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IN VIVO AND IN VITRO STUDIES ON THE ANTI-DNP ANTIBODY RESPONSE

by

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ABBREVIATIONS USED IN THIS THESIS

Å	angstrom (10^{-4} microns)
AFC	antibody forming cell
AFCP	antibody forming cell precursor
Ag	antigen
ARC	antigen reactive cell
B	bentonite
BDB	bisdiazotized benzidine
BRBC	burro red blood cell
BGG	bovine gammaglobulin
BSA	bovine serum albumin
BSS	balanced salt solution
C	complement
CFA	complete Freund's adjuvant
DIP	hydroxy diiodophenyl
DNA	desoxyribonucleic acid
DNP	2,4-dinitrophenyl
FCS	fetal calf serum
HA	hemagglutination
Hcy	hemocyanin
HSA	human serum albumin
[(H,G)-A--L]	poly-lysine backbone with side chains of poly-DL-alanine terminating in short random sequences of histidine and glutamic acid
Ig	immunoglobulin
i.p.	intraperitoneal
IPT	immunoprecipitin tested

i.v.	intravenous
KLH	keyhole limpet hemocyanin
2-ME	2-mercaptoethanol
µg.	microgram
λ	microlitre
µ	micron
mg.	milligram
ml.	millilitre
mM	millimolar
M	molar
MGG	mouse gammaglobulin
MON	monomeric flagellin
NIP	hydroxy nitroiodophenyl
OA	ovalbumin
Penn	potassium benzyl penicillin G
PFC	plaque forming cell
PFU	plaque forming unit
PLL	poly-L-lysine
PPD	purified protein derivative of tuberculin
POL	polymerized flagellin
RGG	rabbit gammaglobulin
RNA	ribonucleic acid
RtGG	rat gammaglobulin
SDNBS	2,4-dinitrobenzene sodium sulfonate
SRBC	sheep red blood cell
[(T,G)-A--L]	poly-lysine backbone with side chains of poly-DL-alanine terminating in short random sequences of tyrosine and glutamic acid
TNP	2,4,6-trinitrophenyl

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SUMMARY

A sensitive reagent for the detection and titration of anti-DNP antibodies in serum and tissue culture medium was developed by coupling the DNP-residue covalently to T_2-coliphage. The resulting DNP-T_2-phage conjugates were found to be completely neutralized by anti-DNP antisera. The optimum conditions for reproducibility of the neutralization assay were determined.

The responsiveness of Balb/c and CBA mice to DNP-BGG was compared *in vitro* and *in vivo*. In both cases, Balb/c mice were found to respond with higher titres to the DNP-residue and were therefore selected for further study.

The requirements conducive to the stimulation of a primary anti-SRBC antibody response *in vitro* were analysed. It was found that Balb/c spleen cells, rotated in the presence of antigen at 1/5 rpm in tissue culture tubes at 37°C, gave the most vigorous responses. Using these conditions, attempts were made to elicit an *in vitro* response to DNP with DNP-BGG coupled covalently to SRBC. The response elicited was low and therefore other antigens were investigated *in vivo*.

These antigens consisted of Balb/c mouse anti-SRBC gamma-globulins (MGG) and rabbit anti-SRBC gammaglobulins (RGG). An average of 2-3 DNP-residues were coupled per molecule of MGG and RGG. Both conjugates were individually allowed to react with SRBC to form DNP-MGG-SRBC and DNP-RGG-SRBC complexes, which were then injected into separate groups of Balb/c mice. The resulting anti-DNP and anti-SRBC antibody responses were analysed for IgM and IgG classes. No significant differences were observed in the

magnitude or antibody class elicited by these two antigens. It was concluded from the data that the sheep erythrocyte functioned as the carrier in the anti-DNP antibody response to the DNP-MGG-SRBC complex.

INTRODUCTION

The elucidation of the mechanism of induction of the antibody response is one of the most pressing problems in contemporary immunology. In order for a molecule to induce such a response, it must be presented to the cells of the immune system in an immunogenic form. Green et al (1968) and Dresser (1963) have illustrated this point with the following experiments. In the experiments of Green et al, working with Hartley strain #2 guinea pigs which are unresponsive to DNP-PLL, it was found that DNP-PLL, when bound noncovalently to methylated BSA, would then stimulate an anti-DNP response. Also, Dresser found that for BGG to be immunogenic, it must be presented in an aggregated form; aggregate-free preparations of BGG not only failed to immunize, but rendered the animals unresponsive (tolerant) to further challenge with the immunogenic form of BGG. The cells involved in the antibody response vary according to the immunogen used (Shortman et al, 1970; Feldmann et al, 1971), and have been grouped into three classes, corresponding to their apparent functions.

A. Macrophages: Shortman et al (1970) and Feldmann et al (1971) have shown that for some antigens, macrophages may play a major role in the initiation of the antibody response. In both studies it was shown that spleen cell suspensions, from which only the macrophages were removed by passing the suspension through a column of glass beads at 37°C, would not mount a normal antibody response to the intact SRBC antigen. In contrast, it was shown that the response to POL can be stimulated in the absence of macrophages; and that the requirement for macrophages could be circumvented by using a soluble

antigen prepared from the sheep erythrocyte (Feldmann et al, 1971). On the basis of these results, Feldmann has suggested that it is the size of the antigen which determines whether or not macrophages are required as accessory cells in the initiation of the antibody response.

In the past ten years, the macrophage has been assigned other roles in this response. For example, Fishman and Adler (1967) suggested that the macrophage induced the formation of antibodies via an antigen-RNA complex, which was formed within the macrophage and subsequently transferred to lymphocytes which were the antibody-forming cell precursors (AFCP). The experimental system consisted of implanting diffusion chambers into irradiated syngeneic rats. The diffusion chamber contained normal lymph node cells and cell-free extracts prepared from macrophages which had been previously incubated with T_2-coliphage. Some chambers contained also ribonuclease. 19S anti-T_2 antibodies were formed only in those rats which contained RNA-Ag complexes extracted from macrophages. Adler et al (1966) found that RNA-Ag complexes extracted from rabbit macrophages, which had been previously incubated with antigen, would induce the formation of antibodies allotypically related to the donor of the RNA-Ag complex. Askonas and Rhodes (1965) and Roelants and Goodman (1969) have a different opinion about the RNA-Ag complex. Both research teams agree that an antigen associated with RNA is more immunogenic than is free antigen, but they do not attribute the formation of the complex to anything more than an artifact of the experimental system. Askonas and Rhodes found that complexes could be formed immediately upon addition of the labelled antigen to the macrophages, and these complexes were as immunogenic

as those extracted after a 2.5 hour incubation period. They have suggested that the RNA is not a new informational unit, but simply a molecule already present in the macrophage which non-specifically imparts heightened immunogenicity to antigens. Roelants and Goodman studied ten different molecules and found that only negatively charged groups were able to form RNA-Ag complexes. They also found that the complexes were formed as readily from homogenates of HeLa cells as from homogenates of macrophages. Since the formation of the RNA-Ag moiety has not been shown to be a product of viable macrophages, they have concluded that the RNA-Ag complexes do not play a physiologically significant role in the immune response. Most of the above experiments do, however, emphasize that macrophages are involved in the initiation of an antibody response to some antigens.

