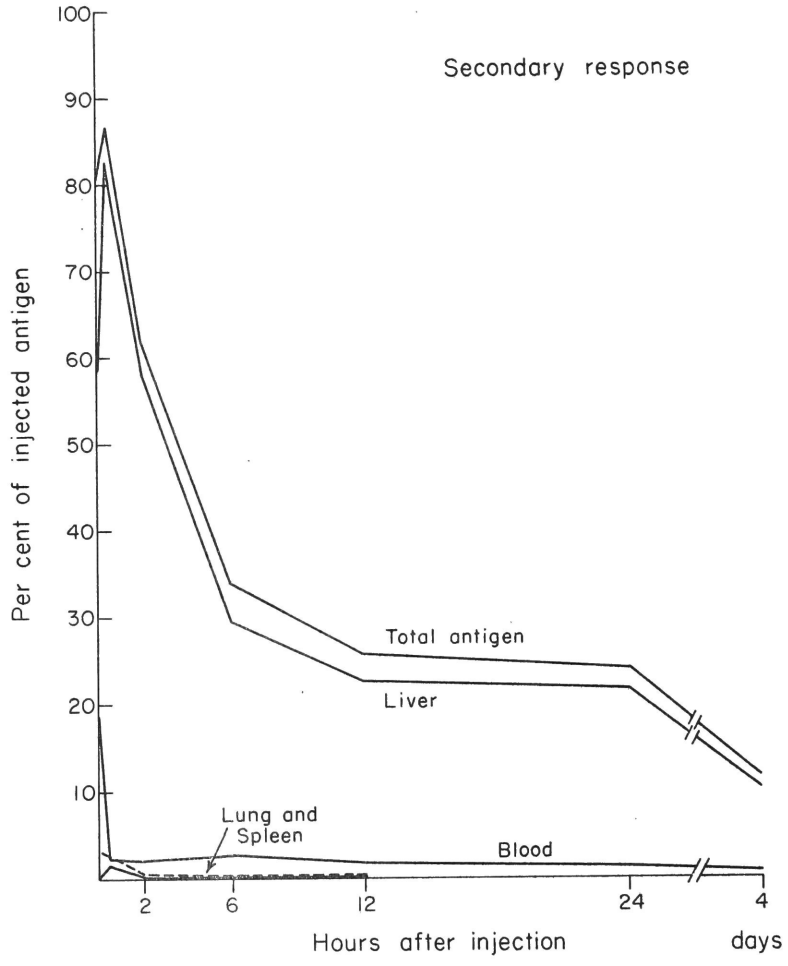


Fig. 30 **The relative percentage of injected antigen recovered in each of the organs and in the blood.**

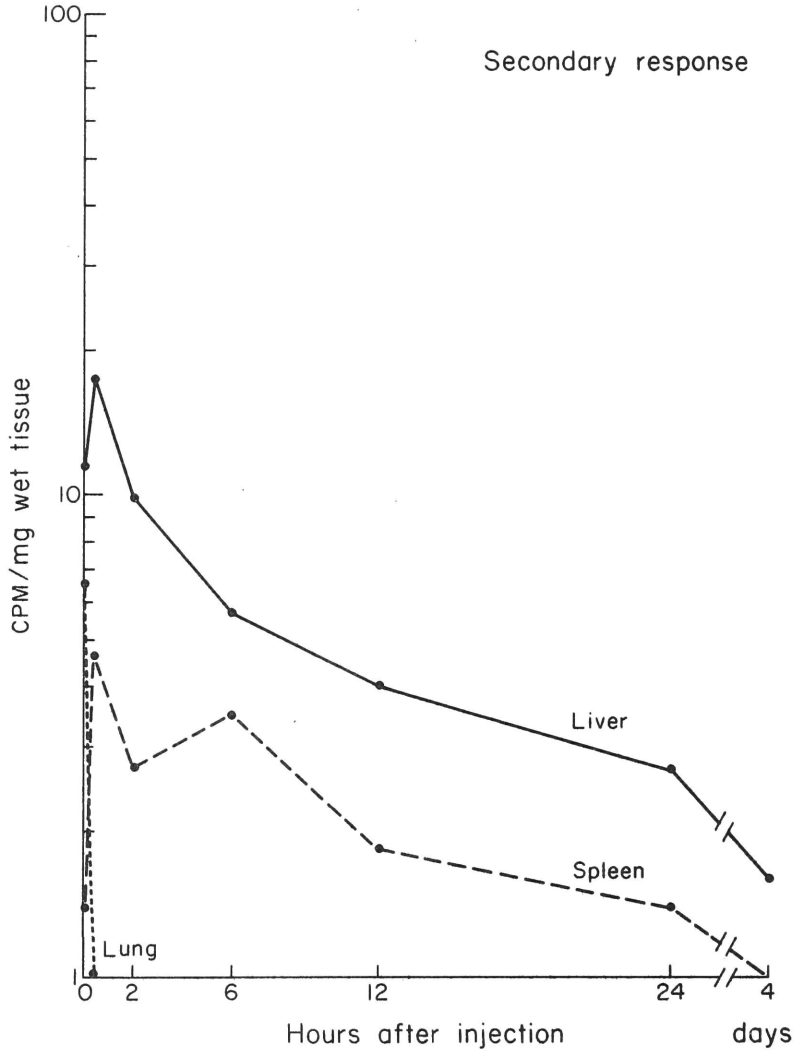


Fig. 31 **The specific radioactivity of organs during the secondary response.**

VI. BIBLIOGRAPHY

1. Ada, G.L., G.J.V. Nossal, J. Pye, and A. Abbot. 1963. Behavior of active bacterial antigens during the induction of the immune response. Part A: The properties of flagellar antigens from Salmonella. Nature. 199:1257.

2. Ashbach, N.E., J. Finstad, J.C. Sarnecki, and B. Pollara. 1964. Phylogeny of immunoglobulins; ultracentrifugal analysis and character of antibody response. Fed. Proc. 23:346.

