DETECTION OF CELLS PRODUCING ANTI-HAPTEN ANTIBODIES

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A technique based on the hemolytic plaque assay has been developed for the detection of cells producing anti-dinitrophenyl antibody. It was found that red cells coupled to protein-dinitrophenyl conjugates by the bis-diazotized benzidine method were unsatisfactory for use in this assay. Indicator red cells could be satisfactorily prepared by a direct reaction between red cells and fluorodinitrobenzene provided that protein was included in the buffer solutions used. Various parameters of the reaction between red cells and fluorodinitrobenzene were investigated and optimum reaction conditions were established. The specificity of the assay was shown by the inhibitory effect of free ε-DNP-L-lysine.

The induction of hemolysin production by RNA extracted from immune tissue was repeated as in the literature. Studies were performed with inbred and outbred animals as well as with degraded and nondegraded RNA.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

GENERAL INTRODUCTION

An animal which has survived a bacterial infection is generally less susceptible to subsequent infection by the same bacteria. Resistance is mediated by serum proteins which are capable of reacting specifically with either the infectious organism or certain of its toxic products. Such reaction results in the loss of pathogenicity or toxicity. The pathogen and its products are referred to as antigens; the serum proteins capable of specific reaction with these foreign substances are called antibodies. Recently it has been suggested that those substances which can elicit the formation of specific antibody be called immunogens, while those which react *in vitro* with specific antibody be designated antigens (1). Should antibody combine with antigen other than the immunogen which elicited its formation *in vivo*, it is said to cross-react with that antigen. The mechanism by which an immunogen elicits the production of antibody which reacts specifically with it (homologous antibody) is a central problem in immunology.

The Nature of Immunogens

It was discovered very early that not only infectious organisms but also their extracts could induce the formation of specific antibodies (2). Furthermore, innocuous materials such as serum proteins and erythrocytes were also immunogenic. It now appears that proteins (3), polysaccharides (4), lipids (5), and even nucleic acids (6) are all immunogenic if properly presented to the animal. A minimal molecular weight of the order of 5000 is thought to be necessary for immunogenicity (1) although synthetic polypeptides with molecular weight of about 1500 have been reported to be immunogenic (7). Immunogens are foreign to the immune mechanism of the animal being immunized;

self-antigens not normally available to the circulation or self-antigens which have undergone slight chemical or physical modifications can be immunogenic just as truly foreign antigens usually are. Another criterion for immunogenicity is the presence of chemical groups which confer rigidity to certain areas of the molecule (8).

The first serious investigation of the chemical nature of immunogens and antigens was the classic work of Landsteiner (9). He showed that small, chemically well-defined molecules, by themselves nonimmunogenic, acquired immunogenicity when coupled to large protein molecules. Such molecules were termed haptens. It became apparent that although large biopolymers were required for immunogenicity, antigenic specificity was determined by small portions of these large molecules. These regions responsible for antigenic specificity are called antigenic determinants. Certain definitive work has given an estimate of the size of an antigenic determinant and, by inference, of the size of the antibody-combining site (i.e., that part of the antibody which reacts specifically with antigen); the use of 1,6-linked oligosaccharide polymers of varying lengths to inhibit dextran-antidextran* reaction revealed that the hexasaccharide gave optimal inhibition (10). It has been shown that purified anti-dextran antibody is really heterogeneous with respect to the size of the antibody-combining site; antidextran antibody may be purified into one component which combines more efficiently with trisaccharides and a second component which combines more efficiently with hexasaccharides (11). It is, therefore, inferred that the size of antigenic determinants may vary within a certain range. In any case, the size of the antigenic determinant is certainly very small relative to the size of the immunogen. An immunogen may, therefore, consist of many antigenic determinants. The phenomenon of cross-reaction has been partially explained on this basis. If two molecules possess a large number of antigenic determinants, it is possible that, either on the basis of

*In this text, for the sake of brevity, the antibody specificity has been used to denote the specific antibody (e.g. "anti-dextran" for "anti-dextran antibody").

primary structure or of steric conformation, they may have certain determinants in common.

The more foreign a molecule is to the host animal, the more immunogenic it will be in that animal. Thus the proteins of one species will usually be immunogenic when injected into another species. The degree of immunogenicity is a reflection of species differences; in general, the further removed two species are, the more immunogenic will be their homologous proteins (12,13). Proteins from individuals of the same species, i.e., allotypic proteins, are more similar in structure and, therefore, less immunogenic to members of that species. Individual differences (isogenic differences) between the proteins of an inbred strain are still less frequent.

The failure to recognize foreign material as immunogenic is the phenomenon of immunologic tolerance. Tolerance exhibits the same degree of specificity as does antibody formation. That an individual does not produce antibody against his own tissue antigens, which are immunogenic in other individuals, is an aspect of tolerance. The autoimmune diseases result from the breakdown of tolerance with the formation of autoantibodies which react specifically with the individual's own antigens (14). It is obvious that in addition to its protective function, the immune mechanism and concurrent tolerance to autoantigens is necessary for maintaining the integrity of the individual. This in fact may be its primary function.

The Nature of Antibodies*

Antibodies are γ-globulins with physical properties similar to those of normal serum globulins (15). The use of more refined techniques has resolved

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* The nomenclature to be used is that outlined in "Bull. World Health Org. 30, 447 (1964)".

antibodies into three main classes, all of which have basically the same structure and are capable of combining with antigen (16). Recent investigations have suggested the presence of two minor globulin fractions (17,18). These molecules are collectively referred to as immunoglobulins (Ig) (16). The properties of the various immunoglobulin classes are summarized in Table I.

Investigations into the structure of immunoglobulins began with attempts to determine the number of polypeptide chains which constituted the molecule. It was shown that on reduction of IgG of several species in the presence of urea the molecular weight of the molecule dropped from 150,000 to 50,000 (19). This suggested the presence of several peptide chains in the intact molecule. The results of enzymatic degradation and further reduction studies led to the postulation of a model of the basic unit of immunoglobulins (20). A representation of this model and the various sites of enzymatic cleavage are shown in Figure 1; the caption indicates regions of IgG immunoglobulins.

Immunoglobulins themselves possess antigenic determinants. Certain of these determinants have been found to be common to the three main classes of immunoglobulins while others are specific for a given class. The common determinants are localized on the light (L) chains (21) and correspond to two distinct antigenic types (22) which are carried on separate molecules (22,23). About 60% of each of the globulin classes is characterized by kappa type L chains, while 30% is characterized by lambda type L chains (24). It has been shown that a single antibody molecule is never hybrid with respect to L chain type; it will contain either two kappa or two lambda L chains (25). The class specific antigens are found on the H chains (21).

Difficulties in determination of the amino acid sequence of immunoglobulins are twofold. Primarily, it has thus far been impossible to isolate a pure, mono-specific antibody fraction and there is no evidence that such a fraction exists. Immunoglobulins are extremely heterogeneous with respect to physical

TABLE I

PROPERTIES AND NOMENCLATURE OF MAJOR IMMUNOGLOBULIN CLASSES*‡

Class	Former Nomenclature	Serum Concentration gm/100 ml	Electrophoretic Mobility pH 8.6 (-10^{-5} cm^2 V^{-1} sec^{-1})	Sedimentation Coefficient (S_{20}, W)	Range of Salting Out $(NH_4)_2SO_4$ pH 7
IgG or γG	γ_2, γ_{8S}, 7Sγ or 6.6Sγ	0.8 to 1.5	-0.6 to + 3.0	6 to 7	1.2 to 1.8 M
IgM or γM	γ_1M, β_2M, 19Sγ	0.05 to 0. 10	about +2	18 to 20	1.1 to 1.6 M
IgA or γA	γ_1A, β_2A	0.05 to 0.20	+1.2 to +3.6	7(80-85%) 10 to 13(15-20%)	1.3 to 1.8 M

*from (1) Cohen, S., in "Immunological Diseases" (Ed. Samter, M.), Little, Brown and Company, Boston (1965).

(2) Fleischman, J.B., Ann. Rev. Biochem. 35, 835 (1966).

‡The immunoglobulin classes IgD and IgE are present in extremely low concentrations and, therefore, are less completely characterized.

FIGURE 1: A model of IgG structure* based on the Porter model.

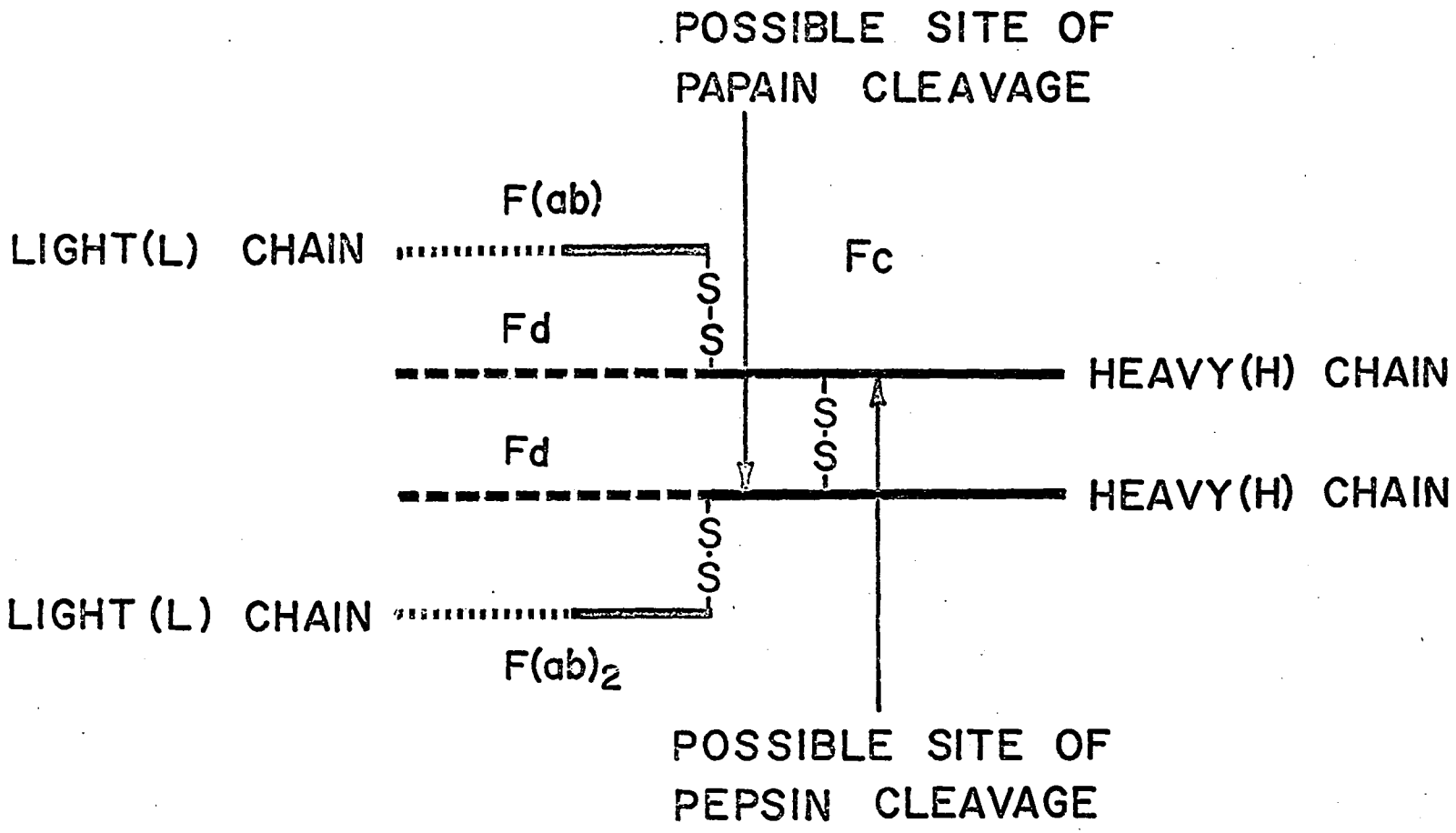
F_{ab} fragment - L chain plus F_d fragment of H chain.

F_c fragment - solid line portions of H chains bound by disulfide bond.

solid line portion of L chain - constant (for given species and antigenic type) region

dashed portion of L chain - variable region

*S. Cohen, Proc. Roy. Soc. B166, 114 (1966).


POSSIBLE SITE OF
PAPAIN CLEAVAGE
F(ab)
LIGHT(L) CHAIN
Fc
S
S
Fd
HEAVY(H) CHAIN
S
S
Fd
HEAVY(H) CHAIN
S
S
LIGHT (L) CHAIN
F(ab)2
POSSIBLE SITE OF
PEPSIN CLEAVAGE

properties (26); antibodies with a single combining specificity exhibit heterogeneity both in size (11) and antigen affinity of the combining site (27). It has been suggested that antibodies with the same primary structure may exist as conformational isomers (28). The problem of heterogeneity of immunoglobulins has for the purpose of structural analyses been circumvented by the uses of myeloma proteins which are structurally analagous to known antibodies. Antibody activity has recently been demonstrated in a myeloma protein for the first time (29). These proteins are the products of plasmacytomas and, due to the clonal nature of such tumours, are relatively homogeneous. Certain myelomas appear to produce an excess of light chains. These homogeneous light chains appear free in the body fluids and are then called Bence Jones proteins (14). Secondly, the size of the immunoglobulin molecules complicates the determination of primary structure.

Recently the primary sequences for several myeloma and purified globulins have been determined. Analysis of the light chains produced by various myelomas indicates that for a given antigenic type the carboxy-terminal half of the molecule is constant, while the amino-terminal half is variable for a given species (3). The degree of homology between the variable portions of mouse and human kappa type L chain has been shown to be greater than the homology between variable portions of two mouse kappa chains (31). Cysteine is the carboxy-terminal amino acid in mouse and human kappa chains and is coupled to the carboxy-terminal amino acid in lambda chains (32). These residues might have been retained due to selective pressure (33).

Studies on the structure of H chains have indicated that about 55% of the Fc fragment is constant to all antibodies (34). A surprising degree of homology has been observed between the carboxy-terminal half of Bence Jones proteins and the Fc fragment. The evolutionary significance of such a finding has been pointed out (35).

The purpose of sequence studies with immunoglobulin H and L chains has been in part to determine the exact structure of the antibody-combining site. The finding that variability in the H and L chains is largely restricted to the amino-terminal ends of these molecules is in agreement with the now generally accepted model of antibody structure. It is thought that the Fd fragment contains the antibody-combining site; the light chain may participate directly in the combining site or may simply act to stabilize it (36).

The Nature of Antigen-Antibody Reactions

The molecular structure of an antigen-antibody complex has not been elucidated but steric complementarity is likely important in complex formation. The weak bonds common to biological interactions, i.e., charge interactions, van der Waals' forces, hydrogen bonds, which might be formed between complementary regions of antigenic determinants and antibody, probably cooperate in stabilizing the complex (37); in recent years hydrophobic bonding has also been implicated.

The overall charge of the population of antibodies specific for a given haptenic group is determined by the net charge of the immunogen (38). This charge effect is apparently independent of the antibody-combining site, since antibodies elicited by oppositely charged immunogens carrying the same haptenic group cross-reacted with the heterologous antigen. It was apparently the charge of the Fab fragment of the antibody molecules which was determined by the charge of the immunogen.

Antibody-combining specificity is predetermined by primary amino acid sequence of the immunoglobulin. Complete reduction of the disulfide bonds and subsequent unfolding and separation of the polypeptide chains of an antibody does not irreversibly destroy its combining specificity (39). Reoxidation of these fragments, notably in the absence of antigen, largely restores the antibody specificity suggesting that specificity is inherent in

the amino acid sequence of the polypeptide chains. The 'thermodynamic hypothesis' postulates that the genetic information which determines primary structure is adequate for the determination of polypeptide conformation (40). The observations outlined above suggest that the same applies for large proteins as well.

Most antigen-antibody reactions proceed with a large positive change in entropy. This has been explained by postulating that the water of hydration around combining sites of both antigen and antibody is released to the solution on complex formation (41). The rate of combination of antibody and homologous hapten is very rapid and since it necessitates little activation energy, apparently does not require significant conformational changes in either antibody or antigenic determinant. The equilibrium constant for complex formation is large, indicating that the complex is very stable (41).

In vitro Manifestations of Antigen-Antibody Reactions

(a) The precipitin reaction

The addition of specific antibody to a solution of soluble antigen results in the formation of a flocculent white precipitate. With the addition of increasing amounts of antigen to a constant amount of antibody, the quantity of precipitate formed increases through the region of antibody excess to a maximum at the equivalence zone. Decreasing amounts of precipitate are formed with the further addition of antigen since excess antigen solubilizes the antigen-antibody complexes. This phenomenon has been explained by the lattice theory (42) which suggested that antibody molecules are bivalent and that antigens are multivalent. In antibody excess, each antigen molecule becomes surrounded by antibody; in antigen excess, soluble aggregates of one antibody molecule and two antigen molecules are formed. In neither of these cases is there interaction between aggregates. At equivalence the proportions

of antigen and antibody are such that very large aggregates are formed. The bivalency of antibody (43) and the multivalency of antigen (44) have been proven more recently. The precipitin reaction may detect in the range of 0.1-1.0 mg antibody/ml (45). The versatility of the precipitin reaction is greatly increased by the fact that it may also be performed in semi-solid agar (46). Gel diffusion techniques, as opposed to precipitation in solution, permit distinction of the several antigen-antibody systems of which a precipitate may be composed. They also give an estimation of the concentrations of a particular antibody present in serum.