B. Thymic derived cells (T-cells): Parrott and de Sousa (1971) implanted [^{3}H]-thymidine labelled neonatal thymus grafts into congenitally athymic mice in order to study the distribution of thymus-derived cells (the labelled lymphocytes) as well as the distribution of the indigenous non-thymic lymphocytes (the unlabelled lymphocytes). These two populations of lymphocytes, which in the blood and lymph are morphologically indistinguishable are, however, different with respect to their place of origin, migratory patterns and lifespan. One population migrates from the thymus to characteristic regions of the immune system, i.e., the periarteriolar sheaths of splenic Malphigian bodies, paracortical regions of lymph nodes, and recirculates in the blood and lymph. The other population of lymphocytes (defined by their lack of label) is found in splenic red pulp, primary follicles and the cortex of the lymph nodes; it is

in fact, the B-cell compartment which is discussed below. Further experiments by Parrott and de Sousa, using thymidine labelling, have shown that the thymic-dependent cell population contained a high proportion of long-lived cells while the B-cell compartment consisted mainly of short-lived cells. Miller and Mitchell (1969) investigated these two different cellular components in terms of immunological function. Their experiments were aimed at defining the cellular basis of immunological deficiencies which resulted from the loss of one of these cell populations. The effect of thymectomy on the antibody response of mice to sheep erythrocytes was established, as well as the ability of various cell pools (thymus cells, thoracic duct cells and bone marrow cells) to restore the anti-SRBC antibody response to such thymectomized animals. Miller et al found that there was a specific cellular defect in neonatally thymectomized mice, such mice being unable to produce antibody-forming cells (AFC) or to show evidence of antigen-reactive cells (ARC) specific for SRBC's. In these experiments, ARC were defined as cells which could be stimulated to divide in the presence of antigen in one host and, when subsequently injected with antigen and normal bone marrow cells into irradiated recipients, give rise to a focus of antibody-forming cells in the spleen (the Kennedy assay). Inoculating neonatally thymectomized mice with normal thoracic duct cells and antigen (SRBC) restored the anti-SRBC antibody response to normal. The ARC was not dependent upon the thymus for reactivity, but was dependent on the thymus for its development. Since bone marrow cells are capable of restoring the whole of the lymphomyeloid complex in heavily irradiated animals (Micklem et al, 1966), the bone marrow was investigated for ARC

precursors (Miller et al, 1969). Bone marrow cells from either normal or thymectomized mice could respond to SRBC's only in those animals having a thymus, i.e., the precursors of the ARC in bone marrow required the thymus to differentiate into ARC (hereafter referred to as T-cells). Experiments by Claman et al (1966) were established to investigate the interactions between bone marrow cells and thymus cells. Thymus cells or bone marrow cells were inoculated with or without SRBC into lethally irradiated syngeneic hosts. One week later, cells from the spleens of these mice were transferred to lethally irradiated secondary hosts together with SRBC alone or SRBC and bone marrow cells. The results were that only secondary hosts receiving bone marrow cells, SRBC and spleen cells from primary hosts injected with SRBC and thymus cells responded. This suggested that the T-cell had to react with the antigen before inter-acting with a bone marrow cell. These experiments have been substantiated by Miller and Mitchell (1969) with SRBC, and Taylor (1969) with two serum albumins.

Antigens which have been found to require the presence of thymus-derived cells to trigger an antibody response are referred to as thymic dependent antigens. It is now known that antigens vary in their thymic dependency. Feldmann et al (1971) have analysed some of the requirements of thymic dependency. They examined, in an in vitro model, antigens (DNP-proteins, and the flagellar antigen of S. adelaide) in various physical forms, in terms of their thymic dependence. They found that polymerized flagellin (POL) induced normal levels of antibody whether the cell suspension contained T-cells or not. The RBC antigen required the presence of T-cells

for a normal response but this requirement could be partially overcome by increasing the concentration of RBC's. Monomeric flagellin (MON), MON-duck erythrocyte conjugates and DNP-duck RBC conjugates all required T-cells for an anti-MON or an anti-DNP antibody response to be mounted. However, the response to the DNP determinant when conjugated to POL was thymic independent. The conclusions drawn from these results by Feldmann et al were that thymic dependence was a physical property of the antigen, not an inherent property of the antigenic determinants. Haptens (determinants) conjugated to thymic dependent carriers elicited responses which were thymic dependent whereas the same determinants conjugated to thymic independent carriers elicited responses which were thymic independent, i.e., T-cells are required for the initiation of an antibody response for only some antigens.

C. Bone marrow derived cells: As mentioned above, the nature of some antigens (e.g., POL, Hcy and E. coli lipopolysaccharide) is such that they can stimulate antibody production without the participation of T-cells. Bone marrow cells were shown to be the precursors of T-cells and antibody forming cells (AFC) by Miller and Mitchell. Miller et al (1967) injected bone marrow cells from normal or neonatally thymectomized mice into irradiated hosts - half of which had been thymectomized one to two months earlier. They killed the irradiated hosts at weekly intervals and examined their spleens for the presence of T-cells by the Kennedy assay described above. They found that T-cells required a thymus for development and that the development was antigen independent. The

cells which gave rise to T-cells were found in the bone marrow, i.e., precursors of T-cells originated in the bone marrow. The possibility also remained that thymus derived lymphocytes were essential for the rapid differentiation of T-cell precursors to active antibody secreting cells. Miller and Mitchell tested this possibility by injecting 10^8 bone marrow cells or 10^8 thymus cells into heavily irradiated CBA mice with or without SRBC. After one week, the spleen cells of these mice were transferred to a second irradiated host together with SRBC. Half of the transferred cells were inoculated together with bone marrow cells. The spleens of the secondary hosts were then assayed for PFC at 4, 6 and 8 days. A significant PFC response was obtained only when the secondary host had received bone marrow cells, SRBC, and stimulated thymus cells. Miller and Mitchell interpreted this result to mean that AFC were descendents either of T-cells (with the injected bone marrow providing only cells essential for the repair of an antigen trapping apparatus), or of bone marrow cells. Further experiments were undertaken by Miller and Mitchell (1968) and by Nossal et al (1968) to distinguish between the two hypotheses, by studying the antibody producing cell population. One example of the type of experiment conducted by these workers is the following. Thoracic duct cells of (CBA x C57BL)F_1 origin were mixed with CBA bone marrow cells and SRBC, and inoculated into thymectomized irradiated CBA hosts. After four days, spleen cells of the host were then incubated with either anti-CBA or anti-C57BL antisera and complement at 37°C before analysis for hemolysin-producing cells (PFC). Treatment of the spleen cells with anti-C57BL antisera had virtually