3. Askonas, B.A. and J.H. Humphrey. 1955. Antibody formation in slices of granulomata produced by adjuvant. Biochem. J. 60:x.

4. Askonas, B.A., and J.H. Humphrey. 1958. Formation of specific antibodies and γ-globulin in vitro. A study of the synthetic ability of various tissues from rabbits immunized by different methods. Biochem. J. 68:252.

5. Askonas, B.A., and R.G. White. 1956. Sites of antibody production in the guinea pig. The relation between in vitro synthesis of anti-ovalbumin and γ-globulin and distribution of antibody-containing plasma cells. Brit. J. Exp. Path. 37:61.

6. Askonas, B.A., J.H. Humphrey, and R.R. Porter. 1956. On the origin of the multiple forms of rabbit γ-globulin. Biochem. J. 63:412.

7. Asofsky, R., and G.J. Thorbecke. 1962. Formation of 7S and 19S globulin in vitro. Fed. Proc. 21:28.

8. Attardi, G., M. Cohn, K. Horibata, and E.S. Lennox. 1959. On the analysis of antibody synthesis at the cellular level. Bact. Rev. 23:213.

9. Bauer, D.C. 1963. Molecular properties of antibodies synthesized in response to simple hatpens. J. Immunol. 91:323.

10. Bauer, D.C., and A.B. Stavitsky. 1961. On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. Proc. Nat. Acad. Sci. 47:1667.

11. Bauer, D.C., M.J. Mathies, and A.B. Stavitsky. 1963. Sequences of synthesis of γ-1 macroglobulins and γ-2 globulin antibodies during primary and secondary responses to proteins, salmonella antigens, and phage. J. Exp. Med. 117:889.

12. Behring, E. von, and S. Kitasato. 1890. Ueber das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. Deutsch. Med. Wschr. 16:1113.

13. Bellanti, J.A., D.V. Eitzman, J.B. Robbins, and R.T. Smith. 1963. The development of the immune response. Studies on the agglutinin response to Salmonella flagellar antigens in the newborn rabbit. J. Exp. Med. 117:479.

14. Bellanti, J.A., S.B. Russ, G.E. Holmes, and E.L. Buescher. 1965. The nature of antibodies following experimental arbovirus infection in guinea pigs. J. Immunol. 94:1.

15. Benacerraf, B., C. Merryman, and R.A. Binaghi. 1964. Studies on the L chains of guinea pig and rat anti-2, 4-dinitrophenyl antibodies. J. Immunol. 93:618.

16. Benacerraf, B., M.M. Sebestyen, and S. Schlossman. 1959. A quantitative study of the kinetics of blood clearance of P^{32} - labeled Escherichia coli and staphlococci by the reticulo-endothelial stystem. J. Exp. Med. 110:27.

17. Benedict, A.A., R.J. Brown, and R. Ayengar. 1962. Physical properties of antibody to bovine serum albumin as demonstrated by hemagglutination. J. Exp. Med. 115:195.

18. Benedict, A.A., R.J. Brown, and R.T. Hersh. 1963a. The temporal synthesis and some chromatographic and ultracentrifugal characteristics of chicken antibodies. J. Immunol. 90:399.

19. Benedict, A.A., R.J. Brown, and R.T. Hersh. 1963b. Inactivation of high and low molecular weight antibodies by mercaptoethanol. Proc. Soc. Exp. Biol. Med. 113:136.

20. Benedict, A.A., R.T. Hersh, and C. Larson. 1963c. The temporal synthesis of chicken antibodies. The effect of salt on the precipitin reaction. J. Immunol. 91:795.

21. Benedict, A.A., C. Larson, and H. Nik-Khah. 1963d. Synthesis of chicken antibodies of high and low molecular weight. Science 139:1302.

22. Berlin, B.S. 1963. The nature of antibodies in mice injected with influenza virus vaccine. Proc. Soc. Exp. Biol. Med. 113:1013.

23. Bernier, G.H., and J.J. Cebra. 1964. Polypeptide chains of human γ-globulin: cellular localization by fluorescent antibody. Science 144:1590.

24. Bjorneboe, M., and H. Gormsen. 1943. Experimental studies on the role of plasma cells as antibody producers. Acta path. et. microbiol. scandinav. 20:649.

25. Bordet, J. 1895. Les leucocytes et les propriétés actives du sérum chez les vacinnés. Ann. Inst. Pasteur. 9:462.

26. Bordet, J. 1898. Sur l'agglutination et la dissolution des globules rouges par le sérum d'animaux injectés de sang défibriné. Ann. Inst. Pasteur. 12:688.

27. Bordet, J., and O. Gengou. 1901. Sur l'existence de substances sensibilisatrices dans la plupart des sérums antimicrobiens. Ann. Inst. Pasteur. 15:289.

28. Boyd, W.C. 1956. Fundamentals of Immunology. Interscience. New York. 3rd edition.

29. Braunsteiner, H., K. Fellinger, and F. Pakesch. 1957. Electron microscopic investigations on sections from lymph nodes and bone marrow in malignant blood diseases. Blood. 12:278.

30. Bridges, R.A., R.M. Condie, S.J. Zak, and R.A. Good. 1959. The morphologic basis of antibody formation development during the neo-natal period. J. Lab. Clin. Med. 53:331.

31. Brown, F. 1960. A β-globulin antibody in the sera of guinea pigs and cattle infected with foot and mouth disease. J. Immunol. 85:298.

32. Bull, C.G. 1915. The fate of typhoid bacilli when injected intravenously into normal rabbits. J. Exp. Med. 22:475.

33. Burtin, P., and D. Buffe. 1963. Immunofluorescent studies of human plasma cells in γ and β_2A myelomas. Proc. Soc. Exp. Biol. Med. 114:171.

34. Campbell, D.H., and J.S. Garvey. 1963. Nature of retained antigen and its role in immune mechanisms. Adv. Immunol. 3:261.

35. Cann, J.R. 1958. The separation and purification of antibodies. In V.A. Najjar, ed., Immunity and Virus Infection. J. Wiley. New York.