(b) Bacterial agglutination and lysis

Particulate antigens, such as bacteria, are agglutinated by reaction with specific antibody. Agglutination or clumping is analagous to precipitate formation with soluble antigen.

The combination of certain bacterial strains with specific antibody in the presence of several constituents of normal serum, which are collectively termed complement, results in lysis of the bacteria. The mechanism by which this occurs is ill understood, but involves the absorption of complement by the antigen-antibody complex (47).

(c) Hemagglutination and hemolysis

The clumping of erythrocytes by antibody specific for antigenic determinants on the red cell surface is called hemagglutination. Red cells, either coated with or covalently linked to additional determinant groups, may also be specifically agglutinated by antiserum specific for the attached groups. This latter phenomenon is known as passive hemagglutination and will detect 0.01 μg antibody and 0.001 μg antigen (48). Combination of erythrocyte and specific antibody or erythrocyte-hapten conjugate and anti-hapten antibody in the presence of complement results in hemolysis or passive hemolysis respectively. There is no visible distinction between these reactions and they have been

reported to be of the same order of sensitivity as hemagglutination in the detection of antibody (49). Hemagglutination and hemolysis are analagous to the reactions involving bacteria outlined above.

Complement fixation by antigen-antibody complexes is used for the detection of antibody or antigen at concentrations where no precipitate or agglutination is visible. It is visualized by the addition of a second standardized antigen-antibody system, usually erythrocyte-anti-erythrocyte (47). The degree of hemolysis in the second system bears an inverse relationship to the quantity of antigen-antibody complex present in the first system.

In Vivo Manifestations of Antigen-Antibody Reactions

(a) Viral and toxin neutralization

Antibodies directed against viral particles will reversibly inactivate the viruses neutralizing or diminishing their infectivity. The mechanism by which this is accomplished is unclear. Antibody may be directed against a critical viral enzyme or may prevent viral penetration of host cells (47). The neutralization of bacteriophage provides an extremely sensitive in vitro antibody assay; it has been calculated that 10^{-7} μg antibody/ml may be detected by this technique (45). On infection of a bacterium, a bacteriophage induces the production of many new phage particles. When the bacterium eventually lyses these new phage are released into the medium and will infect nearby bacteria. The hazy lawn of a bacterial culture becomes dotted with clear areas or plaques for each of the viral particles originally present. Incubation of phage with specific antibody causes a reduction in the number of plaques produced by an aliquot of a given suspension of phage particles.

Neutralization of the toxicity of bacterial products is also mediated by antibody. The mechanism of attenuation is unknown, but it has been suggested that antigen-antibody combination may result in removal of the antigen from circulation or may occur at the biologically active site of

the toxin (47).

(b) Hypersensitivity

Initial exposure to certain antigens may in some individuals result in an increased sensitivity or hypersensitivity rather than immunity to the antigen. Subsequent exposure of the hypersensitive individual to the sensitizing agent may have harmful or even fatal effects. Hypersensitivity may be elicited by normally innocuous substances and is generally considered to be an aberration of the immune mechanism. Hypersensitive reactions are divided into two classes on the basis of the latent period between antigen contact and manifestation of the reaction.

Immediate hypersensitivity occurs very shortly after re-exposure to the antigen and may be transferred by the serum of hypersensitive individuals to normal individuals. Anaphylaxis is an increased reactivity of the whole body to the antigenic stimulus; exposure to the antigen is characterized by violent symptoms and sometimes death. The atopic reaction is a localized anaphylactic reaction often occuring at mucous membranes. Immediate hypersensitivity involves the release of serotonin, histamine and bradykinin. The Arthus phenomenon is the development of an inflammatory reaction often followed by swelling (caused by edema) and eventual necrosis at the site of exposure in sensitized individuals.

Delayed hypersensitivity is slow to reach maximum intensity and may be transferred to normal individuals by lymphoid cells of the hypersensitive individual. Swelling which occurs at the site of the delayed reaction is in part caused by the infiltration of macrophages and lymphocytes. Contact allergies and possibly homograft rejection are other delayed hypersensitive reactions.

A HISTORICAL REVIEW OF THEORIES OF ANTIBODY FORMATION

The mechanism by which a single-celled zygote differentiates into a complex multicellular organism is one of the most intriguing problems in biology. Molecular biology attempts to determine the agent or inducer which causes and perhaps controls the development of many specialized cell lines from common ancestors. Cell lines are characterized by a particular morphology and a phenotypically limited number of cellular products. It has been thought that antibody formation provides a model system for the study of differentiation. In this system antigen is the inducer, although its mechanism of action and whether it acts directly or indirectly is unknown. Induction involves differentiation of lymphoid cells; in the red pulp of the spleen large basophilic blast cells appear which multiply and differentiate into either small lymphocytes or mature plasma cells which are end cells (50). The cellular product is specific antibody. The in vitro induction of a clone of undifferentiated cells to produce specific antibody has not yet been achieved. This is necessary for a precise understanding of the molecular mechanism of the induction of cellular differentiation leading to antibody formation.

The first importance of immunology historically was clinical. It had been early recognized that animals which recovered from infectious disease became resistant or immune to the disease. The growth of bacteria in vitro could exhaust the culture medium and so it was proposed that, in vivo, bacteria equally exhausted some host nutrient essential for their growth (51). The animal, when reinjected with those bacteria, therefore, could not sustain their growth. This theory became untenable when it was shown that resistant animals could be infected by the injection of sufficiently large numbers of bacteria (52). Other workers postulated that antigens became in some way incorporated directly into the antibody molecule, thus accounting for the specificity of the antigen-antibody reaction (53). This view has been discredited by the failure

to find antigen in specific antibody preparations (54) and by the fact that large numbers of antibody molecules are formed when only small numbers of antigen molecules are injected (55).

The first consequential theory of antibody formation was proposed by Ehrlich and is commonly referred to as the "side-chain theory" (56). At this time it was felt that in order for a substance to mediate an effect on a cell the substance had to combine with some integral part of the cell. Ehrlich suggested that the cell surface carried a number of preformed receptors, the primary function of which was combination with cell nutrients. By coincidence these receptors were also toxophiles (antibodies) in that certain of them could combine with toxins. A dual function for these molecules was suggested since it would have been uneconomical for the cell to possess a large number of toxophiles solely for the purpose of combining with the many bacteria not found in the animal's habitat. The combination of toxophile and toxin inactivated the toxophile and prevented it from performing its nutritional function. To compensate for toxophile inactivation (toxicity), the cell produced large numbers of that particular toxophile. Any excess toxophile was released into the blood. Immunity was established by the presence of large amounts of toxophile in the blood, which could combine with toxin before it could react with the cell surface, thereby having its toxic effect. Tolerance consisted in an absence of toxophiles complementary to a specific toxin; such a toxin had no way of affecting the cell. The side-chain theory was the first suggestion that antigen and antibody were complementary and that their interaction might be chemical in nature. It also suggested the existence of preformed antibody, the production of which could be induced by antigen. As such it was the first selective theory of antibody formation.

Landsteiner's observation (9) that antibodies could be formed in response to a large number of synthetic antigens made the side-chain theory less

credible. It was difficult to understand how preformed molecules with a seemingly infinite range of specificities could be synthesized by the body. Breinl and Haurowitz (57) and Mudd (58) presented theories which suggested that antigen participated directly in the formation of the antibody molecule by alteration of the primary protein structure. Antigen acted as a direct template on which the complementary protein molecule was formed. Alexander proposed that antigen might cause the synthesis of new enzymes which could catalyze specific antibody formation either through gene modification or through the modification of some nongenic catalysts (59). Haurowitz later suggested that antigen interfered at the second stage of protein synthesis, the stage when tertiary structure was determined, by electrostatic forces. This theory was stated explicitly by Pauling (60) who suggested that the primary sequence for all gamma-globulins was identical but that if folding of the polypeptide chain occured in the presence of antigen, the tertiary structure would be complementary to that antigen. Karush emphasized the importance of disulfide bonds between half cystine residues in the determination of tertiary structure (61). He suggested that cysteine residues were incorporated in the primary structure of antibody molecules. The determination of tertiary structure involved oxidation of these sulfhydryl groups to disulfide bonds; if the concurrent folding process were to occur in the presence of antigen, the resulting antibody molecule would be complementary to that antigen. These direct template theories were popular since no physical or chemical differences had been observed between normal and immunoglobulins and they explained the formation of antibodies of an infinite range of specificities.

The persistence of labelled antigen in association with nucleic acids in the reticulo-endothelial system, particularly of the liver, gave inferential support to these theories (62). The <u>in vitro</u> induction of antibody formation in normal lymphoid tissue by antigen-containing ribonucleic acid (63) extracted from immune tissue has been reported (63,64).

Direct template theories may be criticized on several points. The dissociation of newly formed antibody from an antigen template following antibody synthesis has not been adequately explained since it is known that antigen-antibody complex formation is favoured thermally (41). Neither the specificity of immunological tolerance nor the specificity of the secondary response (increased efficiency of antibody formation on a re-exposure to a particular antigen) are adequately explained on the basis of the direct template theories (65). The cell division involved in antibody formation would result in dilution of the antigen template among antibody-producing progeny and efficiency would be expected to decrease according to the direct template theories. That denatured, separated H and L chains contain the information for antibody specificity (39) and the fact that the amino acid composition differs among antibodies with different specificities indicate that specificity is determined by primary sequence.

The understanding of protein synthesis in greater detail (66) also presents certain difficulties for direct template theories. It is difficult to imagine how such a large variety of antigenic determinants could influence protein synthesis in essentially the same way. Karush has suggested that amino acid sequence is likely not determined by a miscellaneous group of molecules (67). Antibiotics which influence protein synthesis at the translational level each act in a specific, characteristic manner. To date, it has been impossible to detect the presence of antigen in antibody-forming cells (68,69).

Attempting to draw an analogy between antibody formation and other known biological systems, Burnet and Fenner proposed the Indirect Antigen Template Theory or the Adaptive Enzyme Theory (70), based on contemporary concepts of adaptive enzymes. The basic assumption was that the same cells were involved in the destruction of internal debris without antibody formation

and the removal of foreign material with antibody production. Self-antigens acted as self-markers and could be recognized by the antibody-producing enzymes of that individual. Foreign antigen was capable of adapting these replicable enzymes which once adapted could self-replicate in the absence of antigen. It was not feasible that the body should retain indefinitely pathogenic antigens as specific templates and therefore the animal must be capable of producing specific antibody in the absence of antigen. As more knowledge of enzyme induction and protein biosynthesis became available, Burnet modified his theory to implicate antigen-RNA complex (71) in the formation of the first globulin template. It was only at this stage that antigen was involved. The new template could replicate and was transferred between reticulo-endothelial cells and lymphoid cells. Antigen modification of RNA was also suggested by Goldstein (72).

Schweet and Owen suggested that antigen might have its initial effect at the DNA level, in effect causing mutation of the DNA to an inducible state (73). The resulting inducible cells would be able to accumulate antigen; antigen could then act as inducer eliciting the synthesis of specific antibody. Decline of antibody might be effected if antigen, when combined with antibody, lost its antigenicity (inducer-capacity) or if a build-up of product antibody occurred. Increased knowledge of the mechanism of mutation makes it difficult to explain the molecular mechanism of antigen-induced mutation (74).

The direct and indirect template theories are generically termed the instructive theories; antigen instructs the cell in the production of complementary antibody. Selective theories of antibody formation on the other hand propose that antigen selects complementary performed or "natural" globulin molecules whose synthesis it can in some way induce. The first recent selective theory proposed that the antigen-antibody complex was phagocytosed whereupon it dissociated (75). The presence of the free, selected

globulin in the cell initiated specific replication of that globulin. Recognition is likely to occur outside the cell since antibodies are directed primarily against the surface antigenic determinants of particulate immunogens. The capacity to produce large numbers of different antibodies was not inherent in the zygote but arose early in life by somatic mutation in the thymus. This explained the immunological incompetence of new-born animals and also tolerance to self-antigens. Anti-self antibodies produced by chance could be removed by reaction with the specific auto-antigen. This theory differs from Ehrlich's theory (56) in that it proposes that circulating rather than cell-bound antibody is the unit of recognition. Talmage has suggested that changes within the cell population, rather than intracellular changes in a static cell population, are of primary importance in the increase of specific globulin concentration (76). Secondary response is transferred to normal recipients by cells (77) rather than serum and the rapid rise of antibody titre during the course of immunization is indicative of cell proliferation (69). The importance of cellular selection was also emphasized by Burnet (78). He suggested that potential antibody-forming cells, each capable of producing a limited range of antibody specificities, arose by random mutation in embryonic life. Antigen-antibody combination on the surface of the cell which had produced the antibody would induce differentiation and proliferation of that cell; the concentration of that specific globulin would therefore be increased. The sensitivity and response of antibody-producing cells to specific antigen may vary with the degree of cellular differentiation (78,79). If antigen contacted the antibody-producing cell early in differentiation, further differentiation might be prevented; at later stages of differentiation, contact with antigen might accelerate differentiation and proliferation. Lederberg's elective theory emphasized the importance of gene hypermutability throughout the life of the animal as the source of antibody diversity (80).

Diversity introduced strictly during embryonic life could not account for loss of certain specificities due to random drift nor for the breaking of immunological tolerance. Hypersensitivity to antigen is an integral part of the differentiation of globulin-producing cells since forbidden mutants may always be eliminated in this way. It is unclear whether or not the progeny of a stimulated antibody-producing cell also pass through this hypersensitive stage (81).

The specificity of serological reactions may be a function not of a single type of antibody molecule but rather a characteristic of specifically immune serum (74,79). Normal serum likely contains a large number of "natural antibodies". Among this spectrum of combining specificities there may be several antibodies which will combine with a given antigenic determinant each being complementary to different features of the determinant. Each will have its own characteristic binding affinity for the determinant. A family of such antibodies, each of which combines in its own way with different aspects of antigen, would define the antigen more precisely than would a single antibody conformation.

Monod has compared antibody formation to the inducible permease system of bacteria (82). His antigen-capture model proposes that the cell population capable of antibody production is genetically homogeneous but that during the induction of the immune response certain cells become phenotypically restricted. Antigen contacts only a few potential antibody-forming cells; these cells will produce in addition to classical antibody molecules, cellular (antibody) molecules which are capable of efficiently concentrating antigen into those cells. Re-exposure to antigen results in a rapid increase in the production of antibody since cells initially stimulated will capture increasing quantities of antigen. Cellular antibody will be distributed to progeny and, when antigen is exhausted, the level of this cellular antibody will be diluted out as

division proceeds. Primary response seems to involve more than an alteration in the way antigen is handled since secondary response specific for the original antigen can be elicited by cross-reacting antigen (83). Monod explains these results by suggesting that a given cell can produce only one type of antibody at a time. The antigen capture mechanism of induced cells cannot distinguish between two cross-reacting antigens but antibody response is very specifically determined by preliminary exposure to antigen. Boyden has postulated that the antigen-capturing mechanism is cell-fixed or "cytophilic" antibody (84), and that random specificities are spontaneously produced. Both of these theories imply that antigen enters the cell.

Szilard proposed the first molecular mechanism by which antigen might elicit the production of large amounts of specific antibody (85). Antibody-producing cells had multipotential genotype but unipotential phenotype; antibody genes were normally repressed. Synthesis of a repressor for a specific genome was catalyzed by an enzyme which could be inactivated by specific combination with the homologous antigen. The specific globulin would then be synthesized due to derepression. The repressor which normally inhibited cell division was inactivated in the presence of antigen-antibody complexes so that when sufficient antibody was formed cell division would be initiated. Enzymes show much more heterogeneity than do antibodies and it is difficult to imagine how the strict correspondance between enzyme and the antibody whose synthesis it controlled could be maintained. Alternately, derepression might be caused by antigen-antibody complex through inducer activation or removal of antibody from its site of synthesis (86).

It has been aptly remarked that the existence of a large number of theories of antibody formation indicates that no theory thus far proposed adequately explains this remarkable phenomenon (87).

CHAPTER II

ANATOMICAL INVESTIGATIONS OF ANTIBODY FORMATION

The association of macrophages and lymphatic organs with immunity has been recognized for some time. Macrophages were known to ingest bacteria and other foreign materials and so their importance as cellular elements in the resistance to infection was inferred (88). These cells are components of what has been called the reticulo-endothelial system (89). The reticulo-endothelial system is a classification based on a physiological phenomenon rather than cellular morphology and denotes those cells of the body which are highly phagocytic. It includes the histiocytes or wandering macrophages of the connective tissue, the Kupffer cells found in the sinusoidal linings of the liver, the reticular cells of lymph node sinuses and the microglia of the nervous system (90). There are in lymphoid tissue certain undifferentiated cells, morphologically similar to phagocytes, which do not phagocytose.

The significance of the spleen in antibody formation was demonstrated when spleen cells from an immune donor gave rise to specific antibodies in a nonimmune recipient (91). It was found that the lymph node draining the site of antigen injection contained specific antibody before it could be found in the blood (92). A more detailed investigation correlated certain morphological changes in the lymph node with antibody formation (93) and indicated certain concurrent changes in the small lymphocyte population. Labelled amino acids could be incorporated in specific immunoglobulins by isolated lymph node cells (94).