no effect on PFC recovery, there being only 0-5% reduction; whereas the anti-CBA antiserum had a dramatic effect, reducing by 95% the PFC population. In similar types of experiments utilizing anti-allotype antisera (Miller and Mitchell, 1968) and chromosome markers (Nossal et al, 1968) to detect the origin of AFC, it was also concluded that the AFC were descended from the bone marrow donor. The thoracic duct cells contributed less than 5% of the PFC, and this could be accounted for by the presence of some AFC precursors in thoracic duct lymph (Taylor, 1969). The observation that AFC precursors are found in the bone marrow and not in the T-cell compartment has been substantiated by studies with chromosome markers (Nossal et al, 1968). Nossal and his co-workers found that most of the PFC analysed were carrying the T6/T6 chromosome marker when an irradiated thymectomized recipient was protected by CBA T6/T6 bone marrow and injected with normal CBA thoracic duct cells and SRBC.

D. To summarize: For antibody production to occur, an antigen must stimulate a B-lymphocyte. The physical properties of the antigen (as previously described for each of the functional classes of cells involved in the antibody response) dictate the type of cellular interactions which occur before antibody is produced. A three-cell interaction (macrophage, T-lymphocyte and B-lymphocyte) is required before antibody is produced to antigens such as the sheep erythrocyte (Mosier, 1967; and Tan et al, 1971). POL, on the other hand, does not require macrophages (Feldmann et al, 1971) nor does it require T-lymphocytes (Feldmann et al, 1971),

so it must stimulate B-lymphocytes directly, i.e., some antigens can act directly on B-cells to produce an immune response. Many investigators, [Taylor (1969) using BSA, Chaperon and Glaman (1969) and Miller and Mitchell (1969) using SRBC] discuss the antibody responses to these antigens in terms of a two-cell system but have not considered the macrophage, i.e., there is no documented response to a thymic dependent antigen which occurs in the absence of macrophages.

Hapten-Carrier Relationships

Landsteiner observed that when small, chemically well-defined molecules were injected into animals, for the purpose of studying their immunological properties, these small molecules did not elicit antibodies unless they were first coupled to a heterologous (foreign) macromolecule. The two components of the conjugated immunogen were designated as hapten (the small determinant) and carrier (the remainder of the conjugated molecules). In the case of the protein molecules which have been so studied, it has been possible to cleave the protein into polypeptides and to show that some of the fragments will react with antibodies elicited by the intact protein, and furthermore, that antibodies raised against individual fragments will react with the intact protein. Crumpton et al (1965), for example, partially digested sperm-whale apomyoglobin with chymotrypsin, and showed that five of the eight polypeptides isolated, partially inhibited the precipitation of apomyoglobin by anti-metmyoglobin antisera. These peptides were themselves unable to form detectable precipitates with the antisera as measured by spectrophotometric analysis. By comparing the amino acid analysis

of these polypeptide fragments with X-ray diffraction models of the intact protein, Crumpton was able to define the antigenic regions of the myoglobin molecule, i.e., those regions of the molecule which formed the antigenic determinants. An antigen can, therefore, be defined as a series of antigenic determinants, each one alone functioning as a hapten which is dependent upon the macromoleculer complex for its own antigenicity. This role of the macromolecule with respect to a given determinant is defined as the carrier function. A determinant which serves as the hapten in one experimental design can equally well serve as a carrier determinant in a different experimental design (Iverson, 1970), i.e., the roles of hapten and carrier are interchangeable. For antibody formation, it has been shown that a hapten must be covalently bound to an immunogenic carrier before stimulation of an anti-hapten response will occur. Golan and Borel (1971) have shown that no anti-hapten response will be stimulated if the animal is first made tolerant to the carrier, or if the carrier is not immunogenic [e.g., poly-L(lysine)$_5$ and poly-L(lys)$_{31}$]. In most cases, there seems to be a requirement for more than one type of antigenic determinant for immunogenicity. However, Klinman (1971) has shown recently using an <u>in vitro</u> organ culture system, that the DNP-hapten can function apparently as its own carrier when coupled to poly-D-lysine or poly-L-lysine, if on the average there were at least 22 DNP-residues per molecule.

Rajewsky et al (1969) and Dixon et al (1955) have shown that a secondary immune response to a hapten will not occur unless, for the challenging injection, the hapten is coupled to a carrier to

which the animal has been previously immunized. Initially, this was interpreted to mean that the hapten must be coupled to the same carrier as was used for the primary injection. Later studies have shown that immunity to the carrier can be induced at any time before the secondary injection (Rajewsky, 1969). The hapten must always be covalently linked to a carrier for a secondary response to take place, i.e., if the hapten is injected coupled to a carrier to which the animal has not been immunized, simultaneously with the carrier which had been used for primary immunization, a secondary anti-hapten response will not occur. This dependency of the anti-hapten response on the carrier is called the carrier effect.

The Cellular Basis For The Carrier Effect

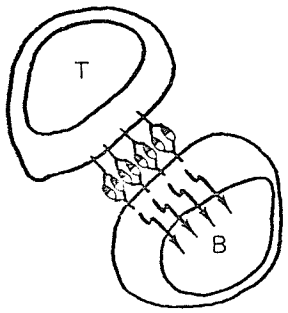
An analogy can be drawn between the carrier-hapten effect and T-cell-B-cell relationships, by consideration of the following data. T-cell dependence and carrier dependence can both be overcome by the use of high-concentrations of the antigen. Steiner and Eisen (1967) showed that a secondary anti-hapten response could be stimulated by using high concentrations of hapten-protein complexes in which the protein carrier used for challenge was different from that used in the primary sensitization. Similarly, Feldmann et al (1971) were able to stimulate an anti-SRBC antibody response in tissue cultures lacking T-cells, by increasing the concentration of SRBC's. T-cell dependence and carrier function have been directly linked in the following study by Feldmann et al (1971). These authors found that tissue cultures devoid of T-lymphocytes were unable to mount an anti-hapten response when the hapten was coupled to a thymic-dependent

carrier, (e.g. SRBC). Haptens coupled to POL (a T-cell independent carrier), however, could stimulate an anti-hapten antibody response. This study demonstrates that some hapten-carrier complexes do not require T-cell intervention for antibody synthesis. When the antibody response is thymic dependent, the T-cell acts by facilitating the interaction of B-cells with the haptenic determinant. It does so by binding presumably to a carrier determinant, thus exposing unbound haptenic determinants to B-cells (see figure 1 below). Therefore, thymic dependent antigens may be regarded as immunogens which, for the elaboration of an antibody response, require the presence of at least two cell types: carrier-specific cells and hapten-specific cells. One experiment which shows the involvement of cells specific for the carrier in the anti-hapten antibody response was carried out by Mitchison (1967). He was able to reduce the magnitude of the anti-hapten response by preincubating spleen cells with unconjugated carrier. This result can be best explained by assuming that cells specific for the carrier determinants were saturated by the free carrier and were unable, therefore, to cooperate with hapten-specific cells for the production of an anti-hapten response.