36. Cann, J.R., R.A. Brown, and J.G. Kirkwood. 1949. The fractionation of bovine serum proteins by electrophoresis-convection. J. Am. Chem. Soc. 71:1609.

37. Cann, J.R., R.A. Brown, J.G. Kirkwood, P. Sturgeon, and D.W. Clarke. 1952. Studies of antibody distribution in the serum proteins of several Rh antisera. J. Immunol. 68:243.

38. Chan, P., and H.F. Deutsch. 1961. Immunochemical studies of human Rh-agglutinins. J. Immunol. 85:37.

39. Chase, M.W. 1953. Immunological reactions mediated through cells, p. 156. In A.M. Pappenheimer, Jr., ed. The Nature and Significance of the Antibody Response. Col. Univ. Press. New York.

40. Chiappino, G., and B. Pernis. 1964. Demonstration with immunofluorescence of 19S macroglobulins and 7S gamma globulins in different cells of the human spleen. Path. Microbiol. 27:8.

41. Chow, B.F., and H. Wu. 1937. Isolation of immunologically pure antibody from immune precipitate of type I pneumococcus. Chin. J. Physiol. 11:139.

42. Cochrane, C.G., and F.J. Dixon. 1962. Antibody production by transferred cells. Adv. Immunol. 2:205.

43. Cohen, S., and R.R. Porter. 1964. Structure and biological activity of immunoglobulins. Adv. Immunol. 4:287.

44. Cohn, E.J., J.A. Leutscher, J.L. Oncley, S.H. Armstrong, and B.D. Davis. 1940. Preparation and properties of serum and plasma proteins. III. size and charge of proteins separating upon equilibration across membranes with ethanol-water mixtures of controlled pH, ionic strength, and temperature. J. Am. Chem. Soc. 62:3396.

45. Cohn, M., H.F. Deutsch, and L.R. Wetter. 1950. Biophysical studies of blood plasma proteins. XIII. analysis of immunological heterogeneity of human gamma globulin fractions. J. Immunol. 64:381.

46. Cohn, Z.A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labelled bacteria by polymorphonuclear leucocytes and macrophages. J. Exp. Med. 117:27.

47. Colvin, J.R., D.B. Smith, and W.H. Cook. 1954. The microheterogeneity of proteins. Chem. Rev. 54: 687.

48. Coons, A.H., E.H. Leduc, and J.M. Connolly. 1955. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102:49.

49. Cruchard, A., F.S. Rosen, J.M. Craig, C.A. Janeway, and D. Gitlin. 1962. The site of synthesis of the 19S gamma globulins in dysgammaglobulinemia of Waldenström. J. Exp. Med. 115:1141.

50. Curtain, C.C. 1961. Localization of 18S gamma-macroglobulin in plasmacytoid cells of bone marrow in a case of macroglobulinemia. Aust. J. Exp. Biol. Med. Sci. 39:391.

51. Curtain, C.C., and J.F. O'Dea. 1959. Possible sites of macroglobulin synthesis: a study made with fluorescent antibody. Aust. Ann. Med. 8:143.

52. Davidsohn, I., C.L. Lee, O. Panczyszyn, and M. Ingall. 1962. Changes in rabbit serum following repeated injections of mouse erythrocytes. Fed. Proc. 21:29.

53. Davidsohn, I., C.L. Lee, and F. Zandrew. 1964. Antibodies in splenectomized rabbits after repeated injections with mouse erythrocytes. Fed. Proc. 23:190.

54. Denys, J., and J. Leclef. 1895. Sur le mécanisme de l'immunité chez le lapin vaccinè contre le streptocoque pyogène. Cellule. 11:175.

55. Deutsch, H.F., and J.I. Morton. 1957. Dissociation of human serum macroglobulins. Science. 125:600.

56. Deutsch, H.F., R.A. Alberty, L.J. Gosting, and J.W. Williams. 1947. Biophysical studies of blood plasma proteins. VI Immunological properties of γ_1-globulin from plasma of normal humans. J. Immunol. 56:183.

57. Deutsch, L. 1899. Contribution a l'étude de l'origine des anticorps typhiques. Ann. Inst. Pasteur. 13:689.

58. Dobson, E.L., and H.B. Jones. 1951. The behavior of intravenously injected particulate material. Acta Med. Scand. (suppl. 144)

59. Doerr, R., and V.K. Russ. 1909. Studien über Anaphylaxie. III Der anaphylaktische Immunkörper und seine Beziehungen zum Eiweissantigen. Z. Immunitäts forsch. 3:181.

60. Dreesman, G. 1963. University of Kansas. Thesis.

61. Dreesman, G., C. Larson, N. Pinckard, R.M. Groyon, and A.A. Benedict. 1965. Antibody activity in different chicken globulins. Proc. Soc. Exp. Biol. Med. 118:292.

62. Dutcher, T.F., and J.L. Fahey. 1959. The histopathology of the macroglobulinemia of Waldenström. J. Nat. Cancer. Inst. 22:887.

63. Dutcher, T.F., and J.L. Fahey. 1960. Immunocytochemical demonstration of intranuclear localization of 18S gamma macroglobulin in macroglobulinemia of Waldenström. Proc. Soc. Exp. Biol. Med. 103:452.

64. Edelman, G.M., and B. Benacerraf. 1962. On structural and functional relations between antibodies and proteins of the gamma stystem. Proc. Nat. Acad. Sci. 48:1035.

65. Edelman, G.M., and M.D. Poulik. 1961. Studies on structural units of the γ-globulins. J. Exp. Med. 113:861.

66. Edelman, G.M., B. Benacerraf, and Z. Ovary. 1963. Structure and specificity of guinea pig 7S antibodies. J. Exp. Med. 118:229.

67. Edelman, G.M., B. Benacerraf, Z. Ovary, and M.D. Poulik. 1961. Structural differences among antibodies of different specificities. Proc. Nat. Acad. Sci. 47:1751.

68. Edelman, G.M., H.G. Kunkel, and E.C. Franklin. 1958. Interaction of the rheumatoid factor with antigen-antibody complexes and aggregaged gamma globulin. J. Exp. Med. 108:105.