Because of the critical role of the macrophage in ridding the body of foreign materials by phagocytosis it was assumed that these cells also synthesized antibody. The persistence of antigen in reticulo-endothelial cells (95) gave rise to the idea that macrophages were directly responsible for antibody

formation (96). Inferential evidence further supported this concept. It was found that although blockading of the reticulo-endothelial system stimulated the liberation of antibody, a greater degree of blockading tended to depress antibody formation (97). Skin graft survival could be prolonged by the injection of trypan blue and since this dye is known to be phagocytosed, it was suggested that macrophage inactivation was occurring (98). A more detailed investigation has shown that such blockading actually stimulates antigen uptake and antibody formation by the spleen (99). The non-phagocytosable portion of an immunogen solution was shown to be less immunogenic than the initial solution before exposure to macrophages. An essential role for phagocytosis in the induction of an immune response was inferred from this "filtering" effect of macrophages (100). Peritoneal exudate cell suspensions (70% macrophages) transferred secondary response to X-irradiated recipients (101).

Cell suspensions from lymph node and sinus consisting essentially of lymphocytes were found to contain more antibody than did serum (102). Lymphocytes were capable of transferring immunity from immune donors to non-immune recipients (103) and immediate hypersensitivity to normal recipients (104). Both macrophages and lymphocytes were believed to differentiate into plasma cells during the course of immunization (105).

Plasma cells (106) were early associated with antibody formation (107); subsequently their importance in antibody formation was implied by other investigators (108) and antibody was extracted from plasma cells (109). In a classic paper, Fagraeus correlated the development of plasma cells in the spleen and antibody production. She proposed that macrophages differentiated into plasma cells which were end cells (110); she was able to demonstrate the <u>in vitro</u> production of antibodies by plasma cells (111).

Cooperation between macrophage and lymphocyte in the immune response has been suggested; the macrophage supposedly recognized and prepared antigenic

material and the lymphocyte produced the specific antibody (112). An intimate association of non-malignant macrophages and lymphocytes was first demonstrated by cinematography (113). In tissue cultures derived from animals which had received a skin homograft or injection of soluble antigen, normal small lymphocytes could be seen to cluster around the surface of a macrophage (113, 114). Both lymphocytes and plasma cells were found to participate in cluster formation and a small number of these cells were seen to form cytoplasmic bridges with the macrophages (115). The possible role of the macrophage in the recognition of foreigness has been suggested by Nossal (116). It is important to note that these workers observed an equal degree of antigen localization or phagocytosis in immune and tolerant animals. Antigen retained over long periods of time in the reticulo-endothelial system has been found to be associated with the ribonucleic acid fraction (117). Specific antibody synthesis has been initiated by ribonucleic acid extracted from the macrophages of immune animals (63,64). To date, the role of the macrophages in antibody formation remains obscure. Newer techniques have clearly demonstrated the production of antibody by plasma cells and, in some cases, by lymphoid cells which have been called plasmablasts (118).

Tissue Culture Studies of Antibody Formation

Tissue culture of antibody-producing tissue has not yet been fully exploited. Tissue culture studies offer the advantages of a well-defined environment and the possibility of clonal investigations. The cell transfer experiments (91,101,103,104) are in fact *in vivo* cultures. Since radiosensitivity of the immune response is limited to the primary response (119), a well-defined cell suspension may be injected into non-immune X-irradiated recipients which serve as "test tube" animals. The animals themselves are incapable of immune response and therefore the effect of antigenic challenge

on the injected cells may be studied. This technique was used to study the development of foci of immune cells when single-cell suspensions from immunized donors were injected into X-irradiated, non-immune recipients (120).

The first *in vitro* induction of antibody synthesis was performed by Carrell (121). Many years later, *in vitro* antibody formation by immune tissues (122) and the *in vitro* induction and maintenance of secondary response (123) were reported. Recently the *in vitro* induction of anti-phage production by lymph node chunks (124) and anti-erythrocyte antibody production by spleen cells (125) has been realized. Mixed cell populations were used for these studies. The adaptation of techniques for the isolation of purified lymphoid cell types (126) to transfer experiments and tissue culture studies should clarify the question of cellular interdependence in antibody formation.

Although the techniques outlined above have contributed to anatomical knowledge of antibody formation, they are essentially refinements of serum studies in that only non-synchronized, mixed cell populations have been used to date. An understanding of basic questions in immunology requires a knowledge of the kinetics and potential of the individual cells responsible for antibody formation. To this end, more definitive techniques have been developed for the identification and study of single cells engaged in antibody formation.

Single Cell Techniques in the Study of Antibody Formation

(a) Labelled antigens and immunofluorescence

It was shown that the coupling of certain fluorescent groups to antibody molecules did not alter antibody specificity (127). It was possible therefore to "stain" tissue sections with fluorescent antibody and thereby visualize the presence of antigen (128). Certain antigens, such as ferritin, may themselves be identified in the electron microscope (129) while others, such as tritiated

haptens, are revealed by autoradiography (130).

Cells containing antigen are not necessarily those producing antibody and so immunofluorescent techniques were adapted to the identification of cells producing antibody. Since the various peptide chains of immunoglobulins are antigenically distinct (131), cells producing various classes of antibodies could be distinguished by immunofluorescence. Antisera specific for each of the various immunoglobulin classes were prepared and linked to different chromophores. Sections of antibody-forming tissue were then stained with these fluorescent antibodies. Plasma cells of the spleen were found to produce either kappa or lambda type L chains (132,133); lymphoid cells of heterozygotes were found to produce only one allotypic specificity (134). A given cell produced one immunoglobulin class exclusively (133,135), but always produced one H chain and one L chain (133). This technique estimates the proportion of cells producing the various molecules but it gives no indication of the antibody specificity being produced. In order to determine the specificity of antibody-producing cells, ferritin was used to stain tissue immune to ferritin. Antigen distribution and, by inference, antibody distribution were then investigated by electron microscopy (136). If iodinated antigen is used in such staining, the grain counts of an autoradiograph may be compared to the histological section in order to identify antibody producing cells (137).

Not all antigens can be easily coupled to the various chromophores so that the "sandwich technique" was developed for identification of cells producing specific antibody (138). Sections of antibody-producing tissue are first incubated with antigen solution, then washed and finally stained with fluorescent homologous antibody. Tissues simultaneously immunized with two antigens were stained sequentially with the homologous antibodies each

coupled to a different chromophore (139). In this case three different types of cells might theoretically be observed. Actually a quasi-clonal distribution of cells producing the different antibodies was observed. An amplification of the sandwich technique involves incubating the tissue section with antigen followed by untreated antibody; the tissue is then stained with antigen-fluorescein complex (140).

The combination of radioautography for the identification of antigen-containing cells and immunofluorescence for identification of antibody-producing cells has recently been reported (69). Antigen was not detected in antibody-forming cells. Labelled antigen and antibody are useful for study of antigen distribution, persistence, degradation and role in antibody formation (141).

(b) Bacterial agglutination or immobilization

Specific antibody can cause agglutination of bacteria both in vivo and in vitro. If a cell suspension is incubated with a solution of bacteria, those cells forming homologous antibody become surrounded by immobilized bacteria (142,143). The antibody-producing cells thus identified may be studied by phase microscopy (142) or by light microscopy, if the proper stain is chosen (143). Bacterial agglutination has also been studied in vivo and in this case antigen was administered through a capillary tube into the dermis. Antibody-forming cells were identified by microscopic examination of tissue sections (144).

Salmonella bacteria are immobilized by anti-flagellar antibody. Single cells of animals simultaneously immunized with two salmonella strains with distinctive flagellar antigens were studied for ability to immobilize either or both of the flagellar types (145). Microdroplets containing 0 to 6 cells were studied in a de Fonbrune oil chamber. Fixed numbers of each test

strain of bacteria were added to each microdroplet and the cells were scored for single or double antibody producers. The production of two antibodies by a single cell was a rare event occuring in only 0.08% of all antibody-producing cells (146). When labelled flagella were used as immunogen, the microdroplet technique and radioautography were used to study antigen distribution in antibody-producing cells (147). No antigen could be detected in antibody-producing cells.

(c) Phage neutralization

Carefully prepared cell suspensions from animals immunized with bacteriophage T2 and T5 were mixed with a suspension of the two bacteriophage (148). Cells were carefully transferred by microdroppers or micropipettes to an oil chamber in which each drop was scored microscopically for the number and type of cells present. After 48 hours incubation the droplets were taken up individually on a strip of filter paper. The solution was eluted with nutrient broth and the eluent was plated on cultures of each of the two indicator bacteria. In contrast to the results presented above, these workers observed that 3.6% of all antibody-producing cells were double producers. This discrepancy may possibly be explained by the greater sensitivity of phage neutralization in the detection of specific antibody. Recently, in the hemolytic plaque assay (see below), 1% of all antibody-producing cells were found to be double producers (149). The question of cell potentiality in antibody-producing tissue is therefore still unresolved, although a certain phenotypic restriction is apparently in effect.

(d) Localized hemolysis or hemagglutination

By immobilizing lymphoid cells immune to erythrocytes and the homologous erythrocytes in a thin semisolid culture medium in the presence of complement, it is possible to localize hemolysis to a plaque or clear area surrounding each antibody-producing cell (150,151). Immunologic specificity is suggested

by the fact that plaques are formed neither in the presence of cyanide nor in the absence of complement. One of the supporting media which has been used is semisolid agar (150); this technique will be discussed at greater length in the following chapter. The other medium which has been used is carboxymethylcellulose (151). Antibody-producing cells, homologous erythrocytes and complement were evenly suspended in a mixture of carboxymethyl cellulose and tissue culture medium. This mixture was incubated on a microscope slide. The red cells around each antibody-producing cell hemolyzed and the cells could then be studied microscopically.

If antibody-producing cells and homologous red cells are incubated in free suspensions, the red cells will tend to form clusters around cells producing hemagglutinins (152). These clusters are not broken up if washed carefully. Cluster formation may be enumerated on a microscope slide or alternately the cell suspension after incubation may be assayed by the localized hemolysis in gel technique (150). Both plaques and clusters are visible after the latter procedure suggesting a functional heterogeneity of the antibody produced (153). 19S antibody is a more efficient hemolysin while 7S antibody is a more efficient hemagglutinin (154). Apparently cluster and plaque formation are manifestations of different antibody-producing cells (153); this observation is in agreement with results outlined previously in which different immunoglobulin classes were shown to be produced by different cells (133).

Since 7S antibodies are inefficient hemolysins, detection of 7S-producing cells by the plaque technique, as initially reported (150,151), is inefficient. 7S* detection has been improved by addition to the test system of antibody specific for the globulins being produced. The reaction of anti-globulin antibodies with antibodies already complexed with antigens on the red cell

*In this text, the abbreviations "7S" and "19S" have been used to denote 7S- and 19S- antibody, respectively.

surface results in a second and perhaps more efficient complement fixing system (155).

Attardi et al have outlined certain criteria for satisfactory single cell techniques (148). Since the quantities of antibody to be detected are very small, such techniques must of necessity be very sensitive and, if possible, quantitative. Phage neutralization is known to be very sensitive (48) and may be quantitated on the basis of the per cent neutralization. Certain assumptions must be made if other single cell techniques are to be quantitative (137,118). The background level of antibody producers should be reliably low and the analysis simple so as to allow the enumeration of large numbers of cells. The hemolytic plaque technique undoubtedly best fulfills this criterion since bacterial agglutination (and immobilization) and phage neutralization assays of single antibody-producing cells all involve tedious micromanipulations. Enumeration in the plaque assay may be performed macroscopically and thus the scanning of a much larger number of cells is feasible.

CHAPTER III

THE ENUMERATION OF CELLS PRODUCING ANTI-DINITROPHENYL ANTIBODY

The use of well-defined immunogens has revealed that antibody response is genetically determined (156). It would appear that haptens could be employed to good advantage in the investigation of antibody formation. The present study was undertaken with a view to adapting the Jerne technique for the detection of cells producing antibodies to the dinitrophenyl residue.

Since Landsteiner's observation that simple well-defined chemical groups were responsible for immunological specificity (9), many classical immunological methods have been adapted for the assay of antihapten antibody. In elucidating the basis of serological specificity, Landsteiner used haptens in the precipitin reaction (157); he was later able to show agglutination by anti-hapten antibodies of red cell stroma coupled to haptens (158).

Passive hemagglutination of intact red cells coupled to azophenylarsonate and ovalbumin was effected by hapten specific antisera (159). Red cells coated with protein could also be agglutinated by antiserum specific for the adsorbed molecules (160). To eliminate the possibility of desorption of the antigen (161), many bifunctional reagents have been used for the coupling of antigens covalently to the surface of red cells to be used in passive hemagglutination; bisdiazotized benzidine, difluorodinitrobenzene and tolylene-2,4-diisocyanate are examples of such coupling agents (162). The use of these reagents permits the conjugation of a wide variety of protein molecules to red cells (159,163,164). Simple haptens have been directly coupled to red cells for use in passive hemagglutination (165) and passive hemolysis (166). Cells to be used in hemagglutination may be stabilized by formalinization prior to conjugation (167) since conjugation tends to increase cell fragility and the possibility of nonspecific hemolysis; however, because formalin treated cells tend to clump, this procedure has not been used

extensively. Finally hapten-coupled bacteriophage may be neutralized by incubation with small amounts of antihapten antibody (168).

Recently the localized hemolysis technique developed by Jerne et al (150) has been adapted for the study of cells forming antibody to polysaccharides (169) and polypeptides (170). These molecules were adsorbed directly onto the red cell surface for the detection of specific antibody-forming cells. While the present investigation was in progress the successful use of arsanilic acid in a similar system was reported; arsanilic acid was diazotized and coupled directly to the red cell surface for detection of anti-arsanilic-producing cells (171). A procedure developed for the preparation of dinitrophenyl-red cell (DNP-red cell) conjugates for use in the plaque technique is described in this chapter.

METHODS AND MATERIALS

1. Immunization

The animals used throughout these experiments were Swiss mice, male or female, from 6-10 weeks of age. Test and control animals were selected randomly from the same group of animals, were housed under identical conditions and were all fed water and Purina mouse chow ad libitum.

Crystallized bovine gamma globulin (BGG) (Sigma Chemical Corporation) and 2,4-dinitrobenzene sulfonic acid ($DNPSO_3H$) (Eastman Organic Chemicals) were coupled according to the method of Eisen et al (172). A saline solution of BGG was made strongly alkaline by the addition of K_2CO_3. A 1000 molar excess of $DNPSO_3H$ was added and the reaction mixture was agitated for 10-11 hours at room temperature. Under these conditions it has been reported that 50-55 DNP residues are coupled to each BGG molecule (172); the number of groups actually coupled in this study was not determined. The mixture was then dialyzed against saline until the dialysate contained no light absorbing material. A 2% solution of the conjugate was passed through a Seitz filter

into 25 cc sterile vials. The pH was then adjusted to 7.5 with sterile hydrochloric acid. This procedure was followed since BGG-DNP tends to precipitate out at physiological pH. Conjugates were stored at 4°C. DNP-human serum albumen (DNP-HSA) was prepared in an identical manner.

2. Immunization Schedule

Preliminary studies involved a comparison of the response to various doses of immunogen injected intraperitoneally, intravenously and subcutaneously. Each injection consisted of 2 mg of BGG-DNP either in saline or emulsified in Freund's adjuvant as indicated in Table IV. One or two injections were given over a period of 4 days and on day 5, just prior to assay, the animals were sacrificed by cervical dislocation.

3. Preparation of Indicator Red Cells

The hapten-erythrocyte conjugates were prepared by an adaptation of Ling's method (164), i.e. the dinitrophenyl residue was coupled directly to the red cell. In this text such conjugated cells are referred to as "sensitized" cells. Erythrocytes were obtained from sterile defibrinated sheep's blood. The blood was refrigerated and the cells were used within two days of collection or alternately they were stored up to one week in Alsever's solution (173). Just prior to sensitization the cells were washed 3 tires in phosphate buffered isotonic saline (pH 7.4) containing 1% chemically pure glucose (w/v). To a solution consisting of 20 ml isotonic ethylenediaminetetraacetic acid buffer (pH 8.4) and 9 ml physiological saline maintained at a given reaction temperature were added 1 ml washed packed sheep red cells and 0.3 ml of fluorodinitrobenzene in acetone (w/v). For termination of the reaction 10-20 ml of phosphate buffered saline were added and the red cells were removed from the reaction mixture by centrifugation at 4°C. The erythrocytes were washed once in 40 volumes of cold phosphate buffered saline, recentrifuged

and then resuspended in 1 ml of cold tissue culture medium 199 (174). Red cells were sensitized shortly before use in the plaque technique. The final red cell concentration was about 7×10^9 cells per ml.

4. Hemagglutination of Sensitized Cells

Microhemagglutination as described in an earlier thesis from this laboratory (175) was performed on certain preparations of sensitized red cells. Conjugates of DNP-HSA and red cells (163) were used to determine antibody titres of serum obtained from rabbits hyperimmunized with BGG-DNP. The amount of specific antibody present in the serum was assayed by the precipitin reaction (173). Attempts were then made to use this serum in the standardization of DNP-red cell conjugates prepared by various methods.