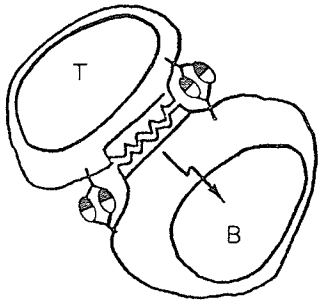
Dutton et al (1971) have proposed several working models to explain cooperation between T-cells and B-cells. The working assumptions of these models are that the T-cells are specific for carrier determinants and the B-cells are specific for haptenic determinants. The four models are represented diagrammatically in Figure 1. All of the models assume that antigen, combined to an antibody-like receptor, plays a key role in stimulation. Model 1

assigns to the T-cell the role of concentrating antigen on its surface to present a number of antigen molecules to a group of receptors on the surface of the B-cell. Multiple activation of B-cell receptors is necessary to stimulate the B-cell. In Model 2, the antigen serves to bring the surface of a T-cell in contact with a B-cell. Contact of a B-cell surface receptor with the corresponding T-cell surface receptor causes stimulation of the B-cell. Models 3 and 4 propose that the B-cell is triggered only when it receives two stimuli at the same time; one being from its receptor molecule, the other from the stimulated T-cell. The stimulus from the T-cell differs in the two models: in Model 3, it is considered to be a special immunoglobulin with anti-carrier specificity, and in Model 4, the activator is described as a diffusible molecule which is not antigen specific. Recent experiments have shown that a non-specific enhancement of the anti-SRBC response may occur if spleen cells in culture are stimulated with an antigen unrelated to the SRBC. Dutton et al (1971) have reported that if irradiated allogeneic spleen cells were present when an anti-TNP and an anti-SRBC antibody response was being elicited in normal spleen cells in vitro, both responses were enhanced. This experiment suggests that a non-specific mediator was produced by the normal cells in culture on contact with the allogeneic cells. This mediator then served to elevate both antibody responses. The concurrent rise in the anti-TNP and anti-SRBC response resulting from the activation of B-cells, which were in contact with antigen, lends support for model 4.

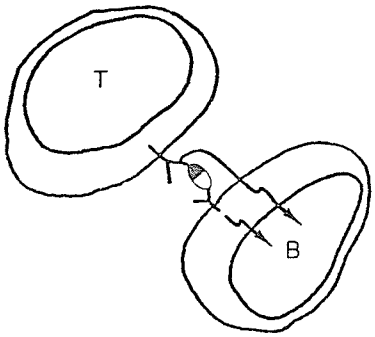
1. ANTIGEN CONCENTRATION



2. SURFACE INTERACTION

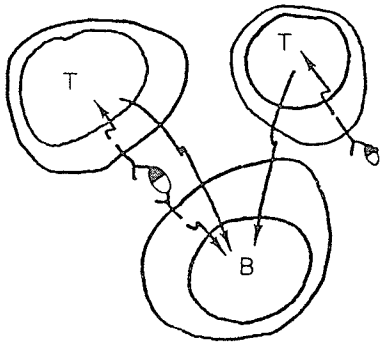


3. CARRIER ANTIBODY



4. THYMUS-DERIVED MEDIATOR

associated or nonassociated



LEGEND: T = T-CELL
B = B-CELL
= ANTIGEN RECOGNITION IMMUNOGLOBIN RECEPTOR
= ANTIGEN--TWO DETERMINANTS, AND
= SIGNAL
= SURFACE RECEPTOR

Figure 1. Models for T-cell-B-cell interaction (Dutton et al, Progress in Immunology 1:353, 1971, by permission of Academic Press Inc.)

From all of the work so far described, it is clear that in order to elicit a response, it is necessary for antigen to be bound to the surface of different classes of lymphocyte (T-cell or B-cell). In the following section, evidence will be reviewed which supports the currently held view that the antigen is bound to the cell membrane by an antibody-like molecule.

Membrane Bound Antibody

Experimental evidence supporting the concept of a specific predetermined antibody-like receptor on ARC has accumulated from a variety of experimental systems.

Information about the antigen binding specificity of the receptor was gained from experiments in which the effects of competitive inhibition on antigenic stimulation were determined. It was observed that an anti-hapten antibody response which continued to be elicited by hapten-protein conjugates was inhibited in the presence of an excess of free hapten, implying that the receptor resembled antibody in terms of its binding properties (Dutton and Eady, 1964; and Mitchison, 1967).

Steiner and Eisen (1967), and Bullock and Rittenberg (1970) using the DNP-hapten and the TNP-hapten respectively, showed that the affinity of the anti-hapten antibody increased with increasing time after the injection of antigen. The hypothesis which was being tested in this experiment was that if the binding properties of the cell-bound receptor were the same as those of the antibody that would be produced by the progeny of any given stimulated cell, then high affinity receptors would be stimulated selectively as the

antigen level decreased with time. The same hypothesis was tested by Mitchison (1967) when he incubated spleen cells from mice which had been immunized with DIP-chicken globulin (CG), first with free NIP, and subsequently with DIP-CG. The affinity of the resulting antibody, produced in irradiated hosts, was lower when the cells were stimulated in the presence of free NIP as compared to cells stimulated in its absence. This result suggested that the closely related NIP determinant combined more efficiently with the high-affinity DIP receptor sites and so blocked the stimulation of those cells which would produce high affinity anti-DNP antibodies.

Evidence to support the suggestion that the receptor is rigidly bound to the cell surface is provided by cell separation experiments. Wigzell and Andersson (1969) passed immune lymph node cells from animals which had been immunized with HSA, BSA or OA through a column of glass or plastic beads which had been coated with antigen (HSA, BSA or OA) and showed that cells of a given specificity were selectively eliminated by passage through the column. Specificity of retention was demonstrated by showing that the addition of free antigens to the suspension passing throughtthe columns would block the retention of specific immune cells.