69. Ehrich, W.E., D.L. Drabkin, and C. Forman. 1949. Nucleic acids and the production of antibody by plasma cells. J. Exp. Med. 90:157.

70. Engle, R.L. 1959. quoted by Kunkel (1960), p. 304.

71. Fagraeus, A. 1948a. The plasma cellular reaction and its relation to the formation of antibodies in vitro. J. Immunol. 58:1.

72. Fagraeus, A. 1948b. Antibody production in relation to the development of plasma cells in vivo and in vitro experiments. Acta. Med. Scand. 130 (suppl. 204):1.

73. Fahey, J.L., and J.H. Humphrey. 1962. Antibodies with differing molecular sizes in mice. Immunology. 5:104.

74. Felton, L.D. 1928. Protective substance in antipneumococcus serum; separation of phosphorus and some inert protein from water-insoluble precipitate of antipneumococcus serum. J. Infect. Dis. 42:248.

75. Felton, L.D. 1932. Dissociation of specific protein precipitate of antipneumococcus horse serum and comparison with protein isolated by chemical means from this immune serum. J. Immunol. 22:453.

76. Felton, L.D., and G.H. Bailey. 1926a. The immunological characteristics of a water-insoluble protein in Type I antipneumococcus sera. J. Immunol. 11:197.

77. Felton, L.D., and G.H. Bailey. 1926b. Specific precipitates obtained from antipneumococcus serum and antibody solution by soluble specific substances of pneumococci. J. Infect. Dis. 38:145.

78. Fink, C.W., W.E. Miller, Jr., B. Dorward, and J. LoSpalluto. 1962. The formation of macroglobulin antibodies. II studies in neo-natal infants and older children. J. Clin. Invest. 41:1422.

79. Finkelstein, M.S., and J.W. Uhr. 1964. Specific inhibition of antibody formation by passively administered 19S and 7S antibody. Science. 146:67.

80. Fishman, M. 1961. Antibody formation in vitro. J. Exp. Med. 114:837.

81. Fishman, M., R.A. Hammerstrom, and V.P. Bond. 1963. In vitro transfer of macrophage RNA to lymph node cells. Nature. 198:549.

82. Fonbrune, P. de. 1949. Technique de Micromanipulation. Masson. Paris.

83. Forssman, J. 1922. L'influence de l'éther sur des anticorps. C.R. Soc. Biol. (Paris) 86:495.

84. Foy, H., and A. Kondi. 1964. Reticulo-endothelial cells as 'nurses' for plasma cells and erythroblasts in baboons. Nature. 204:293.

85. Franek, F., V. Jouja, and J. Kostka. 1962. The nature of pig antibodies against Brucella suis. Folia. Microbiol. 7:1.

86. Franklin, E.C. 1962. The structure, function, and significance of the immune globulins. Vox. Sang. 7:1.

87. Franklin, E.C. 1964. The immune globulins - their structure and function and some techniques for their isolation. Prog. Allerg. 8:58.

88. Franklin, E.C., and J. Lowenstein. 1964. Protein abnormalities associated with proliferative disorders of plasma cells and lymphocytes. Seminars Hemat. 1:144.

89. Franzl, R.E. 1962. Immunogenic sub-cellular particles obtained from spleens of antigen-injected mice. Nature. 195:457.

90. Franzl, R.E. 1964. Immunogenic sub-cellular fractions from spleens and livers of antigen-injected mice. Fed. Proc. 23:344.

91. Freter, R. 1957. Agglutinating efficiency and combining capacity of shigella and vibrio antisera from rabbits at different stages of immunization. J. Exp. Med. 105:623.

92. Fudenberg, H.H., H.G. Kunkel, and E.C. Franklin. 1959. High molecular weight antibodies. In L. Holländer, ed. Proc. VII Cong. Int. Soc. Blood Transf. Karger. Basel.

93. Gitlin, D. 1964. Protein metabolism, cell formation, and immunity. Pediatrics. 34:198.

94. Glenchur, H., H.H. Zinneman, and D.R. Briggs. 1958. Macroglobulinemia: report of two cases. A.M.A. Ann. Internal Med. 48:1055.

95. Good, R.A., and B.W. Papermaster. 1964. Ontogeny and phylogeny of adaptive immunity. Adv. Immunol. 4:1.

96. Goodner, K., and F.L. Horsfall, Jr. 1936. Lipids and immunological reactions II. further experiments on the relation of lipids to the type-specific reactions of antipneumococcus sera. J. Immunol. 31:135.

97. Gowans, J.L., B.M. Gesner, and D.D. McGregor. 1961. The immunological activity of lymphocytes. In G.E.W. Wolstenholme and M. O'Connor, eds. Ciba Foundation Study Group No. 10. Biological Activity of the Leucocyte. J.&A. Churchill, Ltd. London.

98. Graves, J.H., K.M. Cowan, and R.J. Trautman. 1964. Characterization of antibodies produced by guinea pigs inoculated with inactivated foot-and-mouth disease antigen. J. Immunol. 92:501.

99. Greenbury, C.L., D.H. Moore, and L.A.C. Nunn. 1963. Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells. Immunology. 6:421.

100. Grey, H.M. 1963a. Phylogeny of the immune response. Studies on some physical, chemical, and serologic characteristics of antibody produced in the turtle. J. Immunol. 91:819.

101. Grey, H.M. 1963b. Production of mercaptoethanol sensitive slowly sedimenting antibody in the duck. Proc. Soc. Exp. Biol. Med. 113:963.

102. Grey, H.M. 1964. Studies on changes in the quality of rabbit-bovine serum albumin antibody following immunization. Immunology. 7:82.

103. Gurevich, A.E., L.M. Gubernieva, and K. Miasoedova. 1961. Comparison of the enzymatic hydrolyzates of nonspecific γ-globulins and antibodies of rabbits. Biokhimiia. 26:413.