5. Lymphoid Cell Suspension

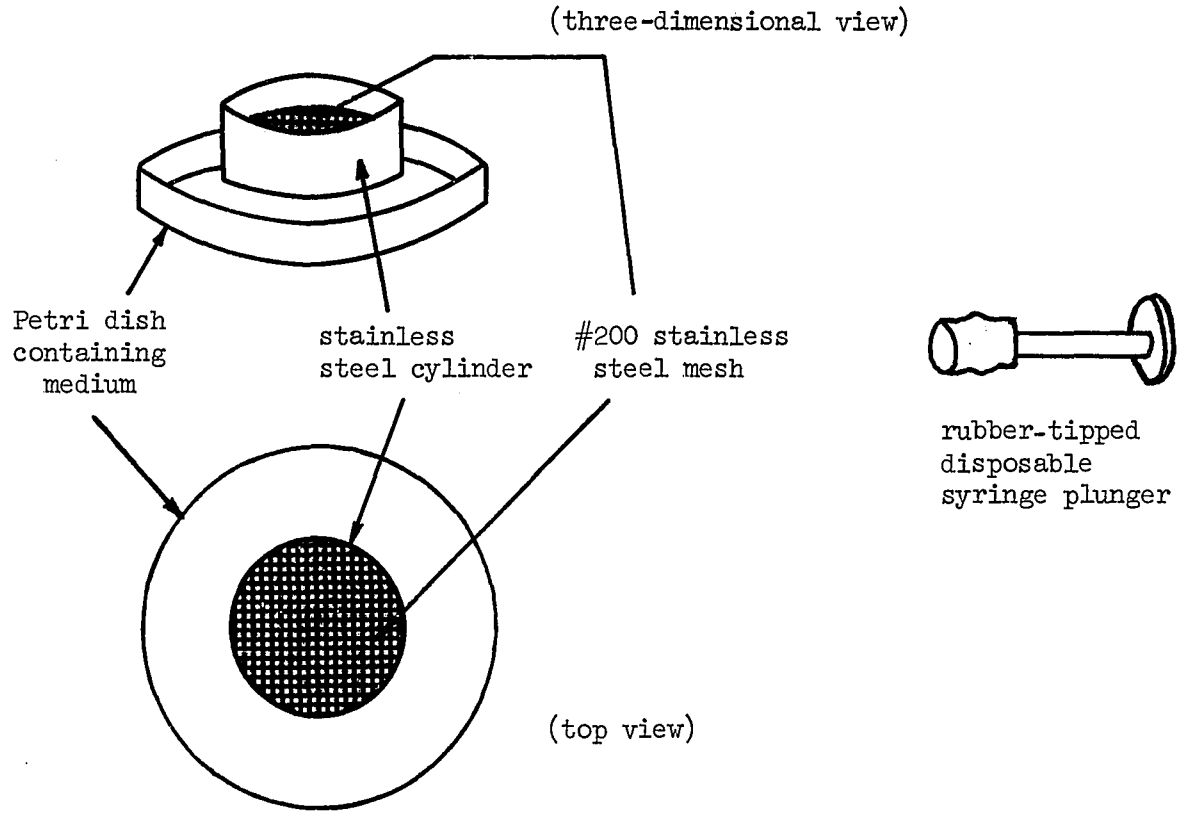
The spleens of animals to be tested were excised and placed immediately into cold medium 199. They were rinsed off and placed into a volume of medium 199, 1 ml per spleen. Each spleen was cut into several fragments and the cells were forced out of the connective tissue by gently rubbing the fragments on a circular stainless steel mesh (# 200) which was welded midway within a stainless cell cylinder. The cell suspension was carefully transferred to a centrifuge tube by a large bore Pasteur pipette. Any remaining large tissue fragments sedimented out of the suspension on standing. The supernatant decanted at this point contained on the average 1.5×10^8 single nucleated cells per spleen (90% viable by Trypan blue exclusion criterion). The spleens and resulting suspensions were kept at $0^\circ C$ at all times. The cell suspensions used were pools from 10 immunized and 10 normal animals; these served as test and control samples respectively. This procedure is summarized in Figure 2.

FIGURE 2: Preparation of spleen cell suspensions.

1. Sacrifice and splenectomy.

2. Spleen cut with scissors into small fragments.

3. Fragments transferred to stainless steel cylinder with stainless steel mesh submerged in medium.



Fragments rubbed against mesh with plunger; cells fall through mesh into medium.

4. Medium transferred to small centrifuge tube by large bore Pasteur pipette.

5. Sedimentation of large tissue fragments.

6. Decantation of cell suspension for plating.

6. The Hemolytic Plaque Assay

The assay for antibody-producing cells was similar to that described by Jerne et al (150). At least 1 day prior to experimentation, a 10-fold concentrated solution of medium 199 was diluted with 9 volumes of an aqueous agar solution (Difco Bacto-Agar) which had been heat sterilized. The final agar concentration was 1.4% and contained 300 units Penicillin G Na (Ayerst, McKenna and Harrison Ltd.) per ml. The bottom of a 10 cm Petri dish was just covered with this solution. Once the agar had solidified, the plates were inverted and were incubated overnight at 37°C to allow evaporation of excess moisture. The dishes were either used immediately or were stored at 4°C in an air-tight box. The Petri dishes were brought to room temperature before use. A second agar solution was prepared as above on the day of an experiment; in this case the final agar concentration was 0.7%; 300 units Penicillin G Na and 0.5 mg DEAE-dextran (Pharmacia) were added per ml. This solution was distributed in 2½ ml portions to 10 x 75 mm test tubes (Kimax) which were kept at 45°C in a water bath. To each tube were added 0.1 ml red blood cells (approximately 4×10^8 cells) and 0.1-0.2 ml lymphocyte suspension (dependent on the cell count). Each tube was immediately inverted three times to mix the contents and was quickly poured onto the base agar layer in a Petri dish. Three or four replicates were plated for each sample. When this layer had solidified, the plates were removed to a 37°C room where they were incubated for one hour. During this time any necessary adjustment of pH, as visualized by the phenol red indicator in the medium 199, was made by the use of a CO_2/air incubator, also at 37°C. After 1 hour incubation the cells were scored for "non-specific plaques". These were infrequent and upon microscopic examination could be ascribed to air bubbles. Two ml of 20% reconstituted lyophilized guinea pig complement (Difco Laboratories) in medium 199 was then added to each dish. Following a further 30 minutes

incubation at 37°C, the Petri dishes were transferred to room temperature. Immune or plaque-forming cells were at this time surrounded by a clear area (plaque) due to localized passive hemolysis. The appearance of a typical plate is illustrated in Figure 3; the microscopic appearance of a single plaque is shown in Figure 4. Plaques were enumerated 2 hours later and the number of nonspecific plaques previously recorded was subtracted from the corresponding number of total plaques.

7. Inhibition Studies

In order to determine the immunological specificity of the plaques observed, ε-DNP-L-lysine was added to lymphoid cell suspensions prepared from immune spleens prior to plating. In preliminary experiments large concentrations of ε-DNP-L-lysine were shown to have no effect on the number of background PFC. The relationship between numbers of observed PFC and concentration of ε-DNP-L-lysine added to the lymphocyte cell suspension was investigated as well as the inhibitory effect of a constant DNP concentration on various DNP-red cell conjugates.

RESULTS

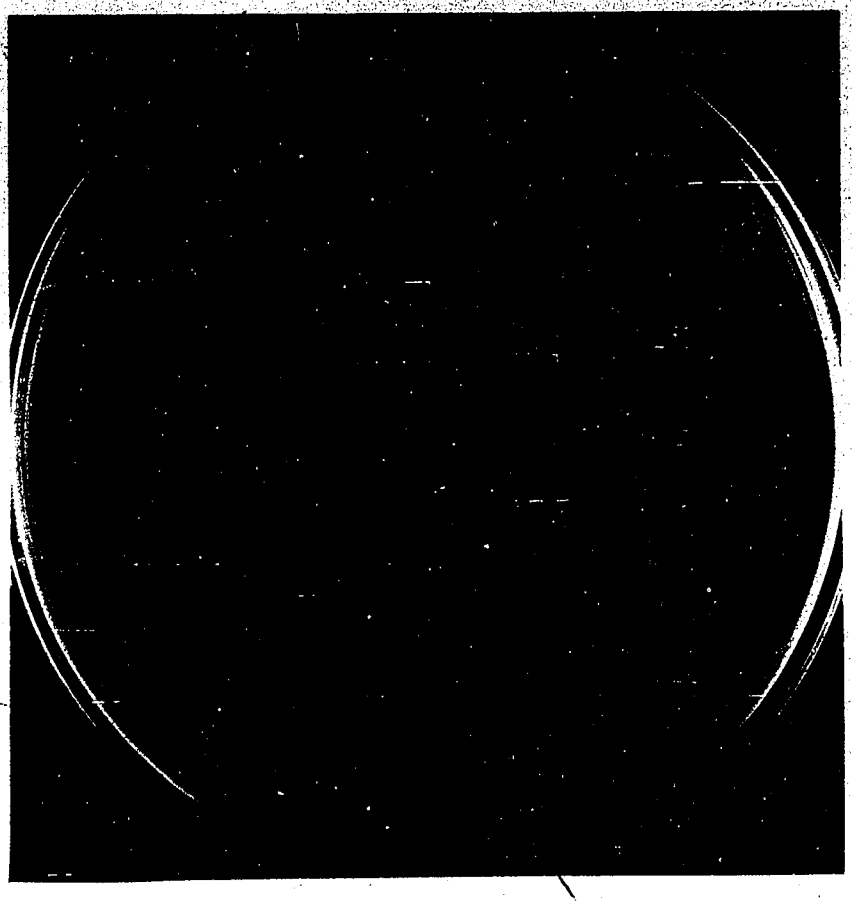
1. Background Level of Plaque Forming Cells

The degree of variation in numbers of background hemolysin-producing cells per animal was small, as illustrated in Table II; the mean was 0.09 $\pm$ 0.028 PFC/10^6 cells. In the plaque technique, cells producing hemolysins and those producing antibody specific for groups artificially coupled to red cells are indistinguishable. It is important that the background level of hemolysin-producing cells as well as the background against DNP-erythrocytes be small and uniform relative to the immune response to be observed. The background indicated in Table II compares favourably with those reported elsewhere (150,176); differences between these results and those published

FIGURE 3: Appearance of typical plates showing localized areas of hemolysis or plaques.

upper photograph - immune spleen cells and sensitized red cells. (4.1 PFC/10^6 spleen cells; 292 PFC/plate)

lower photograph - nonimmune spleen cells and sensitized red cells. (1.4 PFC/10^6 spleen cells; 56 PFC/plate)



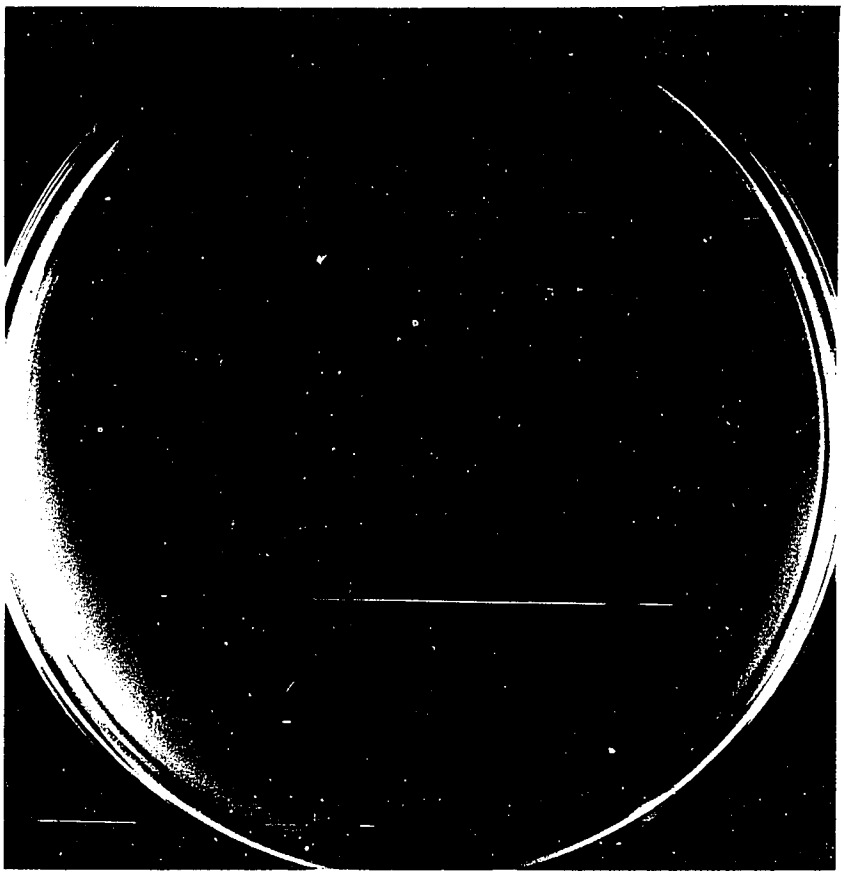
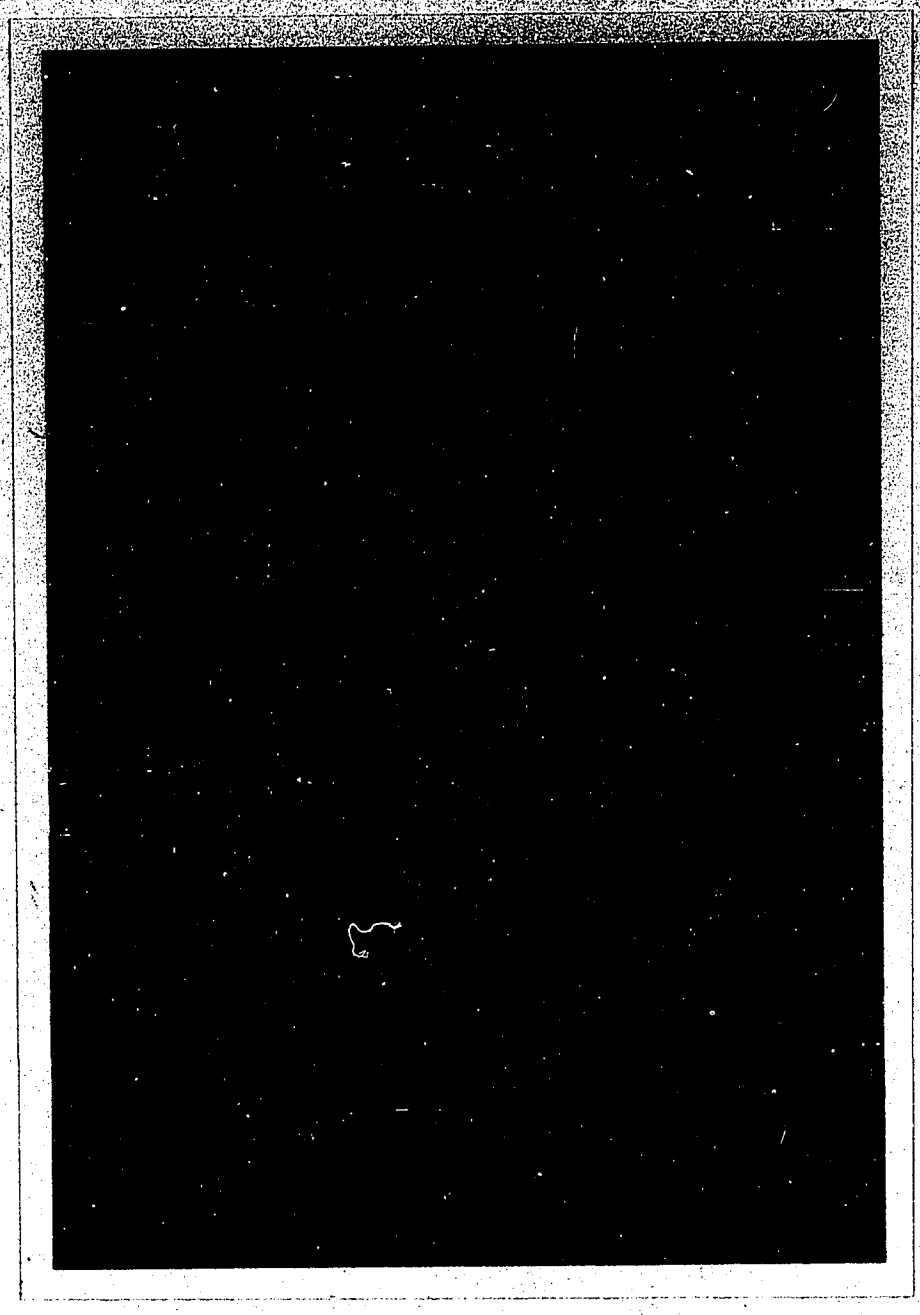
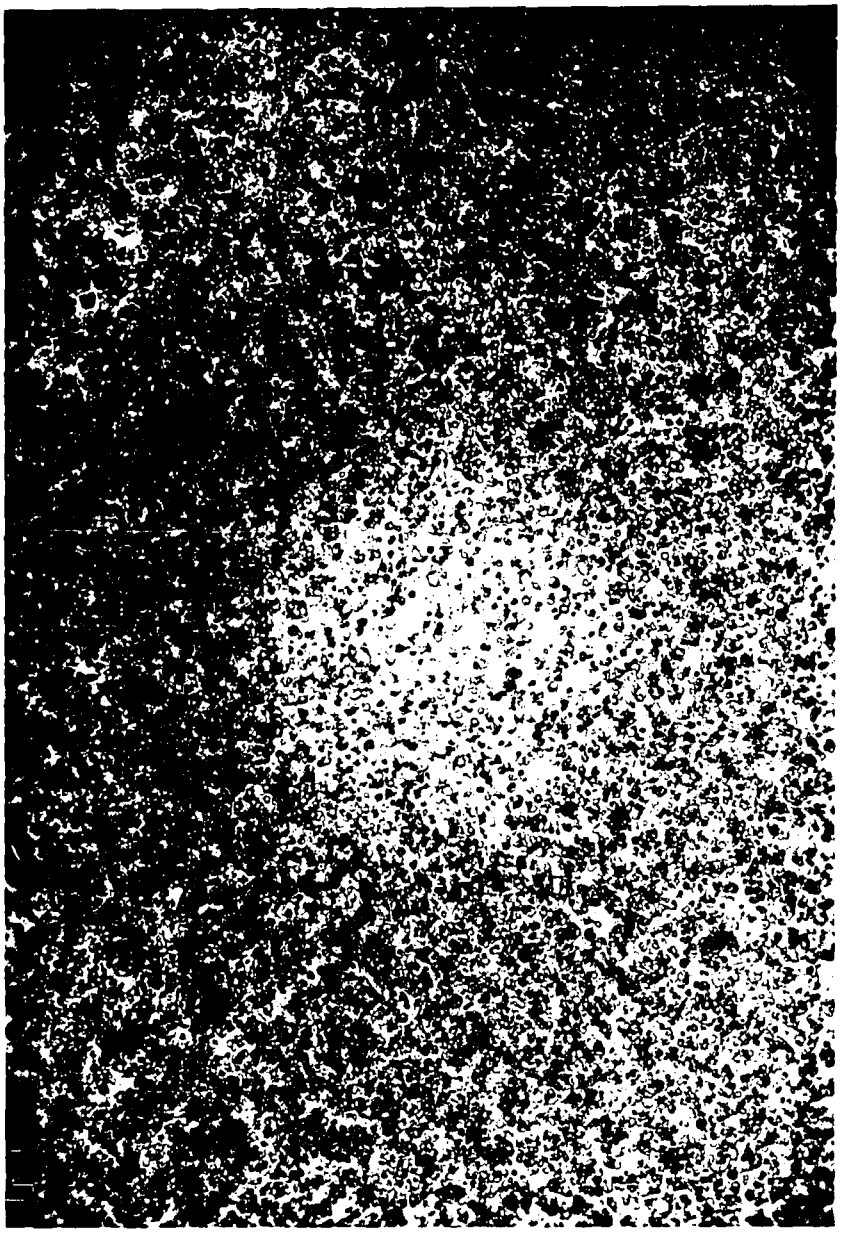