The integral relationship between antigen binding by cells and the stimulation of those cells was shown by two related types of experiments. Segal et al (1969) were able to inhibit specifically the production of anti-DNP antibodies with haptens which would bind covalently to the receptor site. This procedure, known as affinity labelling, utilized the following reagents: BADL(α-N-bromoacetyl-ε-N-DNP-lys); and

BADE(N-bromoacetyl-N'-DNP-ethylenediamine). Each affinity label has two functional sites; one which functions as the hapten, and the other which binds covalently to the antibody molecule. Plotz (1969) confirmed the results of this study by showing that NIP-azide would inhibit an anti-NIP response but not an anti-CGG response if cells were exposed first to the affinity reagent before antigen was introduced. Ada and Byrt (1969) used a radioactive antigen of high specific activity (^{125}I-labelled POL) from Salmonella adelaide which when bound to cells caused destruction by radiation of only those cells which possessed the specific receptor sites. The same cell suspension was able to respond normally to POL derived from Salmonella waycross. These experiments support the suggestion that the antibody-like receptor is an integral part of the plasma membrane of an Ag-reactive cell. One of the most powerful investigative tools which has been used for the analysis of the antibody-like receptor is anti-immunoglobulin antibody. This reagent has provided the most direct information about the nature of the receptor sites involved in antigen binding.

It has been shown by McConnell et al (1969) that anti-μ chain and anti-γchain antibodies will inhibit the binding of heterologous erythrocytes by lymphoid cells (rosette formation). It was shown that the combined effect of anti-μ and anti-γ antibodies resulted in an inhibition equal to the sum of the separate effects. This finding suggested that rosette forming cells fall into at least two classes-- cells whose receptors bear μ-chain determinants and cells whose receptors bear γ-chain determinants. More detailed

studies have been carried out using antisera to immunoglobulin sub-units. Sell and Gell (1967) showed the existence of Ig allotypes on the surface of rabbit immunocytes. These authors found that an anti-allotype antiserum only caused blast transformation in cells from animals whose serum immunoglobulin expressed the homologous allotype. Cells from heterozygous rabbits (expressing two allotypes) could be induced to transform by an antiserum specific for either allotype. The degree of blast transformation (as measured by [^{3}H-]thymidine incorporation) by each anti-allotype antiserum, corresponded to the serum concentrations of these allotypes. It was concluded that each cell has immunoglobulins on its surface, and that each cell bears only one allotype.

Greaves et al (1969) reported that for human lymphocytes, the receptor site could be blocked with an anti-light chain antiserum. Moreover, univalent Fab fragments prepared from the anti-light chain antibodies were not mitogenic (see below) but did inhibit the mixed leucocyte reaction and also PPD induced blastogenesis of cells from tuberculin sensititive individuals. Skamene and Ivanyi (1969) used chicken lymphocytes to demonstrate that anti-μ chain antibodies, but not anti-γ chain antibodies, stimulated nucleic acid synthesis in these cells, at least as judged by ^{14}C thymidine incorporation. However, anti-γ chain antisera were found to stimulate blastogenesis providing the cells were pretreated with an anti-μ antiserum. These experiments suggest that a shift occurs from expression of primarily μ-chains to an expression of γ-chains on the lymphocyte surface. These results also strongly suggest that immunoglobulin determinants

are present on the surface of immunocytes, and that they are indeed part of the receptor site.

Recent advances have been made in the isolation and characterization of receptor molecules from lymphocytes. Eskeland et al (1971) have isolated a 7S IgM molecule and kappa chains from a human cell line derived from Burkitt lymphoma cells. Vitetta et al (1971) have isolated μ-chains also in the form of a 7S IgM monomer from normal mouse spleen cells. The light chain associated with this monomer was not characterized.

The most widely used system which has been used to study the effect of anti-immunoglobulin reagents on the antibody response is an in vitro model used by Mishell and Dutton (1967). It consists of stimulating a spleen cell suspension to produce antibody by incubating the cells with an erythrocyte antigen. With this in vitro system, Hartmann et al (1970) were able to inhibit the primary immune response to sheep erythrocytes with anti-IgG, anti-IgM and anti-Fab(γG_{2a}). Anti-allotype or anti-Fc antibodies did not cause inhibition of the response. Sjöberg and Greaves (1971) used the same test system but obtained different results. Anti-IgG suppressed only the IgG antibody response. This data was derived for only the secondary response, since no primary IgG response to the SRC could be elicited. Anti-IgM antibodies suppressed only the IgM response. Fuji and Jerne (1969) using a multivalent anti-immunoglobulin antiserum and Lesley and Dutton (1970) using an anti-kappa chain antiserum also have demonstrated the inhibitory powers of anti-γ immunoglobulin antisera. In contrast to the data obtained from

isolation studies, the in vitro work just described implicates the participation of IgG antibody-like receptors in an immune response, as well as IgM receptors.

No indication has been given yet about the action of anti-Ig antibodies on the actual lymphocytes involved in an immune response. Mention was made previously of only the mitogenic properties of certain anti-Ig preparations. Greaves et al (1969) have shown that low concentrations of anti-Ig and Fab monomers prepared from this antiserum do not cause blastogenesis. This suggests that the linking of two or more receptor sites is necessary before blastogenesis will occur. Because of the blastogenic potential of anti-immunoglobulin antibody, investigators who have used this reagent to study the inhibition of an immune response have sought to determine whether the cells are in fact being activated non-specifically by the combination of anti-antibody and the receptor site (sterile activation) and are, therefore, rendered incapable of reacting specifically to the antigen. Sjöberg et al (1971) have compared the extent of incorporation of ^{14}C thymidine by cultures which were incubated with anti-Ig to that of normal cultures, i.e., not stimulated either by antigen or by anti-immunoglobulins. The degree of stimulation in the cultures incubated with anti-Ig antibodies was found to be no higher than that obtained for the unstimulated cultures, implying that non-specific blastogenesis had not occurred. Lesley and Dutton (1970) demonstrated the reversibility of anti-Ig inhibition by showing that if mouse cells were incubated first with anti-Ig antiserum for one hour, and then washed and resuspended in fresh

medium which was free of antiserum, there was no inhibition of the PFC response to SRBC. Also, incubating the anti-Ig coated cells in medium containing normal mouse immunoglobulin (Ig) would reverse the inhibitory effect of anti-Ig. The antibody to the receptor site must, therefore, dissociate from the cells and combine with the mouse gammaglobulin (MGG) in solution in the medium; as soon as the receptor site becomes accessible, it can be stimulated then with antigen. The inhibition described in this latter study is presumably the result of steric effects; if the antibody coated cells were incubated at 37°C in the presence of complement, the response to antigen was completely irrecoverable, i.e., the cells were then killed by complement induced lysis.