104. Gustafsson, B.E., and C.B. Laurell. 1958. Gamma-globulins in germ-free rats. J. Exp. Med. 108:251.

105. Harris, T.N., and S. Harris. 1949. Histochemical changes in lymphocytes during the production of antibodies in lymph nodes of rabbits. J. Exp. Med. 90:169.

106. Hawkins, J.D., and F. Haurowitz. 1961. The recovery of injected antigens from rat spleens. Biochem. J. 80:200.

107. Heidelberger, M., and E.A. Kabat. 1936. Chemical studies on bacterial agglutination; identity of precipitin and agglutinin. J. Exp. Med. 63:737.

108. Heidelberger, M., and K.O. Pedersen. 1937. The molecular weight of antibodies. J. Exp. Med. 65:393.

109. Hektoen, L. 1920. Further observations on the effects of roentgenization and splenectomy on antibody-production. J. Infect. Dis. 27:23.

110. Hill, W.C., Jr., and J.J. Cebra. 1965. Retention of antibody activity by γM-subunits. Fed. Proc. 24:634.

111. Horsfall, F.L., Jr., K. Goodner, C.M. MacLeod, and A.H. Harris. 1937. Antipneumococcus rabbit serum as therapeutic agent in lobar pneumonia. J.A.M.A. 108:1483.

112. Humphrey, J.H., and R.R. Porter. 1956. An investigation on rabbit antibodies by the use of partition chromatography. Biochem. J. 62:93.

113. Ishizaka, K., T. Ishizaka, and T. Sugahara. 1961. Molecular basis of passive sensitization I. role of disulfide linkages in γ-globulin molecule. J. Immunol. 87:548.

114. Jacobson, L.O., and M.J. Robson. 1952. Factors affecting X-ray inhibition of antibody formation. J. Lab. Clin. Med. 39:169.

115. Jacobson, L.O., M.J. Robson, and E.K. Marks. 1950. The effect of X-irradiation on antibody formation. Proc. Soc. Exp. Biol. Med. 75:145.

116. Jaffé, R.M. 1931. The reticulo-endothelial system in immunity. Physiol. Rev. 11:277.

117. Jager, G.V., E.L. Smith, M. Nickerson, and D.M. Brown. 1948. Immunological and electrophoretic studies on human γ-globulins. J. Biol. Chem. 176:1177.

118. Jerne, N.K., A.A. Nordin, and C. Henry. 1963. The agar plate technique for recognizing antibody producing cells. In B. Amos and H. Koprowski, eds. Cell Bound Antibodies. Wistar Institute Press. Philadelphia.

119. Kabat, E.A. 1939. The molecular weight of antibodies. J. Exp. Med. 69:103.

120. Kaplan, M.H., A.H. Coons, and H.W. Deane. 1950. Localization of antigen in tissue cells. III. cellular distribution of pneumococcal polysaccharides types II and III in the mouse. J. Exp. Med. 91:15.

121. Karush, F. 1959. Quantitative measurement of heterogeneity among antibodies. In J.H. Shaffer, G.A. LoGrippo, and M.W. Chase, eds. Mechanisms of Hypersensitivity. Little, Brown, and Co. Boston.

122. Kekwick, R.A., and B.R. Record. 1941. Some physical properties of diphtheria antitoxic horse sera. Brit. J. Exp. Path. 22:29.

123. Kerby, G.P., B.C. Holland, and S.P. Martin. 1950. The quantitative estimation of the removal of bacteria from the blood by the various organs of the immunized animals. J. Immunol. 64:123.

124. Kim, Y.B., S.G. Bradley, and D.W. Watson. 1964a. Characterization of early 19S and late 7S immunoglobulins in the mouse. J. Immunol. 93:798.

125. Kim, Y.B., S.G. Bradley, and D.W. Watson. 1964b. Development of immunoglobulins in germfree and conventional colostrum-deprived piglets. Fed. Proc. 23:346.

126. Kong, Y.M., and A.G. Johnson. 1963. Factors affecting primary and secondary antibody production by splenic tissues. J. Immunol. 90:672.

127. Kraus, R. 1897. Ueber specifische Reactionen in keimfreien Filtraten aus Cholera, Typhus und Pestbouillonculturen, erzeugt durch homologes Serum. Wien. Klin. Wschr. 10:736.

128. Krumbhaar, E.B. 1926. Functions of the spleen. Physiol. Rev. 6:160.

129. Kuhns, W.J. 1954. Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid; differences between human precipitating and non-precipitating skin-sensitizing diphtheria antitoxin as shown by electrophoresis. J. Exp. Med. 99:577.

130. Kunkel, H.G. 1960. Macroglobulins and high molecular weight antibodies. In F.W. Putnam, ed. The Plasma Proteins. Vol. I. Academic Press. New York.

131. Kunkel, H.G., and R.J. Slater. 1952. Zone electrophoresis in a starch supporting medium. Proc. Soc. Exp. Biol. Med. 80:42.

132. Kunkel, H.G., M. Mannik, and R.G. Williams. 1963. Individual antigenic specificity of isolated antibodies. Science. 140:1218.

133. Kunkel, H.G., J.H. Rockey, and T. Tomasi. 1961. Methods of separation and properties of antibodies of high molecular weight. In M. Heidelberger and O.J. Plescia, eds. Immunochemical Approaches to Problems in Microbiology. Rutgers University Press. New Brunswick.

134. Kurnick, N.B. 1955. Pyronin Y in the methyl-green-pyronin histological stain. Stain Technology 30:213.

135. Landsteiner, K. 1945. The Specificity of Serological Reactions. 2nd. ed. Harvard University Press. Cambridge.

136. Landsteiner, K., and J. van der Scheer. 1938. On cross reactions of immune sera to azoproteins; antigens with azocomponents containing 2 determinant groups. J. Exp. Med. 67:709.

137. Langevoort, H.L., R.M. Asofsky, E.B. Jacobson, T. de Vries, and G.J. Thorbecke. 1963. γ-globulin and antibody formation in vitro. II. parallel observations on histologic changes and on antibody formation in the white and red pulp of the rabbit spleen during the primary response, with special reference to the effect of endotoxin. J. Immunol. 90:60.