FIGURE 4: Microscopic appearance of a single plaque.

Adjacent to PFC are two autologous (mouse) erythrocytes. X 150.





may be ascribed to the different strains of animals used. The background level of pooled spleen cell PFC when plated with DNP-erythrocytes was very similar to that with erythrocytes, as can be seen by comparing Tables II and III. It was, therefore, impractical to investigate individual background levels to DNP-erythrocytes.

2. Immunization Schedule

A comparison of several 5 day immunization schedules, tabulated in Table IV, revealed that maximum numbers of spleen plaque-forming cells on the fifth day followed a single intravenous injection of immunogen on day 1. However, as large numbers of animals were to be used in further experimentation, a schedule of intraperitoneal injections on day 1 and day 4 with sacrifice on day 5 was selected for subsequent investigations. The response to varying doses of immunogen using this schedule is outlined in Table V. The optimum doses were 6 mg immunogen injected on day 1 followed by 2 mg injected on day 4. Animals were routinely sacrificed on day 5; it was found that no significant change in numbers of PFC occurred when the assay was performed on days 6 or 7. This point was not investigated in detail since it was the assay system rather than the response of the animals which was under study at this time.

3. Preparation of Indicator Red Cells

Several methods for the conjugation of DNP to red cells to be used in the plaque technique were attempted. It was found that cells coupled to DNP-human serum albumen by bisdiazotized benzidine (BDB) (163), under conditions used for hemagglutination, were too fragile for use in the plaque technique. They underwent spontaneous generalized lysis when added to the warm agar solution and this obscured any areas of localized immune hemolysis. Attempts to couple the DNP residue directly to the red cell by reaction of

TABLE II

HEMOLYSIN-PRODUCING SPLEEN CELLS IN NON-IMMUNIZED SWISS MICE

WET WEIGHT OF SPLEEN (gm)	NUCLEATED CELLS PER PETRI DISH (millions)	PFC/PLATE*	$\overline{PFC}$/PLATE	PFC/10^6 NUCLEATED CELLS
0.1	4.68	1,0 - -	0.50	0.11
0.2	5.72	0,1,1,0	0.50	0.09
0.1	6.89	0,0,2,0	0.50	0.07
0.2	6.63	1,1,1,0	0.75	0.11
0.1	6.50	0,1,0,1	0.50	0.08
0.2	4.42	0,1,1 -	0.67	0.15
0.2	7.80	1,0,0 -	0.33	0.04
0.2	3.90	0,1,1,0	0.50	0.13
0.1	4.16	0,0,1,0	0.25	0.06

*Quadruplicate plates were prepared from each spleen.

TABLE III

ANTI-DNP AND HEMOLYSIN-PRODUCING SPLEEN CELLS IN NON-IMMUNIZED SWISS MICE‡

ANIMALS	NUCLEATED SPLEEN CELLS/ PLATE	PFC/PLATE*	$\overline{PFC}$/PLATE	PFC/10^6
1-5	35.8×10^6	5,4,3,4,2,0,4,3,4,10	3.9	0.11
6-10	20×10^6	2,4,2,4,6,2,0,4,2,2	2.8	0.14

‡DNP-red cell conjugates used as indicator cells.

*10 plates were prepared from each of 2 pools of spleen cells. These pools were each prepared from spleens of 5 mice.

TABLE IV

COMPARISON OF SEVERAL IMMUNIZATION SCHEDULES IN MICE

Immunogen: DNP-Bovine Gamma Globulin conjugate

IMMUNIZATION ROUTE	BGG-DNP INJECTED ON DAY 1	BGG-DNP INJECTED ON DAY 4	DAY 5	PFC/10^6 NUCLEATED SPLEEN CELLS*
	mg	mg		
Intraperitoneal	2	2	Sacrifice	5.1
Subcutaneous (Emulsified in Freund's Adjuvant)	2	-	"	0.8
Subcutaneous	2	-		
Intravenous	-	2	"	0.7
Subcutaneous	2	-	"	1.0
Intraperitoneal	-	2		
Intravenous	2	-	"	8.8
Intraperitoneal (Emulsified in Freund's Adjuvant)	2	-		
Intraperitoneal		2	"	2.4
Non-immunized				0.5

*The average of quadruplicates each plated from a pool of spleen cells prepared from 5 animals.

TABLE V

IMMUNE RESPONSE TO VARIOUS DOSES OF DNP-BOVINE GAMMA GLOBULIN CONJUGATES ADMINISTERED INTRAPERITONEALLY IN MICE

BGG-DNP INJECTED ON DAY 1	BGG-DNP INJECTED ON DAY 4	DAY 5	PFC/10^6 NUCLEATED* SPLEEN CELLS
mg	mg		
6	0	Sacrifice	7.1
4	0	"	2.9
2	0	"	5.1
6	2	"	17.9
4	2	"	16.7
2	2	"	7.3

*The average of quadruplicates plated from pools of cells, each prepared from spleens of 5 animals.

$DNPSO_3H$ were also unsuccessful.

When FDNB was reacted with red cells, under the same conditions but for shorter reaction times than had been used for DFDNB (164), the sensitized cells could be used successfully as indicator cells in the plaque assay. Initially, attempts were made to standardize these cells by passive hemagglutination. The rabbit anti-DNP serum used for hemagglutination revealed titres of 3200-6400 when tested with red cells sensitized with HSA-DNP by the BDB technique (163); cells sensitized directly with DNP by the Ling method were not agglutinated by this serum. Attempts to couple larger amounts of DNP to the red cells (by prolonging the reaction time) resulted in hemolysis and so this standardization approach was abandoned. The ineffectiveness of FDNB in preparation of red cells for use in hemagglutination has been reported elsewhere (165).

4. Variables of the Conjugation Reaction

The effects of various reaction parameters on the efficiency of the detection of PFC were studied as indicated in Figures 5-8.

(a) Concentration of reactants: It was found that concentrations of DNP higher than that routinely used (0.3 ml of a 2% acetone solution of FDNB in 30 ml of the reaction mixture or 0.2 mg/ml final concentration) were not soluble in the reaction mixture. Because of the relatively large numbers of cells required for the assay, it was impractical to decrease the cell number per reaction mixture, thereby increasing the relative FDNB concentration. For these reasons, investigation of the effects of concentration changes was not feasible.

(b) Reaction time and temperature: The optimum reaction time was 9 minutes at $37^{\circ}C$ when bovine serum albumen was omitted from the washing

FIGURE 5: Detection of PFC with indicator red cells sensitized at 22°C or 30°C.

Washing buffer and media contained 1% BSA.

broken and dashed lines - immune spleen cell suspension.
solid line - nonimmune spleen cell suspension.

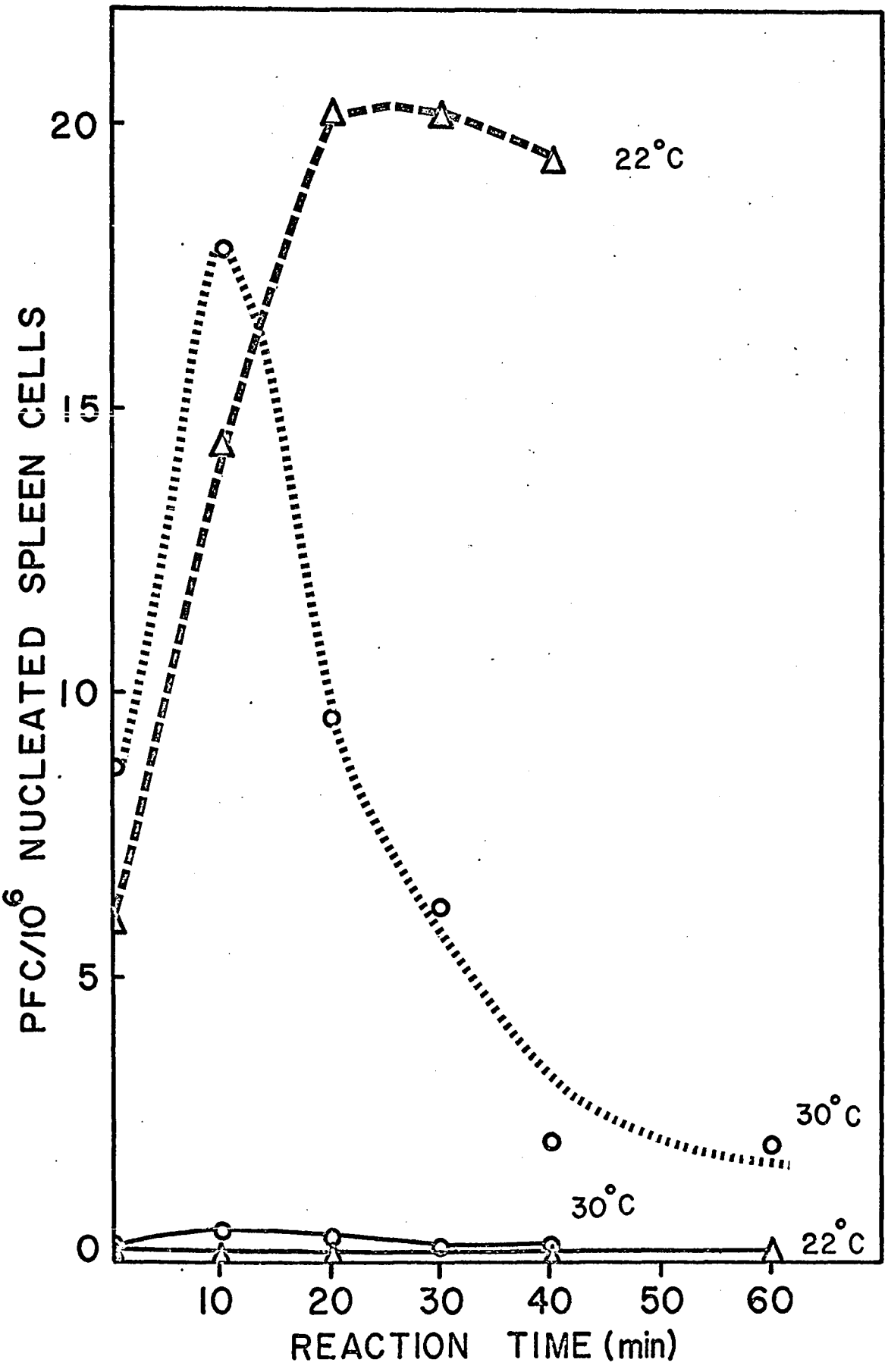

20
15
10
5
0
PFC/10^6 NUCLEATED SPLEEN CELLS
22°C
30°C
30°C
22°C
10
20
30
40
50
60
REACTION TIME (min)

FIGURE 6: Inhibition of plaque formation by free ε-DNP-L-lysine.

(5 mg/ml lymphocyte suspension)

Indicator cells were prepared at 37°C. Washing buffer and media contained 1% BSA.

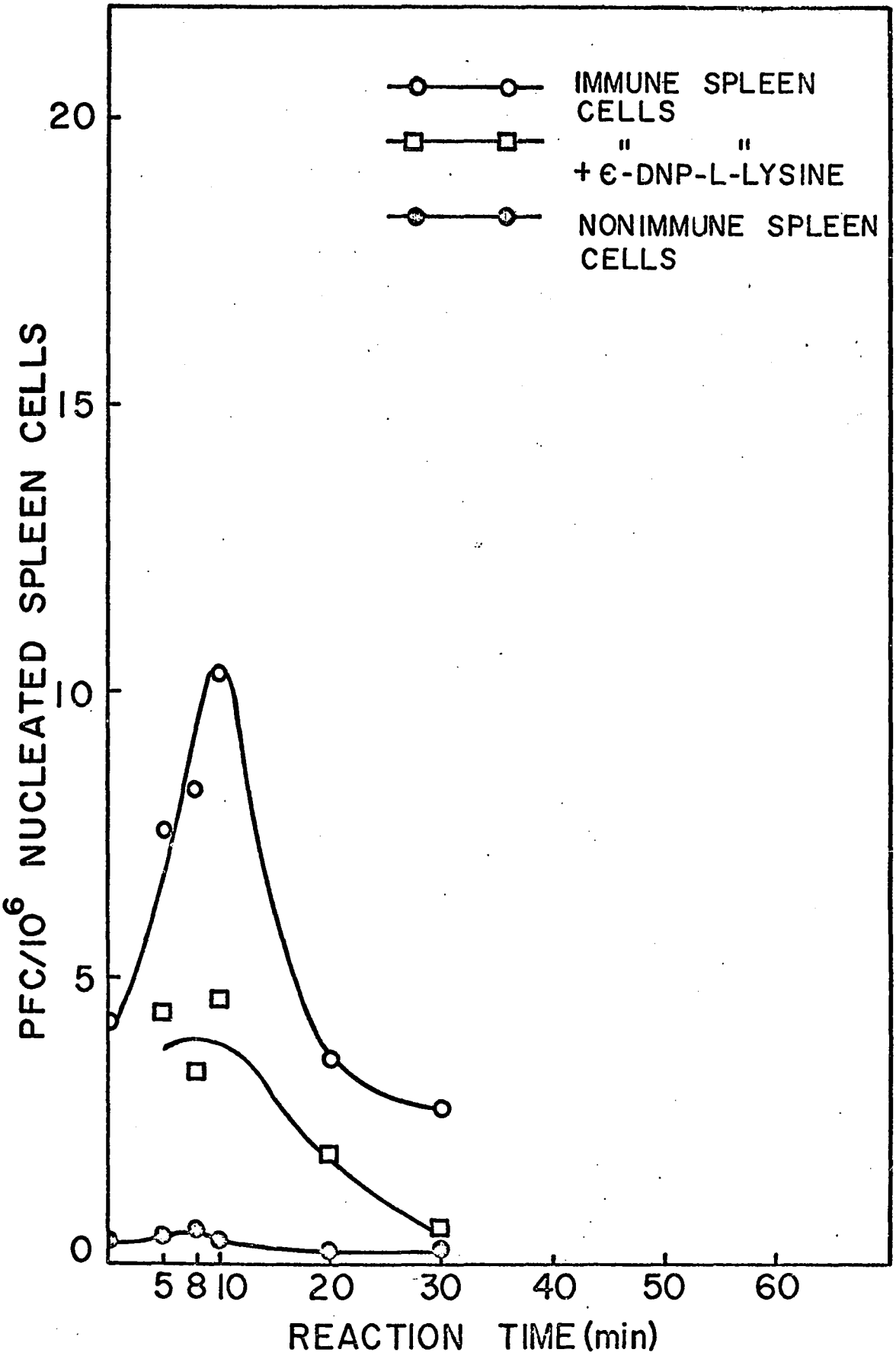

IMMUNE SPLEEN CELLS
" "
+ ε-DNP-L-LYSINE
NONIMMUNE SPLEEN CELLS
PFC/10^6 NUCLEATED SPLEEN CELLS
0
5
10
15
20
5 8 10 20 30 40 50 60
REACTION TIME (min)

FIGURE 7: Detection of PFC with red cells sensitized at 25°C or 30°C. No BSA added to washing buffer or media.

broken and dashed lines - nonimmune spleen cell suspension.
solid line - immune spleen cell suspension.