Evidence thus indicates that anti-Ig and antibody to Ig subunits can combine with cell-associated antigen binding receptor sites and inhibit the combination of antigen with these receptor sites. Since isolation studies have indicated the presence of only IgM monomers on the surface of normal immunocytes, and in vitro work has demonstrated that IgM is not the only immunoglobulin represented in receptor sites, it is clear that the basic problem of immunoglobulin representation on these cells remains unsolved.

The Relationship Between Lymphocyte Receptors and Humoral Antibodies

In studying the relationship between the density of haptenic determinants (NIP) on a conjugated protein and the class of antibody produced, Mäkelä (1970), using NIP_8-bacterial cell conjugates and NIP_8-OA, has obtained results (Figure 2) which imply that polyvalent conjugates induce significantly higher IgM responses, than

do mainly monovalent conjugates. Since polyvalent conjugates were generally stronger immunogens, the magnitude of the IgG responses of these conjugates were reduced by dose adjustment, to make results comparable. The IgM/IgG ratio was determined by carrying out triplicate titrations of all sera. The titre in the presence of a reducing agent (0.1 M 2ME) was mainly due to IgG antibodies; that in the presence of 10^{-6}M hapten was mainly due to IgM antibodies. The third replica had no inhibitor. Mäkelä has formulated a hypothesis by assuming that IgM and IgG producing cells are triggered by the way in which the receptor site interacts with the antigenic determinants, and that the receptor site on the lymphocyte is the same class as that ultimately synthesized by the stimulated plasmacyte. Another basic assumption is that since IgM and IgG molecules differ in size (IgG has a full stretch of 120 Å and IgM has a stretch of 350 Å), an IgM receptor site can be preferentially stimulated when haptens (like determinants) are spaced further than 120 Å, i.e., if the distance between two like determinants on a carrier is 150 Å, an IgG molecule cannot bridge two of them, but an IgM molecule can--in this case IgM anti-hapten antibody will be preferentially synthesized. If only one IgM receptor site binds antigen, the situation favours IgG production (Figure 2-A). When the IgG receptor molecule can form more than one bond with antigen, then the titre of the IgG antibodies increased (Figure 2-B, and 2-C). If multiple bonds can be formed only by IgM receptor sites, the response elicited would be in favour of higher IgM production (Figure 2-D). One consequence of Mäkelä's hypothesis is that hapten density will affect the ratio of IgM:IgG specific to the type of IgG/IgM

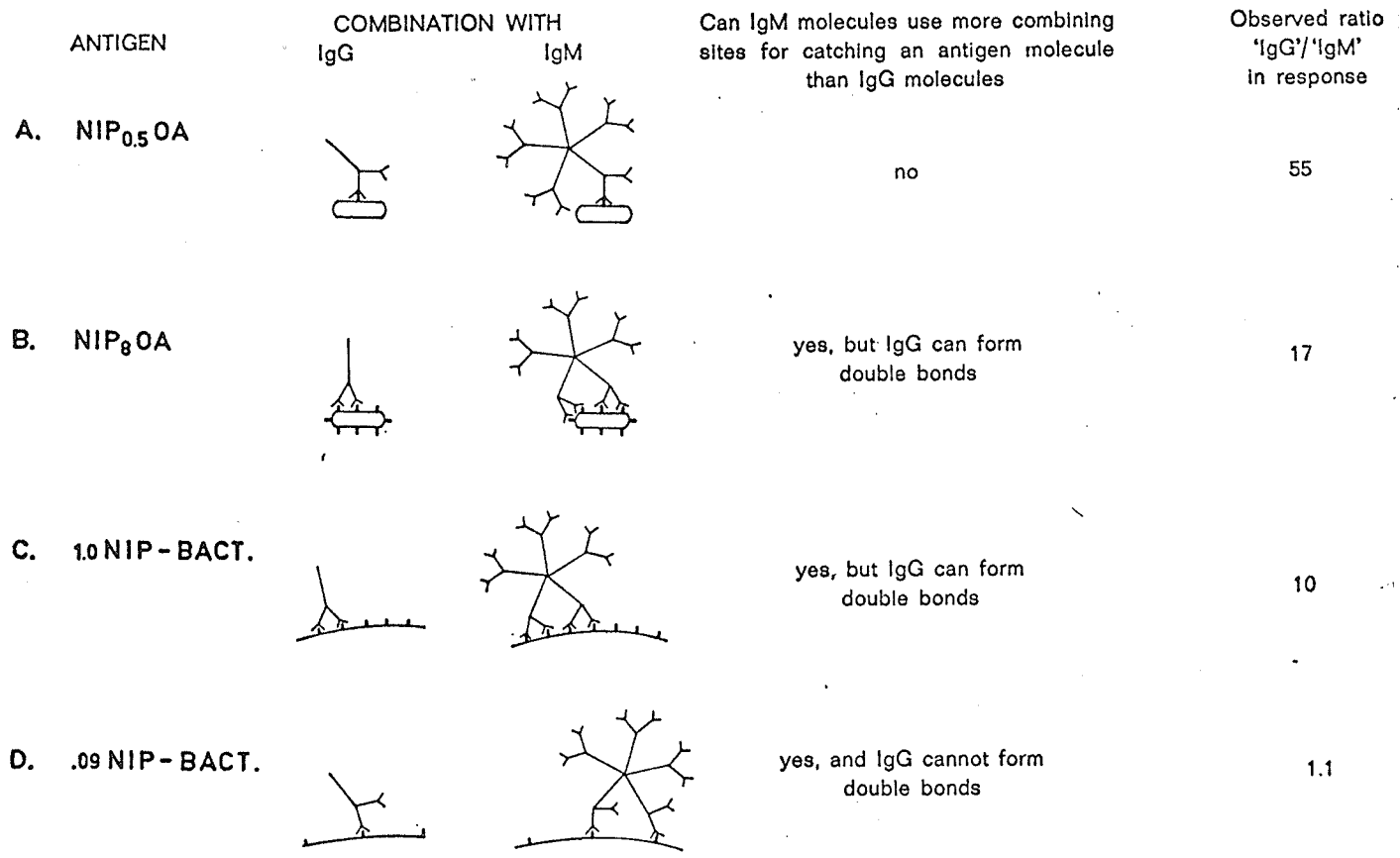


Figure 2. A hypothetical illustration of how IgG and IgM anti-NIP receptor antibodies may combine with the hapten conjugates used. Structures with forks are receptor antibodies combining with molecules of NIP (stubs) on the surface of the immunogen (by permission of the author, Dr. O. Mäkelä, Transplantation Reviews 5:3, 1970)

response elicited. Mäkelä further suggested that the type of antigen required to elicit a response of equal IgM and IgG titres, is one of low hapten density and high molecular weight. Mäkelä, being aware that the rules governing free antibody molecules may not be applicable to the cellular receptors, has added that tight packing of IgG receptors may imitate a polyvalent IgM molecule. For this thesis, it is the density of receptors as well as the density of like determinants which dictates the class of antibody elicited by a hapten-carrier conjugate.