138. LaVia, M.F., S.A. Uriu, and L.A. Ferguson. 1960. Studies on antibody production by spleen explants maintained in vitro. J. Immunol. 84:48.

139. Lederberg, J. 1956. Linear inheritance in transductional clones. Genetics. 41:845.

140. Lee, C.L., T. Takahashi, and I. Davidsohn. 1960. Electrophoretic separation of certain in vitro and in vivo reactions of rabbit anti-mouse erythrocyte serum. Nature. 187:157.

141. LoSpalluto, J., W. Miller, Jr., B. Dorward, and C.W. Fink. 1962. The formation of macroglobulin antibodies. I. studies on adult humans. J. Clin. Invest. 41:1415.

142. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265.

143. Luckhardt, A.B., and F.C. Becht. 1911. The relation of the spleen to the fixation of antigens and the production of immune bodies. Am. J. Physiol. 28:257.

144. McCormick, J.N. 1963. An immunofluorescence study of rheumatoid factor. Ann. Rheum. Dis. 22:1.

145. Makela, O., and G.J.V. Nossal. 1961. Study of antibody-producing capacity of single cells by bacterial adherence and immobilization. J. Immunol. 87:457.

146. Makinodan, T., M.A. Kastenbaum, and W.J. Peterson. 1962. Radiosensitivity of spleen cells from normal and pre-immunized mice and its significance to intact animals. J. Immunol. 88:31.

147. Makinodan, T., R.F. Ruth, and H.R. Wolfe. 1954. Precipitin production in chickens. X. cellular changes in the spleen during antibody production. J. Immunol. 72:39.

148. Marshall, A.H.E., and R.G. White. 1950. Reactions of the reticular tissues to antigens. Brit. J. Exp. Path. 31:157.

149. Mellors, R.C., and L. Korngold. 1963. The cellular origin of human immunoglobulins (γ_2, γ_{1M}, γ_A). J. Exp. Med. 118:387.

150. Mellors, R.C., R. Heimer, J. Corcos, and L. Korngold. 1959. Cellular origin of rheumatoid factor. J. Exp. Med. 110:875.

151. Mellors, R.C., A. Nowoslawski, and L. Korngold. 1961. Rheumatoid arthritis and the cellular origin of the rheumatoid factors. Am. J. Path. 39:533.

152. Michael, J.G., and F.S. Rosen. 1963. Association of "natural" antibodies to gram-negative bacteria with the γ1-macroglobulins. J. Exp. Med. 118:619.

153. Miller, J.J. III, and G.J.V. Nossal. 1964. Antigens in immunity. VI. the phagocytic reticulum of lymph node follicles. J. Exp. Med. 120:1075.

154. Motohashi, S. 1922. The effect of splenectomy upon the production of antibodies. J. Med. Research. 43:473.

155. Müller-Eberhard, H.J. 1960. A new supporting medium for preperative electrophoresis. Scand. J. Clin. Lab. Invest. 12:33.

156. Norton, S., H.R. Wolfe, and J.F. Crow. 1950. Effect of injections of soluble antigen on the spleen size and antibody production in chickens. Anat. Rec. 107:133.

157. Nossal, G.J.V. 1958. Antibody production by single cells. Brit. J. Exp. Path. 39:544.

158. Nossal, G.J.V., A. Szenberg, G.L. Ada, and C.M. Austin. 1964. Single cell studies on 19S antibody production. J. Exp. Med. 119:485.

159. Onoue, K., Y. Yagi, P. Stelos, and D. Pressman. 1964. Antigen-binding activity of 6S subunits of β_2 macroglobulin antibody. Science. 146:404.

160. Ortega, L.G., and R.C. Mellors. 1957. Cellular sites of formation of gamma-globulin. J. Exp. Med. 106:627.

161. Pappenheimer, A.M., H.P. Lundgren, and J.W. Williams. 1940. Studies on the molecular weight of diphtheria toxins, antitoxins, and their reaction products. J. Exp. Med. 71:247.

162. Pfeiffer, R. 1894. Weitere Untersuchungen über das Wesen der Choleraimmunität und über specifisch baktericide Processe. Z. Hyg. Infektionskr. 18:1.

163. Pfeiffer, R., and E. Friedberger. 1903. Weitere Beiträge zur Theorie der bacteriolytischen Immunität. Zbl. Bakt. Abt. I. Orig. 34:70.

164. Pfeiffer, R., and D. Marx. 1898. Untersuchungen über die Bildungsstätte der Choleraantikörper. Deut. Med. Wschr. 24:47.

165. Pike, R.M., and M.L. Schulze. 1964. Production of 7S and 19S antibodies to the somatic antigens of Salmonella typhosa in rabbits. Proc. Soc. Exp. Biol. Med. 115:829.

166. Porter, K.A., and E.H. Cooper. 1962. Transformation of adult allogeneic small lymphocytes after transfusion into newborn rats. J. Exp. Med. 115:997.

167. Porter, R.R. 1955. The fractionation of rabbit γ-globulin by partition chromatography. Biochem. J. 59:405.

168. Race, R.R. and R. Sanger. 1954. Blood Groups In Man. 2nd. ed. Blackwell Scientific Publications. Oxford.

169. Rath, D. 1899. Ueber den Einfluss der blutbildenden Organe auf die Enstehung der Agglutinine. Zbl. Bakt. Abt. I. Orig. 25:549.

170. Rawson, A.J., N.M. Abelson, and D.J. McCarty. 1961. The isohemagglutinin partition in rheumatoid arthritis. Arthritis Rheum. 4:463.

171. Robbins, J. 1965. In Molecular and Cellular Basis of Antibody Formation. Czechoslovak Academy of Science. Prague. In Press.

172. Robbins, J., and R.T. Smith. 1964. The effect of X-ray irradiation upon the sequence of immune globulins following initial immunization in the rabbit. J. Immunol. 93:1045.