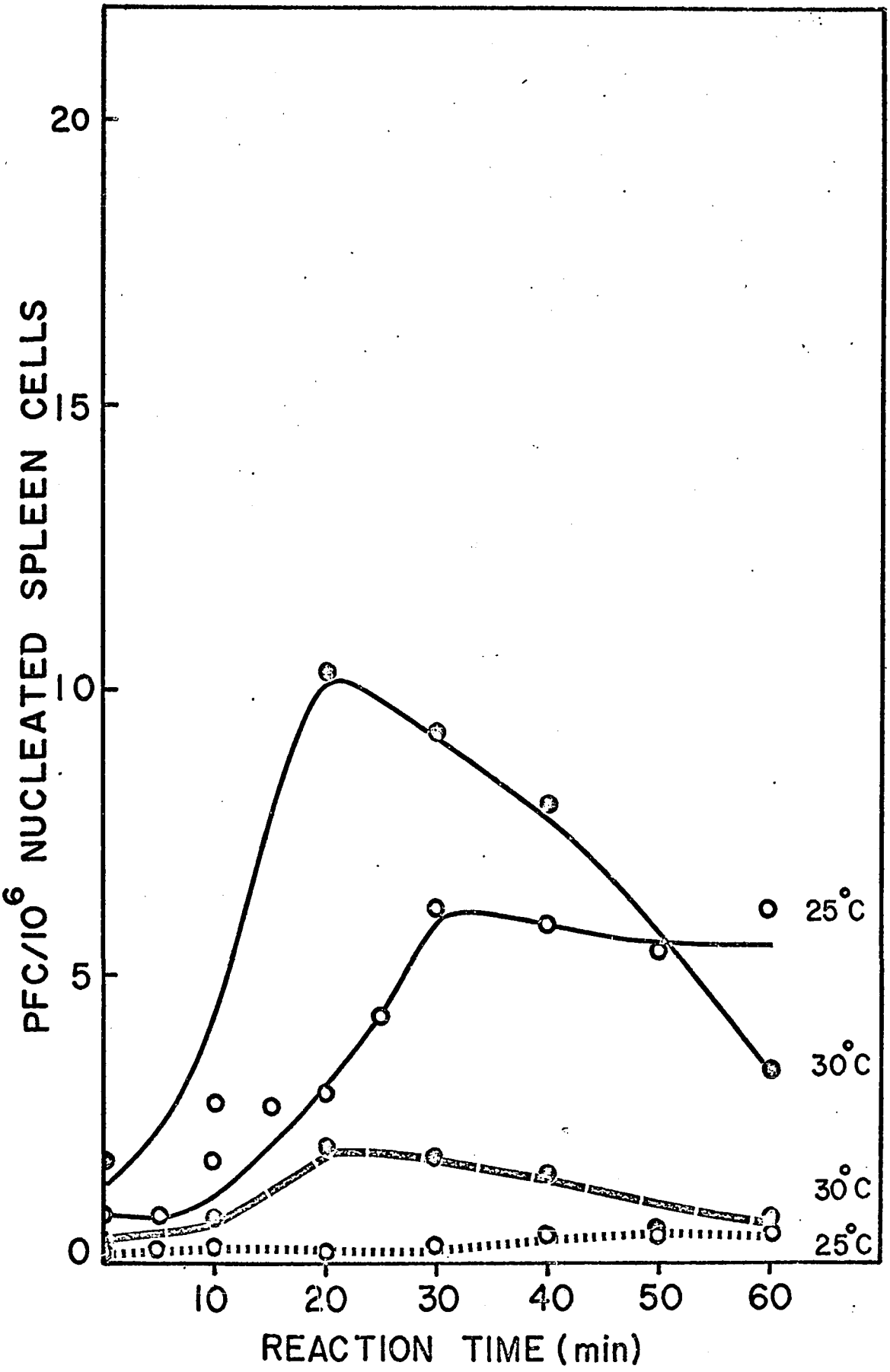

20
15
10
5
0
PFC/10^6 NUCLEATED SPLEEN CELLS
25°C
30°C
30°C
25°C
10
20
30
40
50
60
REACTION TIME (min)

FIGURE 8: Detection of PFC with red cells sensitized at 37°C. No BSA added to washing buffer or media.

circles - immune spleen cell suspension.
triangles - nonimmune spleen cell suspension.

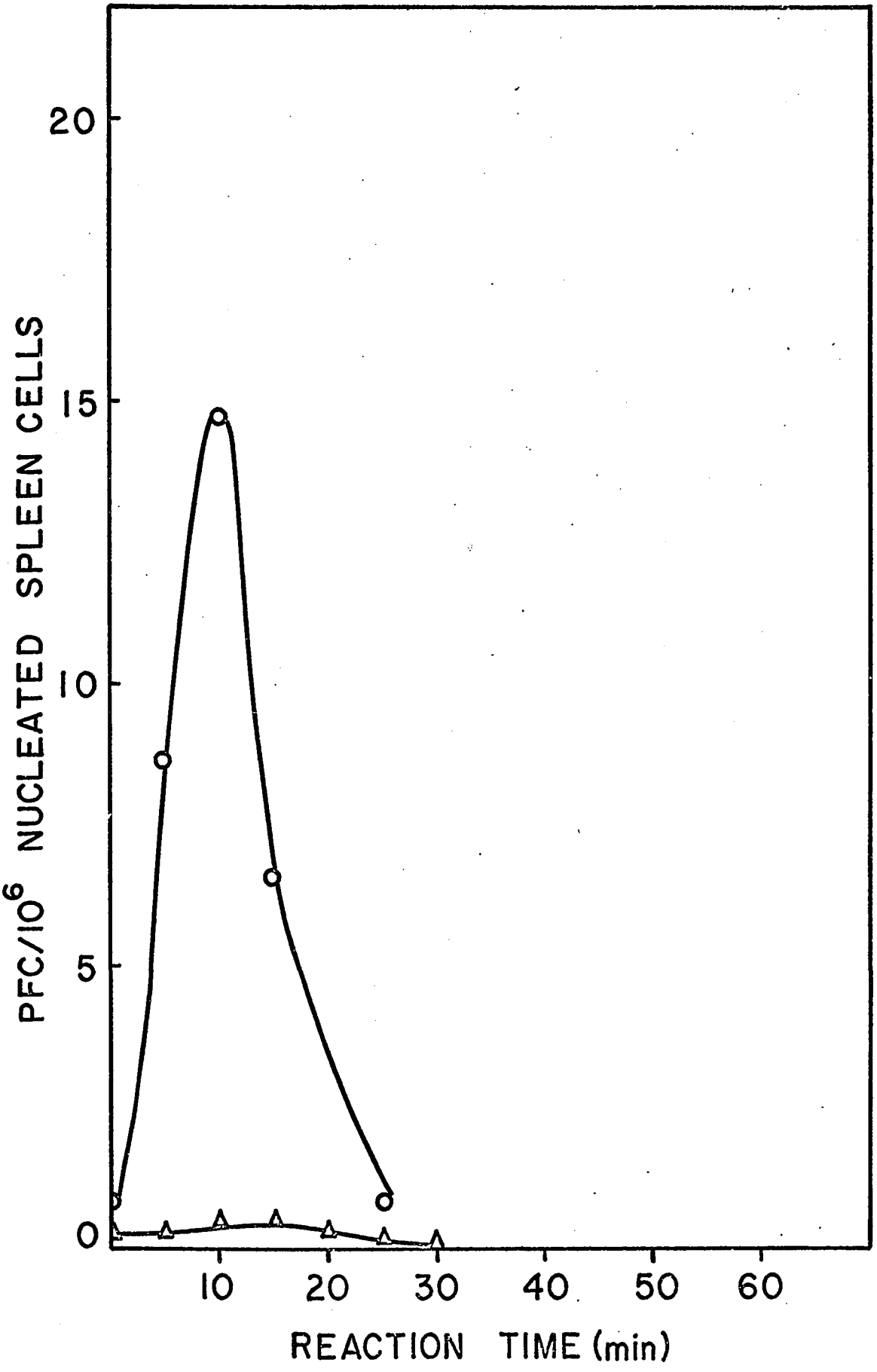
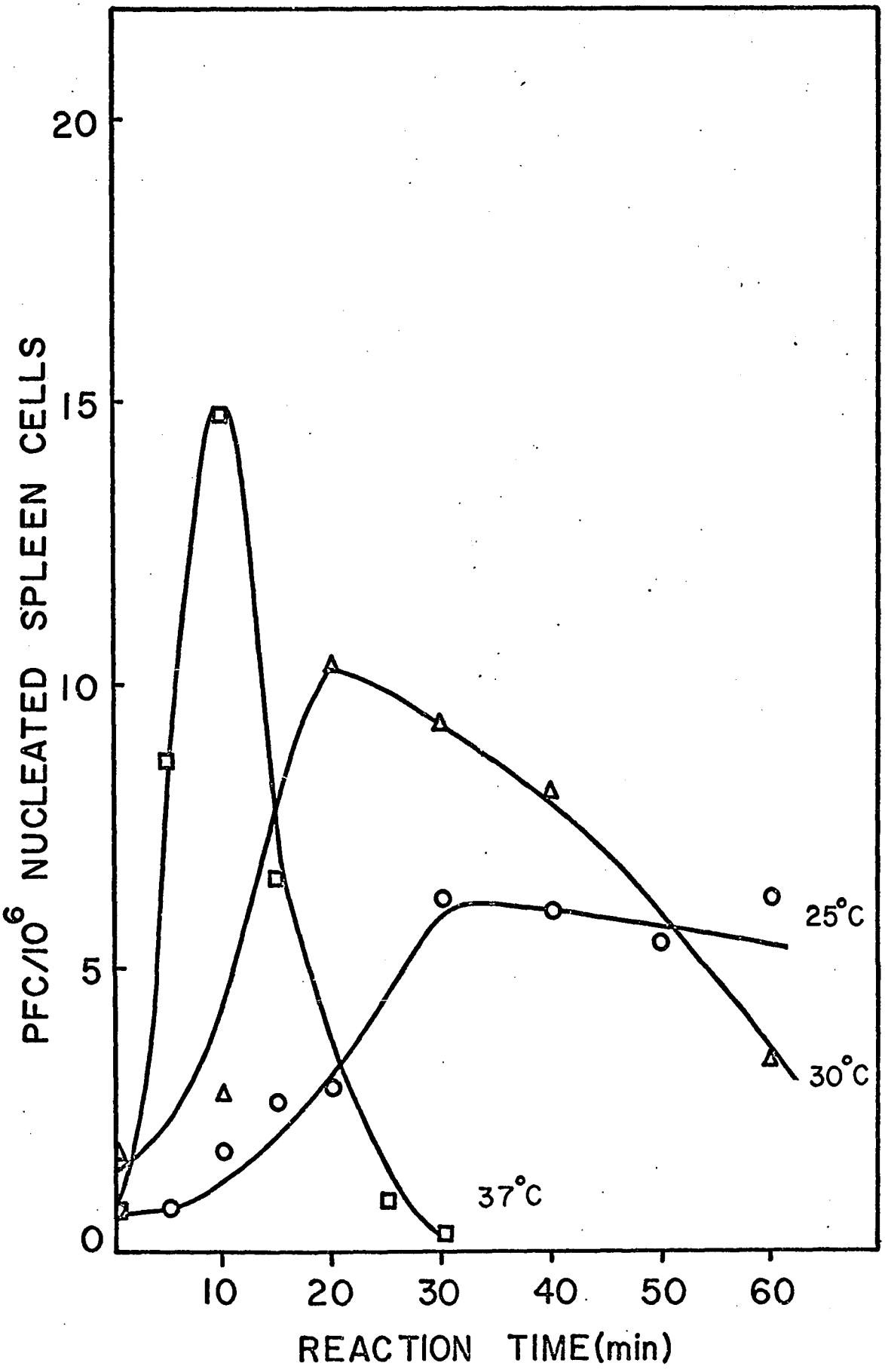

20
15
10
5
0
PFC/10^6 NUCLEATED SPLEEN CELLS
10
20
30
40
50
60
REACTION TIME (min)

FIGURE 9: Effect of reaction temperature on:

(i) PFC detection by sensitized red cells.
(ii) fragility of sensitized red cells.

Immune spleen cell suspensions were used.


20
15
10
5
0
PFC/10^6 NUCLEATED SPLEEN CELLS
10
20
30
40
50
60
REACTION TIME(min)
25°C
30°C
37°C

buffer and medium. Under these conditions the reaction time was critical since fragility of the resulting sensitized red cells increased markedly. By contrast, when sensitized cells were washed and suspended in solutions containing 1% BSA their stability was greatly increased; under these conditions, the optimum reaction time was 20 minutes at 22°C. Since the number of antibody-producing cells detected might be related to the number of haptenic groups coupled to the red cell surface and this, in turn, is determined by the rate of reaction, it is evident that the number of PFC detected will be affected by the temperature of conjugation.

Increasing cell fragility and lysis become important after the optimum reaction time. This effect is also temperature dependent, as illustrated by the slope of the curves in both Figures 5 and 9. A summary of studies on reaction time and temperature is given in Table VI.

5. Inhibition Studies

The addition of free ε-DNP-L-lysine to the lymphoid cell suspension was shown not to have a deleterious effect on the PFC of normal spleen cell suspensions, but it did depress significantly the number of plaques formed by immune spleen cell suspensions. These observations, outlined in Table VII and Figure 6, are indicative of the specificity of the assay method.

6. Number of Hemolysin-Producing Cells in Immunized Mice

Immune cell suspensions showed a higher number of PFC when plated with normal red cells than did non-immune cells. This phenomenon, as shown in Figures 7 and 8, is apparently enhanced in the presence of BSA, as indicated by Figures 5 and 6.

TABLE VI

SUMMARY OF RED CELL SENSITIZATION DATA

BSA ADDED TO MEDIA	REACTION TEMPERATURE	OPTIMAL REACTION TIME	IMMUNE PFC/10^6 NUCLEATED SPLEEN CELLS / NON-IMMUNE PFC/10^6 NUCLEATED SPLEEN CELLS (both at optimal reaction time)
	°C	Minutes	
+	25	20	40
+	30	10	35
+	37	10	20
-	25	30	12
-	30	20	5
-	37	8-10	30

TABLE VII

SPECIFIC INHIBITION OF PLAQUE FORMATION BY ε-DNP-LYSINE*

LYMPHOID CELL SUSPENSION 2.5 ml (10^8 cells)	ε-DNP-LYSINE ADDED	PFC/10^6 NUCLEATED SPLEEN CELLS
	mg	
Immune	0	9.31
"	1	3.72
"	10	2.89
Non-immune	0	0.35

*No BSA added to media.

It was suspected that BSA might be adsorbed to the red cells and thus detect anti-BSA antibody-producing cells which had been stimulated by BSA contaminant present in the immunogen BGG-DNP. However, when washing solutions and media contained 0.1% BSA or 0.1% normal mouse albumen, cell suspensions from immune animals again revealed more PFC when plated with nonsensitized (normal) sheep erythrocytes than did cell suspensions from non-immune animals.

DISCUSSION

These findings are essentially similar to those reported recently by others (169-171) in that they demonstrate that the hemolytic plaque technique can be adapted for detection and enumeration of cells producing antibodies specific for haptens, provided that such molecules can be coupled to erythrocytes without damaging their membranes.

Since hemolysin-producing cells cannot be distinguished from anti-hapten-producing cells by the plaque technique, each DNP-red cell preparation was always plated with both normal and immune spleen cell suspensions. Plating efficiency for a given conjugate preparation was considered to be the ratio between PFC revealed by spleen cells from immune animals and PFC revealed by spleen cells from non-immune animals. Immune animals used in standardization of the sensitization procedure were all treated in an identical manner and their spleen cells were pooled so that effects due to any individual variation in antibody response would be obviated. The background level appeared to increase very slightly as more DNP groups were coupled to the red cell surface. Although the significance of this observation is unknown, it is possible that more spontaneously arising "natural" anti-DNP-producing cells may be detected as more DNP groups are conjugated to the red cell surface.

1. Immunization Schedules

The lymphoid tissue draining the site of immunogen administration is

thought to respond first with antibody formation. Thus immunogen injected intravenously would pass directly to the spleen and antibody response should be visualized early in the spleen; immunogen administered intraperitoneally should also reach the spleen quite early in the course of immunization and spleen response should be evident early. This was in fact borne out by the immunization schedules considered, as shown in Table IV. Immunogen diffuses slowly to the draining lymph node following subcutaneous injection and in the short run, little antibody response is observed. Immunogen emulsified in adjuvant is released still more slowly from the site of injection and response is still later, as shown in Table IV; adjuvant also apparently slows the availability of immunogen injected intraperitoneally, although the mechanism by which this occurs is not evident.