Several investigators have shown that it is possible to obtain an anti-hapten antibody response in vitro by coupling the hapten to immunogenic complexes. For example, Strausbauch et al (1970), Kettman and Dutton (1970) and Katz et al (1971) have all shown that a hapten-SRBC conjugate can be used to induce an anti-DNP or anti-TNP antibody response in tissue culture. Bullock and Rittenberg (1970) have used TNP-bentonite, and Feldmann et al (1971) have used DNP-POL to accomplish the same objectives in that both groups were able to induce an anti-hapten antibody response in vitro. It is, therefore, theoretically possible to obtain the desired type of IgG and IgM anti-hapten antibody response in tissue culture, i.e., a hapten-carrier conjugate having the appropriate properties, can be synthesized to elicit the desired response.

For efficient in vitro stimulation of antibody formation, it has been shown repeatedly that, within certain limits, the immunogen must consist of an antigenic carrier to which the hapten has been coupled. Kettman and Dutton (1970), for example, have found that in their TNP-SRBC system, lightly substituted erythrocytes were only weakly

immunogenic for an anti-TNP response. The type of response elicited by an immunogen in tissue culture does not always parallel that obtained *in vivo*. With the SRBC antigen, for example, it has not been possible, using the Jerne plaque assay, to demonstrate a primary IgG response (Sjöberg et al, 1971; and Mishell and Dutton, 1967). The primary IgG response has been observed only by the use of the bacteriophage neutralization assay: Segal et al (1970) used DNP_8-Hcy as the immunogen and DNP-T_2-phage in the antibody assay, Tao (1968) used ϕX-174 bacteriophage for both immunization and assay. Apart from these reports, the IgG antibody response has been observed *in vitro* only in the secondary response. In some studies on the anti-hapten antibody response *in vitro* (Trowbridge et al, 1970; Naor et al, 1971; and Katz et al, 1971), it has been found necessary to pre-immunize the mice with the carrier molecule before consistently positive results were obtained *in vitro*.

Using a hapten-SRBC antigen, it has been shown by Kettman and Dutton, 1970; Trowbridge et al, 1970; and others, that maximal antibody production occurs at four to five days after the primary stimulus, and two to three days after the secondary stimulus. Most other antigens (ϕX-174, and KLH-Bentonite, for example) produce a similar pattern of antibody formation, i.e., maximal response four to five days after primary stimulus and two to three days after secondary stimulus, but there are two published reports of a secondary response *in vitro* which peaked seven days or later after establishing the cultures. McArthur et al (1969), studying the anti-KLH response, showed that the total incorporation of ^{14}C-amino acids was maximal around day 10 following antigenic stimulation. Klinman (1971)

obtained essentially similar results, by showing that the peak anti-DNP response occurred on day 14, with antibody being detectable only after day 8. On the basis of these results, it is clear that a culture must survive *in vitro* for more than one week, in order to establish unequivocally the nature of the response.

OBJECTIVES OF THE THESIS

The initial objective of the studies described in this thesis was to investigate the class of antibody comprising the immunoglobulin-like receptors involved in the anti-DNP antibody response. An *in vitro* system was chosen to facilitate this investigation based on the work of Sjöberg et al (1971), Fuji and Jerne (1969), and Skamene and Ivanyi (1969). Justification for choosing this system was based on the fact that the primary *in vitro* antibody response to the DNP-determinant has been shown to consist of both an IgM and an IgG antibody response (Segal et al, 1969), thus affording a unique opportunity of studying *in vitro* the production of two classes of antibody. This study required the synthesis of a sensitive reagent with which to detect the small amounts of anti-DNP antibody produced in tissue culture, and so DNP-T_2-phage was synthesized (Carter et al, 1968). The study also required the establishment of tissue culture conditions adequate to support an antibody response. A tissue culture system was therefore evolved to allow the production of an anti-DNP antibody response. Since this response was subsequently found to be of insufficient magnitude to allow further study of the membrane bound receptor sites *in vitro*, an indirect approach was undertaken *in vivo* using as a working hypothesis the model proposed by Mäkelä (1970). One of his basic assumptions was that recognition by an IgM receptor site

required a fragment of SRBC membrane bearing at least two antigenic determinants which were no less than 120 Å and no more than 350 Å apart. The experiments in this phase of the study were designed to provide specific information on the size of the immunogen at the time of recognition by B- and T-lymphocytes. The principle of the experiments was based on the use of complex antigens which consisted of an immunogenic carrier separated from haptenic determinants by a non-immunogenic protein. By establishing experimentally the role of the various immunogens, it proved possible to calculate the approximate dimensions of the immunogen at the time of recognition.

METHODOLOGY

I. BACTERIOPHAGE NEUTRALIZATION

Introduction

Each class of bacteriophage is infectious to a specific group of host bacteria. T_2-coliphage infect predominantly E. coli B, but can be absorbed to other classes of bacteria (Adams, 1959). The T_2-phage DNA, once inside a compatible host bacterium, will multiply and then cause lysis of the cell to occur, with the consequent release of 200 to 300 progeny phage particles (Adams, 1959). The progeny will continue to infect any surrounding bacterial cells until an equilibrium state is reached. If there is an excess of phage in the medium, all of the bacteria are lysed. In the case of a low initial concentration of phage, the bacteria themselves will stop metabolizing when they reach a concentration of approximately 5×10^8 cells per ml. (Adams, 1959) before complete lysis has occurred. Since the phage require an actively metabolizing bacterium for propagation, no more phage are produced.