173. Roberts, A.N., and F. Haurowitz. 1962. Intracellular localization and quantitation of tritiated antigens in reticuloendothelial tissues of mice during secondary and hyperimmune responses. J. Exp. Med. 116:407.

174. Rockey, J.H., and H.G. Kunkel. 1962. Unusual sedimentation and sulfhydryl sensitivity of certain isohemagglutinins and skin sensitizing antibody. Proc. Soc. Exp. Biol. Med. 110:101.

175. Rosen, F.S. 1962. The macroglobulins. New Eng. J. Med. 267:491,546.

176. Rowley, D., and K.J. Turner. 1964. Increase in macroglobulin antibodies of mouse and pig following injection of bacterial lipopolysaccharide. Immunology. 7:394.

177. Rowley, D.A. 1950a. The effect of splenectomy on the formation of circulating antibody in the adult male albino rat. J. Immunol. 64:289.

178. Rowley, D.A. 1950b. The formation of circulating antibody in the splenectomized human being following intravenous injection of heterologous erythrocytes. J. Immunol. 65:515.

179. Schlossman, S.F., and E.A. Kabat. 1962. Specific fractionation of a population of anti-dextran molecules with combining sites of various sizes. J. Exp. Med. 116:535.

180. Schoenberg, M.D., V.R. Mumaw, R.D. Moore, and A.S. Weisberger. 1964a. Cytoplasmic interaction between macrophages and lymphocyte cells in antibody synthesis. Science. 143:964.

181. Schoenberg, M.D., J.C. Rupp, and R.D. Moore. 1964b. The cellular response of the spleen and its relationship to the circulating 19S and 7S antibody in the antigenically stimulated rabbit. Brit. J. Exp. Path. 45:111.

182. Schrohenloher, R.E., H.G. Kunkel, and T. B. Tomasi. 1964. Activity of dissociated and reassociated 19S anti-γ-globulins. J. Exp. Med. 120:1215.

183. Schur, P.H., and G.D. Christian. 1964. The role of disulfide bonds in the complement fixing and precipitating properties of 7S rabbit and sheep antibodies. J. Exp. Med. 120:531.

184. Shulman, S., L. Hubler, and E. Witebsky. 1964. Antibody response to immunization by different routes. Science. 145:815.

185. Silverstein, A.M., J.W. Uhr, K.L.Kraner, and R.J. Lukes. 1963. Fetal response to antigenic stimulus. II. antibody production by the fetal lamb. J. Exp. Med. 117:799.

186. Smith, E.L., and B.V. Jager. 1952. The characterization of antibodies. Ann. Rev. Microbiol. 6:207.

187. Smith, R.T. 1960. Response to active immunization of human infants during the neo-natal period. In G.E.W. Wolstenholme and M. O'Conner, eds. Ciba Foundation Symposium. Cellular Aspects of Immunity. Churchill Ltd. London.

188. Solomon, A., J.L. Fahey, and R.A. Malmgren. 1963. Immunohistological localization of γ1 macroglobulins, β2A myeloma proteins, 6.6S myeloma proteins and Bence-Jones proteins. Blood. 21:403.

189. Stavitsky, A.B. 1961. In vitro studies of the antibody response. Adv. Immunol. 1:211.

190. Stelos, P. 1958. Comparative study of rabbit hemolysins to various antigens. I. hemolysins to beef red cells. J. Infect. Dis. 102:103.

191. Stelos, P., and W.H. Taliaferro. 1959. Comparative study of rabbit hemolysins to various antigens. II. hemolysins to Forssman antigen of guinea pig kidney, human type A red cells and sheep red cells. J. Infect. Dis. 104:105.

192. Stelos, P., and D.W. Talmage. 1957. The separation by starch electrophoresis of two antibodies to sheep red cells differing in hemolytic efficiency. J. Infect. Dis. 100:126.

193. Stelos, P., L.G. Taliaferro, and P.A. D'Alesandro. 1961. Comparative study of rabbit hemolysins to various antigens. III. Chromatographic analysis of Forssman hemolysins induced by various antigens. J. Infect. Dis. 108:113.

194. Stoner, R.D., and V.P. Bond. 1963. Antibody formation by transplanted bone marrow, spleen, lymph nodes, and thymus cells in irradiated recipients. J. Immunol. 91:185.

195. Sullivan, F.L., E.F. Nekerman, and P.R. Cannon. 1934. The localization and fate of bacteria in the tissues. J. Immunol. 26:49.

196. Süssdorf, D.H., and L.R. Draper. 1956. The primary hemolysin response in rabbits following shielding from X-rays or X-irradiation of the spleen, appendix, liver or hind legs. J. Infect. Dis. 99:129.

197. Svehag, S.-E. 1964a. The formation and properties of poliovirus-neutralizing antibody. III. sequential changes in electrophoretic mobility of 19S and 7S antibodies synthesized by rabbits after a single virus injection. J. Exp. Med. 119:225.

198. Svehag, S.-E. 1964b. The formation and properties of poliovirus-neutralizing antibody. IV. normal antibody and early immune antibody of rabbit origin: a comparison of biological and physicochemical properties. J. Exp. Med. 119:517.

199. Svehag, S.-E., and B. Mandel. 1964a. The formation and properties of poliovirus neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction. J. Exp. Med. 119:1.

200. Svehag, S.-E., and B. Mandel. 1964b. The formation and properties of poliovirus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis, and sensitivity to X-irradaition. J. Exp. Med. 119:21.

201. Taliaferro, W.H., and L.G Taliaferro. 1950. The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Dis. 87:37.

202. Taliaferro, W.H., and L.G. Taliaferro. 1951. The role of the spleen in hemolysin production in rabbits receiving multiple antigen injections. J. Infect. Dis. 89:143.

203. Taliaferro, W.H., and L.G. Taliaferro. 1952. The role of the spleen and the dynamics of hemolysin production in homologous anamnesis. J. Infect. Dis. 90:205.

204. Taliaferro, W.H., and L.G. Taliaferro. 1956. X-ray effects on hemolysin formation in rabbits with the spleen shielded or irradiated. J. Infect. Dis. 99:109.