2. Method of Conjugation of DNP to Red Cells

Erythrocytes coupled to HSA-DNP by the BDB procedure were used satisfactorily in the detection of humoral anti-DNP antibodies by passive hemagglutination, but such erythrocytes proved highly fragile in the hemolytic plaque assay undergoing generalized hemolysis. In the plaque technique, red cells were added to a 45°C agar solution and were subsequently incubated 1½ hours at 37°C while cells used in hemagglutination were never subjected to temperatures higher than 22°-25°C. Red cells used in the plaque technique must of necessity be more stable than those used in hemagglutination.

Erythrocytes to which the hapten was coupled directly by reaction with FDNB were unsatisfactory for the detection of humoral anti-DNP-BGG antibodies by passive hemagglutination. However, detection of antibodies by red cells directly coupled to diazotized arsanilic acid (159) or DFDNB (165) has been reported. In the latter case it was pointed out that FDNB was not efficient in detection of humoral anti-DNP antibodies and furthermore that handling of the cells subsequent to their sensitization was critical to their preservation (16

The coupling of DFDNB to red cells in buffer systems other than EDTA yields an unstable product (177).

The failure of cells reacted with FDNB to detect humoral antibodies is unexplained. Perhaps hapten residues when linked directly to the red cell surface have a tendency to become hidden in the convolutions of the cell wall and, therefore, are sterically less available for reaction with humoral antibody; consequently, the red blood cells could not be cross-linked as effectively into detectable aggregates. However, if a very large number of groups could be linked directly to the cell surface under conditions which did not render the cell too unstable, such a cell might be useful in hemagglutination, as indicated by other workers (159,165). The claim that only one antigen-antibody complex per erythrocyte in the presence of complement may be sufficient for hemolysis (118) would support the idea that lightly coupled red cells (and, therefore, more stable), while ineffective in hemagglutination, might be effective in the hemolytic plaque assay.

A single BDB molecule is capable of linking a large protein molecule carrying many haptenic residues to the red cell. Such a complex, perhaps inherently unstable, is very effective in hemagglutination. The use of modified reaction conditions might yield BDB sensitized red cells stable enough for use in the plaque technique. The importance of buffer systems in the FDNB coupling reaction has already been noted (164). A similar phenomenon may exist in the BDB system. Alternately, if hemolytic efficiency were dependent on the nearness of the antigen-antibody reaction to the red cell wall, DNP-erythrocyte conjugates would lyse more readily on combination with anti-DNP antibody than would BGG-DNP-erythrocyte conjugates. This is reasonable since antigen-antibody interaction close to the cell surface could conceivably cause the conformational changes in the cell wall necessary to hemolysis. If this were the case, BGG-DNP-erythrocyte conjugates would

be inherently ineffective in the detection of anti-DNP antibodies in hemolytic systems.

Two opposing reactions are taking place in the conjugation system. The first is the reaction of FDNB with nucleophilic groups on the red cell surface. Ideally, the more DNP residues which react with the red cell, the more sensitive will the cell become as an indicator but, due to steric considerations, certain DNP groups on the cell surface will be unavailable for reaction with antibody. The number of residues coupled would be expected to increase with reaction time and the rate of the reaction should be temperature dependent. In all cases studied, this is apparently true up to a certain optimal reaction time, as seen in Figure 9. At this point, the second, probably complex reaction, manifested as increased red cell fragility, becomes significant. Fragility of the DNP-erythrocyte conjugates is also temperature dependent; it increases with the reaction time and temperature.

Hemolysis results from the disruption of the "state of order" of components of the red cell surface and perhaps the interior (178). Red cells show various degrees of resistance to hemolysis (179) and the degree of this resistance is effected by changing the nature of the cell suspension, pH or the temperature of the suspension (178). The effects of temperature on hemolysis are more complex than would be the case for a simple reaction. Under certain conditions, heating may result in irreversible changes in the resistance to lysis (180). Studies on normal human blood indicated that heating to $46^{\circ}C$ for up to one hour did not result in any visible abnormalities, but between $47^{\circ}C$ and $50^{\circ}C$ certain changes occurred in the red cells which depended on the duration of the heating (181). The actual mechanism of these changes was unclear but they were accompanied by changes in the shape of the red cell. It is possible that the reaction of a chemical group with the red cell surface might lower the threshold for heat induced hemolysis

of the cell by alteration of the conformation of certain critical structural proteins.

3. Efficiency of Detection

Under optimal detection conditions, the spleen PFC of animals which had received two intraperitoneal injections of BGG-DNP were increased 40-fold over background levels. The number of rabbit spleen PFC was 50-fold greater on day 4 after a single subcutaneous injection of arsanilic acid-BSA conjugate (171). These results are very similar although BGG-DNP injected into mice under these conditions does not give such a large early response. This difference may be due to differences in the immunogenicity of the conjugates used or to the species difference between test animals. On day 4, after a single intravenous injection of sheep red cells into AKR mice, a 10,000-fold increase in the number of PFC (or 200 PFC/10^6 spleen cells) was observed (150); other workers have reported similar values when red cells were used as antigen (182). It is understandable that the response to a single antigenic determinant would be markedly less than the response to red cells which carry a large number of different antigenic determinants. A hapten would be capable of eliciting response in proportionally fewer immunologically competent cells than would multideterminant antigens.

4. Specificity of Detection

The inhibition of plaque formation by free ε-DNP-L-lysine indicates that this detection method is specific. Although the degree of inhibition increased with increasing free ε-DNP-L-lysine, inhibition was not complete. Complete inhibition may be achieved *in vitro* by free hapten, but the system under study here is a dynamic one in which the quantity of antibody present is not constant; antibody is continually being synthesized by immune cells. Furthermore, combination of hapten and antibody is reversible, whereas antibody-red

cell combination results in hemolysis, which is an inherently irreversible process. Therefore, even with large hapten concentrations, inhibition is not expected to be complete.

5. Stabilization of Cells

The protective effect of BSA, illustrated by comparison of Figures 5 and 7, is unclear. It has been reported that the proper pH of solutions used for the washing of sensitized cells is very important for cells stability (165). BSA is capable of interaction with many ions so the observed effect might be one of pH of tonicity. It should be pointed out, however, that under no conditions thus far investigated could sensitized cells be prepared in advance and stored. In all results presented here, they were prepared just prior to use in the plaque assay.

6. Hemolysin-Producing Cells in Immunized Mice

When either BSA or mouse serum albumen was added to the washing solution and media, the cell suspensions from immunized animals on incubation with unsensitized erythrocytes revealed a significantly larger number of PFC than did non-immune cell suspensions (cf. Figure 5). A smaller but similar result was observed when BSA was omitted from solutions and media (cf. Figure 7). Although splenic bacterial infection could cause localized hemolysis, it is highly unlikely that such a result could be consistently due to bacterial infection in the spleens of immunized animals; bacterial infection should be expected to occur randomly in both immunized and non-immunized animals. Furthermore, the precaution of adding penicillin to all media minimized the possibility of observing any localized red cell hemolysis due to bacteria. Perhaps BSA, by stabilizing the sensitized red cells, and thereby increasing the sensitivity of the assay, has magnified an effect which was also present in the absence of BSA.

Since BGG and erythrocytes both possess many antigenic determinants, it is possible that they contain a few identical or similar groups which could cross-react. If this were the case it would be expected that such an effect would be decreased if a well-defined immunogen such as DNP-poly-L-lysine were used, since cross-reaction in this case would be minimal.

A third explanation might be the nonspecific adsorption of BSA onto the red cell surface. The immunogen BGG-DNP no doubt contained small amounts of BSA contaminant which would have also elicited an immune response; any BSA adsorbed onto red cells could conceivably detect anti-BSA-producing cells. Although it is generally necessary to tan erythrocytes before proteins can be adsorbed onto them for use in hemagglutination or the plaque assay (170), adsorption might occur in this system since the number of BSA molecules per red cell is very large. Consequently, however, there must also be a large number of free BSA molecules in the agar layer and these would be expected to react with anti-BSA antibody before it could react with BSA adsorbed to the erythrocytes.

A generalized stimulation of the immune mechanism is known to occur during active immunization; this phenomenon is ill understood but could be responsible for the increased number of anti-erythrocyte-producing PFC resulting from immunization with BGG-DNP. That the same effect is observed with low concentrations of BSA and mouse serum albumen appears to support this suggestion.

CONCLUSIONS

As pointed out earlier, the plaque assay, like other single cell techniques, is not quantitative in terms of the number of antibody molecules being produced and detected by a single cell. Furthermore, since the assay requires the sacrifice of the animals being studied, it is less satisfactory than hemag-

glutination for investigations using large animals or time course studies which require large numbers of animals.

As originally presented (150), the assay detects primarily 19S-producing cells which are predominant in the early course of immunization. Recent modifications of the technique (155) which permit the detection of 7S-producing cells, predominant later in the immune response, were not used in this study since the assay was routinely performed four days after the initial immunization; at this stage few 7S-producing cells would be present in the spleen.* The possibility of detecting 7S-producing cells greatly enhances the versatility of the plaque assay. For example, it is now possible to compare the 19S-and 7S-producing cell populations in the same animal or group of animals at a given time in immunization schedule.

Adaptation of the Jerne technique for the enumeration of anti-DNP antibody-producing cells indicates the versatility of this powerful technique and further extends its utility. Because of the fragility of the sensitized red cell, satisfactory coupling conditions must be worked out for each hapten. As indicated, background levels are consistent and low and this provides for a very sensitive enumeration of antibody-producing cells. Although the test is not as sensitive as phage neutralization, it has found more general use in immunological single cell studies because of its simplicity and rapidity.

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*Supporting evidence for the predominance of 19S hemolysins at this stage might have been obtained by the incorporation of mercaptans into the medium; these compounds are known to degrade 19S antibody to 7S moieties devoid of antibody activity. This aspect was not explored in this investigation.

CHAPTER IV

THE INDUCTION OF ANTIBODY FORMATION BY AN INFORMATIONAL MACROMOLECULE

The specific, defining characteristics of differentiated cells have been sought for a long time. Since the organism's genotype must remain essentially constant throughout all body cells, phenotypic restriction is ascribed to selective gene activation as might be manifested by characteristic populations of RNA in different cells of the body. An informational RNA has been implicated as the inducer of differentiation since incubation of undifferentiated tissue with RNA isolated from differentiated cells causes differentation (183). Lack of tissue specific effects in these experiments has suggested to some that this RNA on hydrolysis merely yields an increased supply of nucleotides for the undifferentiated cells (184).

The concept of an informational macromolecule in antibody formation has suggested a means by which both macrophages and lymphoid cells might participate in the immune response. In this model the macrophage supposedly processes foreign antigens differently from self-antigens and prepares a specific macromolecule or macromolecule-antigen complex which is then transferred to immunologically competent cells. These cells are thus induced to divide and differentiate, simultaneously producing specific antibody. The intimate association of certain lymphocytes and macrophages in immune non-malignant tissue has suggested a means by which information transfer might occur (113). The identification of cytoplasmic bridges between macrophages and lymphocytes in such cell clusters has led to the suggestion that ribosomes may even be transferred between these cells (115). The long term retention of antigen in association with RNA and the heightened antigenicity of such complexes further support these ideas (185).

Fishman et al have reported the formation of specific antibody by

lymphocytes following the incubation of these cells with ribonuclease sensitive material extracted from macrophages which had been exposed to antigen in vitro (186). RNA extracted from lymph nodes draining the site of a rejected homograft transferred transplantation immunity to non-immune spleen cells (187). The ribonuclease sensitive extract from immune spleens was capable of immunizing normal mice (188). Askonas and Rhodes found that RNA capable of inducing antibody formation in non-immune tissue was associated with antigen. In one case, this RNA was extracted from macrophages immediately after their exposure to antigen; this would seem to indicate that the synthesis of a specific RNA is not necessary for the occurrence of this phenomenon. Incubation of non-immune spleen cells with RNA isolated from immune spleens has been reported to give rise to strain specific induction of antibody formation as assayed by the hemolytic plaque assay (176).

Because the plaque assay provides a uniquely simple and sensitive assay for induced antibody-producing cells, it was proposed to study the induction of anti-hapten formation by a similar procedure. Studies on the erythrocyte system in both random bred and highly inbred mice indicated that induction was very inefficient. Since haptens are much less immunogenic than polyvalent immunogens, such as erythrocytes, similar studies on the hapten-anti-hapten system were not considered feasible. Results of the erythrocyte system are outlined below.

METHODS AND MATERIALS

1. Immunization

Either Swiss or $B6AF_1$ (Jackson Laboratories, Bar Harbor, Maine) mice, 6-10 weeks old, were used for these experiments. The animals from which RNA was to be extracted were given intraperitoneal injections of 0.1 ml of 25% suspension of washed sheep erythrocytes on day 1 and day 4; they were killed on day 5 and their spleens were excised and immediately frozen on dry ice in

preparation for RNA extraction. Hemolytic plaque assays performed on sample animals from both Swiss and $B6AF_1$ groups at this time indicated that both strains responded very well to sheep erythrocyte immunization.

2. RNA Extraction

While still frozen the spleens were homogenized in equal parts of freshly distilled, buffer-saturated phenol (Fisher Certified Reagent) and 0.01 M acetate buffer (pH 5.0) containing 0.5% sodium dodecyl sulfate (Matheson, Coleman and Bell) recrystallized from ethanol and 2 mg Bentonite (190). About 15 ml each of buffer and phenol were used for every 10 mouse spleens being extracted. Following homogenization the solution was heated to 65°C for 6 minutes in a water bath. It was then immediately chilled to room temperature in an alcohol-dry ice mixture. After centrifugation at 25,000 g (10°C) for 3 minutes, the aqueous layer was removed. The phenol layer and interface were re-extracted at 20°C for 5 minutes with an additional half volume of fresh buffer. The aqueous layer resulting from recentrifugation was pooled with the first aqueous layer. The pooled aqueous layers were extracted twice with a half volume of phenol for 5 minutes at room temperature. All extractions were performed in iodine flasks with continuous shaking. The final aqueous layer was precipitated at -20°C by adding 2.5 M sodium acetate to a final concentration of 0.3 M and 2 volumes of absolute alcohol. After two hours the flocculent white precipitate was packed by centrifugation and was washed 6-8 times with 66% ethanol at 0°C. The precipitate was kept under ethanol until use; it was never stored for longer than two days. This is similar to the procedure reported by Cohen et al (176). The purity of the extracted RNA was estimated by the ratio of its optical density at 260 mμ to that at 280 mμ and the nature of the RNA was usually determined by the sucrose density gradient pattern of the sample. Just before use in the the incubation step, the RNA was dissolved in medium 199.

3. Sucrose Density Gradient Patterns

From a stock solution of 40% sucrose (Merck and Co., Inc., Rahway, N.J.) and acetate buffer which consisted of 0.01 M sodium acetate and 0.1 M sodium chloride adjusted to pH 5.0 with 1 M acetic acid, sucrose solutions ranging from 4-20% were prepared. The density gradients were prepared by carefully layering volumes of 0.2, 0.8, 1.0, 1.0, 1.0 and 0.8 ml of 40%, 20%, 16%, 12%, 8% and 4% sucrose solutions respectively into 5 ml nitrocellulose tubes. These gradients were allowed to stand overnight at 4°C and the following day 90 γ of RNA in not more than 0.2 ml volume of buffer were layered onto the top of each gradient. Following two hours centrifugation (6°C) at 60,000 rpm in a Beckman L2-65 ultracentrifuge, in a swinging bucket rotor (SW-65), a syringe needle (No. 22) was inserted into the bottom of the tube and the gradient was collected dropwise in 0.1 ml fractions. The optical density at 260 mμ was read in a Beckman DU spectrophotometer.

4. Incubation Procedure

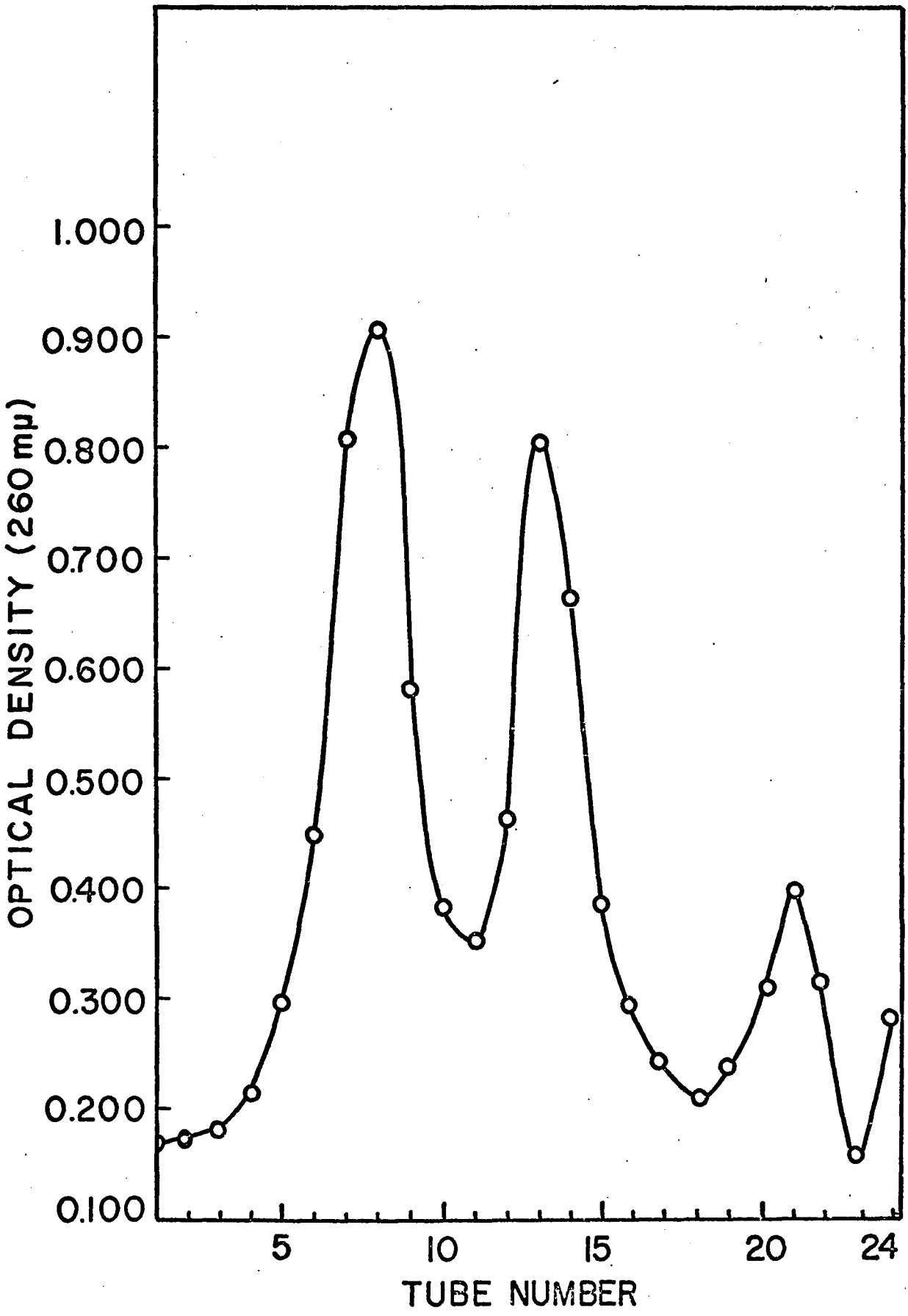
Recipient spleen cells were collected from non-immune mice as shown in Figure 2. They were teased into medium 199 and were kept at 0°C until the addition of RNA following which they were incubated at 37°C for 15 minutes. An appropriate number of lymphoid cells was then plated with washed sheep erythrocytes in a soft agar layer, as outlined in the previous chapter. The plaque assay was identical to that performed previously except that incubation at 37°C was carried out for 3 hours; complement was then added and incubation at 37°C continued for an additional 30 minutes.

RESULTS AND DISCUSSION

The density gradient pattern shown in Figure 10 indicates that the method of RNA extraction employed can yield RNA which is relatively undegraded. The bulk of the RNA is in the two most rapidly sedimenting peaks which are

FIGURE 10: Sucrose density gradient pattern of RNA extracted as outlined in text - undegraded.

Tube 1 represents the bottom of the gradient.


1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
OPTICAL DENSITY (260 mμ)
5
10
15
20
24
TUBE NUMBER

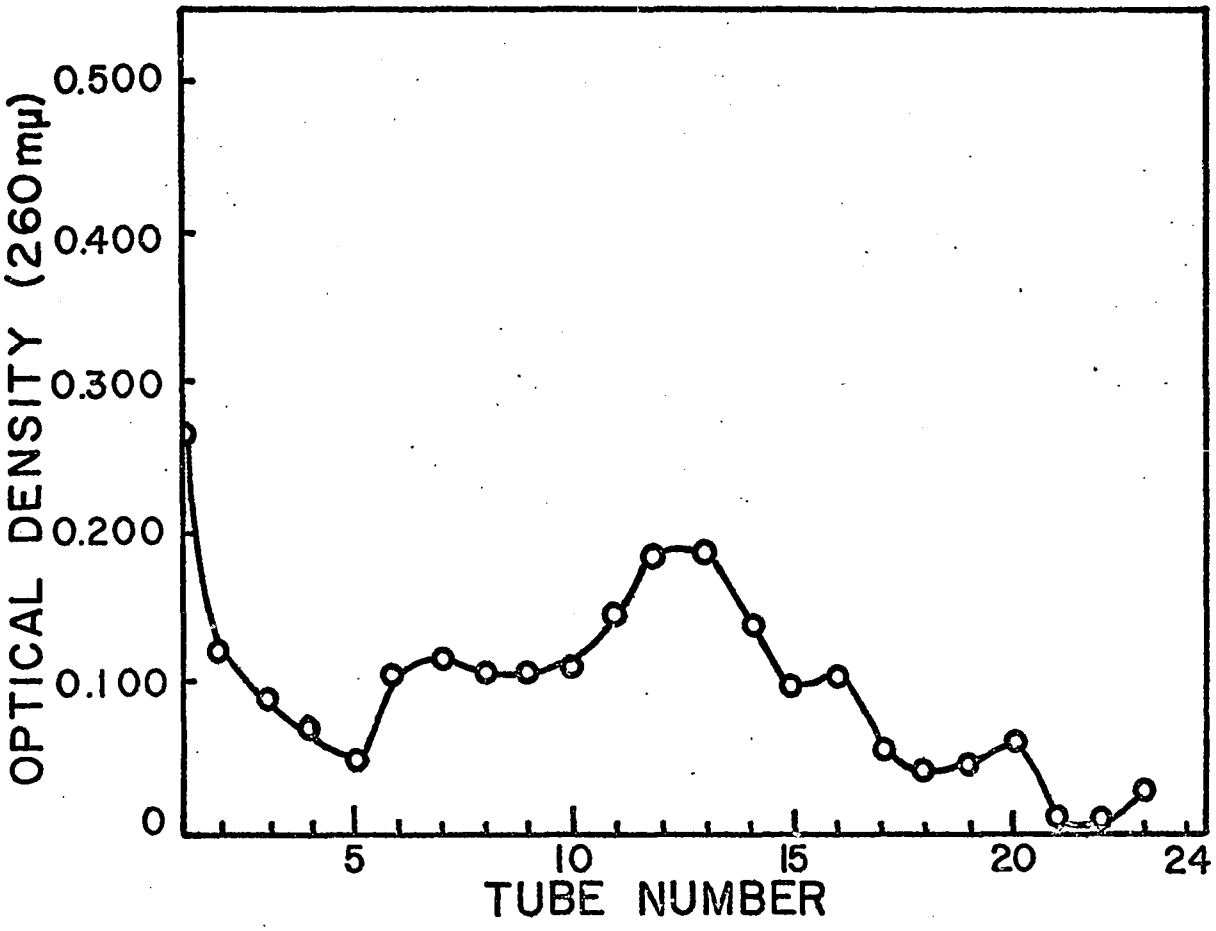


FIGURE 11: Sucrose density gradient pattern of RNA extracted as outlined in text - degraded.

Tube 1 represents the bottom of the gradient.

presumably ribosomal RNA peaks. Only a small proportion of the RNA is found in the slow sedimenting, low molecular weight peak, suggesting that a minimum of degradation has taken place during extraction. Degradation did occur occasionally, as shown in Figure 11; however, in this work the efficiency of induction of the immune response was not affected by this degree of degradation. A similar finding has been reported by others (191);when the ratio of optical densities at 260 and 280 mμ is about 2, the RNA sample is considered to be relatively protein-free and this ratio is, therefore, an estimate of sample purity. The OD ratio of RNA samples used in these experiments is indicated in the respective tables.

Tables VIII and IX show that there is little individual variation in the induction phenomenon. Furthermore, cells from outbred mice are induced equally well as those from inbred mice. This finding is in apparent contradiction with the finding that there is a strain specificity in RNA induced antibody formation (176). Evidently the induction phenomenon is quite marginal even though erythrocytes, known as potent immunogens, were used. The PFC values reported in the Tables for sample immune animals from the various groups of RNA donors indicated that these animals were at least as immune to the antigen as the animals used by others. Cohen reports a 3-10 fold increase in the number of induced PFC over the background value of PFC in spleens of normal mice (176). Friedman reports a 5-10 fold increase for a 30-60 minute incubation of immune RNA with non-immune cells; when incubation is extended up to 24 hours as much as a 50-fold increase is observed (192). The results presented here represent a 2-fold increase (cf. Table X) over background which is of the same order of magnitude as that reported for the same reaction conditions (176). Because of the marginality of the induced response to erythrocytes, a potent immunogen, it was highly unlikely that any meaningful data could have been obtained from the DNP system since the in vivo response to haptens studied here is 200 times smaller than the response

TABLE VIII

THE INDUCTION OF ANTI-ERYTHROCYTE FORMATION IN INDIVIDUAL SPLEENS OF SWISS MICE BY RNA EXTRACTED FROM IMMUNE ANIMALS

ANIMALS	RNA ADDED	CELLS/mgRNA ADDED	CELLS/PLATE	$\overline{PFC}$/PLATE	PFC/10^6	$\overline{PFC}$/10^6
	mg	Millions	Millions			
1	2	49	24	3.00	0.13	
2	2	56	17	3.67	0.22	
3	2	60	18	4.00	0.22	0.21
4	2	69	20	10.33	0.52	
5	2	113	49	5.67	0.12	
6	2	190	52	2.67	0.05	
7	0		20		0.17	
8	0		66		0.07	
9	0		14		0.19	0.16
10	0		37		0.21	

*RNA extracted from 19 immune Swiss mice (899 PFC/10^6 spleen cells by plaque assay on 4 such animals); 260:280 OD ratio = 1.88

TABLE IX

THE INDUCTION OF ANTI-ERYTHROCYTE FORMATION IN INDIVIDUAL SPLEENS OF 9 B6AF$_1$ MICE BY RNA EXTRACTED FROM 14 B6AF$_1$ MICE IMMUNIZED WITH SHEEP ERYTHROCYTES

ANIMALS	RNA ADDED TO SPLEEN CELL SUSPENSION	CELLS/mgRNA ADDED	CELLS/PLATE	$\overline{PFC}$/PLATE	PFC/10^6	$\overline{PFC}$/10^6
	mg	Millions	Millions			
1	0		3.1	0.66	0.21	
2	0		2.7	0.33	0.12	
3	0		1.6	0.66	0.41	0.4
4	0		1.5	1.33	0.89	
5	0		8.0	0.33	0.41	
6	1	9	1.1	1.66	1.51	
7	1	17	2.2	2.66	1.21	0.7
8	1	25	3.3	0.66	0.20	
9	1	28	3.6	1.00	0.28	

RNA sample 260:280 OD ratio = 2.08

TABLE X

THE INDUCTION OF ANTI-ERYTHROCYTE FORMATION IN POOLED SPLEEN CELLS OF 10 $B6AF_1$ MICE BY RNA EXTRACTED FROM IMMUNE $B6AF_1$ MICE*

ALIQUOT	CELL CONCENTRATION	RNA ADDED TO 1ml CELL SUSPENSION	CELLS/PLATE	PFC/ PLATE	$\overline{PFC}$	PFC/10^6
	Millions/ml	mg				
1	510	5	50	60,65,59,78	66	1.3
2	510	0	50	36,51,35,33	39	0.8

*RNA extracted from 43 $B6AF_1$ mice immune to sheep erythrocytes; 260:280 OD ratio = 1.85

3	850	3	17	28,18,40,15,34	27	1.59
4	850	0	17	11,13,17,17,15	15	0.88

*RNA extracted from 77 $B6AF_1$ mice immune to sheep erythrocytes (677 PFC/10^6 nucleated spleen cells by plaque assay on 5 such animals); 260:280 OD ratio = 1.9

to erythrocytes. Moreover, the in vivo response to immunogen is expected to be more efficient than an in vitro response.

The role of the macrophage in antibody formation is still an open question. Some experiments have indicated that the macrophage may be essential to the immune response (193). Alternately the role of the macrophage may simply be the clearance of foreign material and cell debris from the system; phagocytosis of antigen by macrophage is increased when antigen is complexed with antibody.

The macrophage may be capable of synthesizing an informational macromolecule specific for those antigenic determinants which it phagocytoses. In this case ribonuclease would eliminate macrophage-RNA induced antibody formation in non-immune tissue by destroying a critical portion of the messenger code. On the other hand, RNA complexed with antigen may act as an adjuvant or "super-antigen". Ribonuclease might reduce this complex below the minimum size required for immunogenicity. In this case the RNA responsible for the induction of in vitro antibody formation could be nonspecific (191). Such RNA complexed to antigen may concurrently stimulate cellular anabolism and differentiation by providing metabolic substrates to the cell (184). In this regard it should be pointed out that Cohen has reported the resistance to proteolytic enzymes of his antibody-inducing RNA preparations (176). It is possible that an antigenic determinant which has resulted from enzymatic degradation of an immunogen by macrophage or other cell may no longer contain groups which render it susceptible to further proteolysis. Alternately, RNA complexed to antigen may sterically inhibit proteolytic enzymes.

Although certain qualitative differences may exist between the RNA extracted from macrophages which have been exposed to antigen and RNA extracted from immune lymphoid tissue, it might be thought that RNA extracted from immune tissue would be more efficient than macrophage RNA in the induction of antibody formation. RNA extracted from immune tissue was shown here to be extremely

inefficient in the induction of primary antibody formation. Furthermore, since the system in question is certainly a highly artificial one, many questions may be raised as to its significance *in vivo*.

CONCLUSIONS

Because of the marginality of RNA induced antibody formation with the highly immunogenic sheep erythrocyte, it was considered impractical to study further the DNP hapten in the same system. Preliminary experiments corroborated this view-point. Furthermore, the results of such transfer experiments will remain equivocal until such time as pure cell populations can be used as RNA donors. Should the "informational macromolecule" concept be established and characterized *in vitro*, it will then remain to propose and prove a parallel *in vivo* system. At the present time it appears unlikely that such a scheme provides the principal *in vivo* means of recruiting new antibody-producing cells.

GENERAL DISCUSSION

A large number of questions remain unresolved in the field of immunology. Elucidation of the structure of immunoglobulins has, for example, raised many perplexing questions about their biosynthesis. The synthesis of peptide chains of which one half is constant and the other variable is a genetic phenomenon without precedence; the mechanism by which such a synthesis could be accomplished remains unclear. Furthermore, as a generic group of protein molecules, immunoglobulins are also unique in the degree of heterogeneity which they possess. The control mechanism for the assembly of these peptide chains into the completed immunoglobulin molecule is also obscure. Such questions require investigation by the most sophisticated techniques of molecular biology.

Many other immunological problems, however, will have to be answered by the use of cellular techniques. The mechanism of antigen action on cells during the inductive phase of the immune response is unknown. In fact, it remains to be resolved whether macrophage or lymphocyte is the primary cell type involved in the inductive phase. Since all cells of an individual must possess genetic information for the synthesis of all immunoglobulin classes but, at a given time, synthesize only one of these classes (and probably only one antibody specificity), a phenotypic restriction must be operative. Early in the course of immunization, 19S is the principal antibody being produced, while later antibody is composed largely of 7S. Whether this sequence represents the "switch" of single cells from 19S to 7S production or the sequential biosynthetic activity of two distinct groups of cells is unknown. An intricate feedback control mechanism by the antibody concentration in the circulation could explain both these phenomenon. Certain preliminary studies have indicated that such a mechanism is operative but as yet it remains essentially undefined. Single cell studies potentially could shed much light

on these cellular questions. Certain elaborations may be required in order that single cells or their descendant clones may be studied over a time course.

The plaque technique, by virtue of its simplicity and concrete reference point (figures are given in terms of a fraction of cells producing specific antibody), offers a rapid means of assessing the immunological state of an animal. Its adaptation to the use of various haptens opens a vista of possible genetic and biochemical investigations which may be performed with responder and non-responder animals (156).

To date the plaque technique has contributed significantly to the understanding of the cellular anatomy of antibody formation; the definition of certain lymphocytes and plasma cells as antibody-producing cells was made possible through use of the plaque technique. Because it provides a most satisfactory, simple assay for both in vitro tissue culture and in vivo systems and may now be used for the study of well defined immunochemical systems such as arsanilic acid and dinitrophenyl, the plaque technique is certain to prove invaluable to immunology in the future.

SUMMARY

1. The hemolytic plaque assay for the detection of cells producing anti-erythrocyte antibody was established. This technique was then adapted for the detection of anti-dinitrophenyl antibody-producing cells.

2. Red cells coupled to protein-dinitrophenyl conjugates by the bis-diazotized benzidine method, although satisfactory for hemagglutination studies, were unsatisfactory in the hemolytic plaque assay. A direct reaction between fluorodinitrobenzene and red cells was found to yield satisfactory albeit somewhat unstable sensitized red cell. Various parameters of this reaction were investigated and optimum conditions were established.

3. The difficulty of cell instability was overcome by the inclusion of protein in certain of the buffer solutions used.

4. Specificity of the hemolytic assay for anti-DNP producing cells was shown by the inhibitory effect of free ε-DNP-L-lysine.

5. An attempt was made to induce hemolysin production by RNA extracted from immune tissue as reported in the recent literature. Studies with both inbred and outbred mice gave marginal results, thus placing in doubt the claim of any strain specificity of such RNA. Furthermore, use of degraded and nondegraded RNA resulted in the same degree of induction.

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