205. Taliaferro, W.H., and D.W. Talmage. 1956. Antibodies in the rabbit with different rates of metabolic decay. J. Infect. Dis. 99:21.

206. Taliaferro, W.H., L.G. Taliaferro, and B.N. Jaroslow. 1964. Radiation and Immune Mechanisms. Academic Press. New York.

207. Talmage, D.W. 1959. Immunological specificity. Science. 129:1643.

208. Talmage, D.W., G.G. Freter, and W.H. Taliaferro. 1956a. The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums. J. Infect. Dis. 98:293.

209. Talmage, D.W., G.G. Freter, and W.H. Taliaferro. 1956b. Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation. J. Infect. Dis. 98:300.

210. Thorbecke, G.J., and E.C. Franklin. 1961. Antigenic cross-reactivity between 7S and 19S rabbit γ-globulin. J. Immunol. 87:753.

211. Thorbecke, G.J., and F.J. Keuning, 1953. Antibody formation in vitro by haemopoietic organs after suncutaneous and intravenous immunization. J. Immunol. 70:129.

212. Thorbecke, G.J., and F.J. Keuning. 1956. Antibody and gamma globulin formation in vitro in hemopoietic organs. J. Infect. Dis. 98:157.

213. Thorbecke, G.J., R.M. Asofsky, G.M. Hochwald, and G.W. Siskind. 1962. Gamma globulin and antibody formation in vitro. III. induction of secondary response at different intervals after the primary; the role of secondary nodules in the preparation for the secondary response. J. Exp. Med. 116:295.

214. Tiselius, A., and E.A. Kabat. 1939. An electrophoretic study of immune sera and purified antibody preparations. J. Exp. Med. 69:119.

215. Tizzoni, G., and G. Cattani. 1892. Ueber die Wichtigkeit der Milz bei der experimentellen Immunisirung des Kaninchens gegen den Tetanus. Zbl. Bakt. Abt. I. Orig. 11:325.

216. Tomasi, T.B., Jr. 1965. Human gamma globulin. Blood. 25:382.

217. Topley, W.W.C. 1930. The role of the spleen in the production of antibodies. J. Path. Bact. 33:339.

218. Turk, J.L., and S.H. Stone. 1963. Implications of the cellular changes in lymph nodes during the development and inhibition of delayed type hypersensitivity. In B. Amos and H. Koprowski, eds. Cell Bound Antibodies. Wistar Institute Press. Philadelphia.

219. Uhr, J.W. 1964. The heterogeneity of the immune response. Science. 145:457.

220. Uhr, J.W., and M.S. Finkelstein. 1963. Antibody formation. IV. formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ØX 174. J. Exp. Med. 117:457.

221. Uhr, J.W., J. Dancis, E.C. Franklin, M.S. Finkelstein, and E.W. Lewis. 1962a. The antibody response to bacteriophage ØX 174 in newborn premature infants. J. Clin. Invest. 41:1509.

222. Uhr, J.W., M.S. Finkelstein, and E.C. Franklin. 1962b. Antibody response to ØX 174 in non-mammalian vertebrates. Proc. Soc. Exp. Biol. Med. 111:13.

223. Van der Scheer, J., J.B. Lagsdin, and R.W.G. Wyckoff. 1941. Electrophoretic and ultracentrifugal analysis of antipneumococcal horse sera. J. Immunol. 41:209.

224. Van der Scheer, J., R.W.G. Wyckoff, and F.H. Clark. 1940. The electrophoretic analysis of several hyperimmune horse sera. J. Immunol. 39:65.

225. Vaughan, J.H., A.H. Dutton, R.W. Dutton, M. George, and R. Q. Marston. 1960. A study of antibody production in vitro. J. Immunol. 84:258.

226. Waldenström, J. 1958. Die Makroglobulinämie. Ergebn. Inn. Med. Kinderheilk. 9:586.

227. Wasserman, A. 1898. Weitere Mittheilungen über "Leitenketten-Immunität". Berlin Klin. Wschr. 35:209.

228. Weidanz, W.P., A.L. Jackson, and M. Landy. 1964. Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. Proc. Soc. Exp. Biol. Med. 116:832.

229. Weinrach, R.S., and D.W. Talmage. 1958. The role of antibody in immune hemolysis. J. Infect. Dis. 102:74.

230. White, R.G. 1963. Functional recognition of immunologically competent cells by means of the fluorescent antibody technique. In G.E.W. Wolstenholme ed. Ciba Foundation Symposium. The Immunologically Competent Cell. Little, Brown, and Co. Boston.

231. Wiedermann, G., P.A. Miescher, and E.C. Franklin. 1963. Effect of mercaptoethanol on complement binding activity of human 7S gamma globulin. Proc. Soc. Exp. Biol. Med. 113:609.

232. Williams, J.W., R.L. Baldwin, W.M. Saunders, and P.G. Squire. 1952. Boundary spreading in sedimentation velocity experiments. I. the enzymatic degradation of serum globulins. J. Am. Chem. Soc. 74:1542.

233. Wissler, R.W., M.J. Robson, F.W. Fitch, W. Nelson and L.O.Jacobson. 1953. The effects of spleen shielding and subsequent splenectomy upon antibody formation in rats receiving total-body X-irradiation. J. Immunol. 70:379.

234. Wolfe, H.R., S. Norton, E. Springer, M. Goodman, and C.A. Herrick. 1950. Precipitin production in chickens. V. the effect of splenectomy on antibody formation. J. Immunol. 64:179.

235. Wust, C.J., and G.D. Novelli. 1962. Organ weight changes and protein synthesis in mice after immunization to the enzyme triose phosphate dehydrogenase. J. Immunol. 89:285.

236. Zinsser, H. 1931. Resistance To Infectious Diseases. Macmillan. New York.

237. Zucker-Franklin, D., E.C. Franklin, and N.S. Cooper. 1962. Production of macroglobulins *in vitro* and a study of their cellular origin. Blood. 20:56.

End