Full advantage may be taken of this natural amplification to study some characteristics of anti-phage antibody by stabilizing the phage-bacterial cell interactions in a gel matrix. With an appropriate dilution of bacteriophage and a bacterial suspension in the logarithmic phase of growth, it is possible to visualize the result of a single phage infection of a host cell. Initially, each phage is surrounded by about 10^5 bacteria, so the probability of every phage infecting a bacterium is high. Phage progeny particles continue to infect bacteria in close proximity to the central initially lysed bacterium. The ultimate result is a visible area

of lysis (a plaque) in a lawn of bacteria. It is, therefore, possible to describe a phage stock in terms of the number of plaque-forming units (PFU) it contains. The determination of PFU does not provide a direct count of the absolute number of phage particles, since many of the phage can absorb to bacterial debris; the number of plaques observed is, therefore, less than the absolute number of phage. In the case of antibody to the T_2-coliphage, antibody combining with antigenic determinants on the phage tail-fibers sterically inhibits the interaction between the phage and receptor sites on the host bacterial cell wall. Antibody to other regions of the T_2-phage have been reported to only cause minimal neutralization (Franklin, 1961). Neutralization is manifested by a comparison of the plaque count in plates containing phage which had been incubated in the presence of anti-T_2 antibody, to the plaque count on plates containing phage which were incubated without antibody. The reduction in plaque number may be expressed as an index of neutralization (%N). The time of incubation required for the antigen-antibody reaction to reach equilibrium is defined by the following equation:

$$t = \log \frac{P}{P_o} \cdot \frac{2.3\ D}{K}$$

K = rate constant
D = dilution of serum
t = time of incubation
P_o = PFU at t_o
P = PFU at t

The effect of time was determined in a preliminary study and it was found that an equilibrium was reached within 45 minutes for all of the antibody preparations used (see results p. 55). The

percent neutralization is computed by use of the following formula:

$$\%N = \frac{\text{PFU without antibody - PFU in the presence of antibody}}{\text{PFU without antibody}} \times 100$$

The previous equation shows that the %N is related to the concentration of antibody present. The higher the concentration of antibody, the higher the percent neutralization. When the antibody is of very low affinity, it can dissociate from the tail-fiber on dilution, and so allow the phage to infect a bacterium. Jerne and Avegno (1956) have developed an assay to detect this low affinity antibody, which has been termed the 'decision technique'. In the present study, that dilution of antiserum which caused 50% neutralization ($\%N_{50}$) is defined as the end point of an antibody titration. The actual titre of the antiserum is represented by the reciprocal of the end-point dilution (Figure 3).

Experimental Procedures

The basic technique of phage neutralization has been recently adapted to the study of antibodies to a wide range of antigens (Mäkelä, 1966; Carter et al, 1968; Taussig, 1970; and Haimovich et al, 1969). This has been accomplished by coupling the particular antigen or hapten covalently to the bacteriophage. In this investigation, the 2,4-dinitrophenyl (DNP) residue has been coupled to T_2-coliphage to study anti-DNP antibody production.

A. Preparation of a high titre phage stock: A high titre T_2-phage stock was prepared as described by Adams (1959); two methods have been used. In the first method (Method I), 1 ml. of T_2-phage (1×10^{10}) was added to 5 ml. of a growing E. coli B

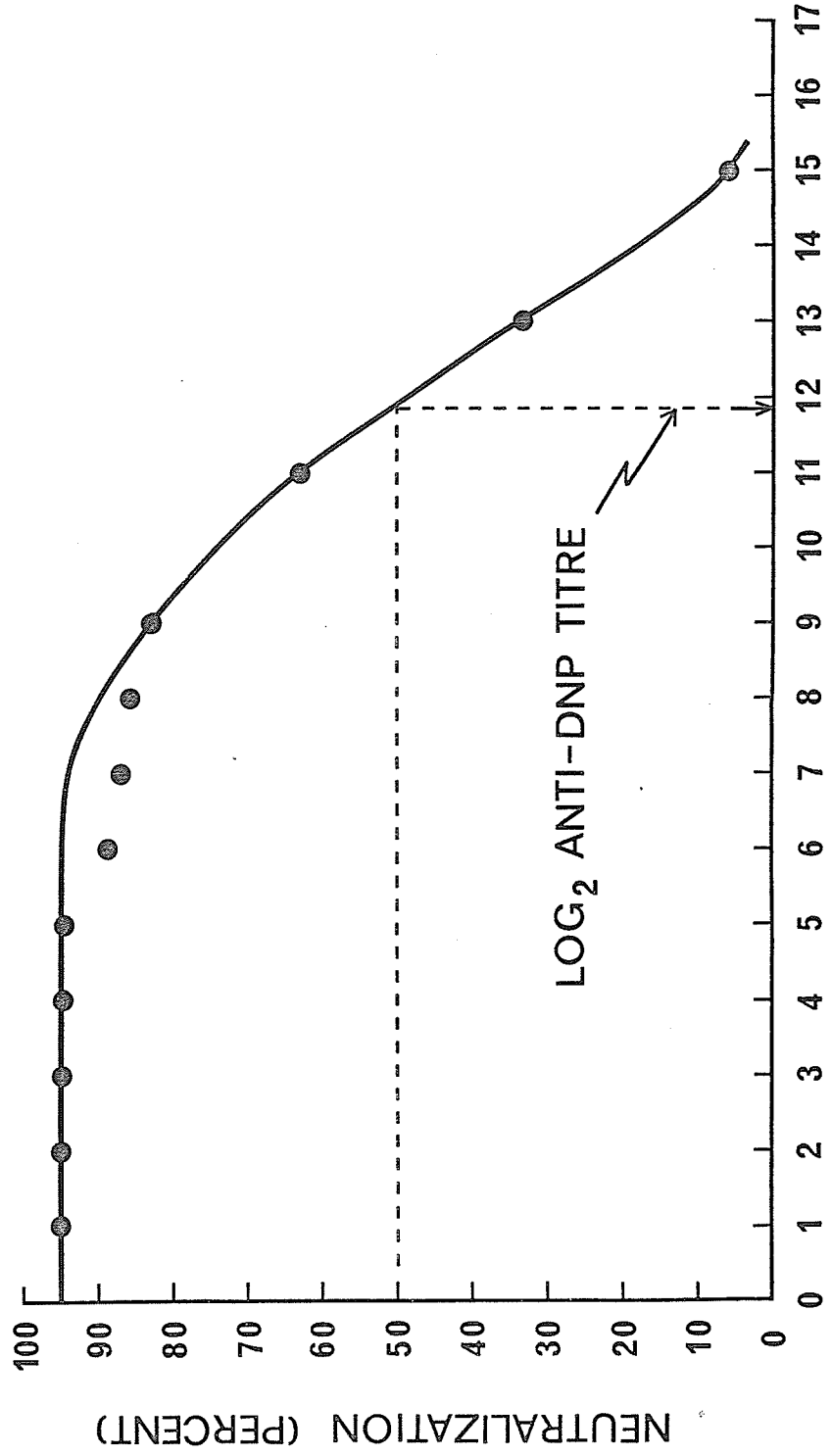


FIGURE 3. TITRATION OF AN ANTI-DNP ANTISERUM BY NEUTRALIZATION OF DNP-T_2-PHAGE. The titre of the antibody is defined as the reciprocal of that dilution which causes 50% neutralization of the DNP-phage.

suspension, and the suspension was left to incubate overnight at room temperature. Debris was then removed by centrifugation at 2,500 rpm for 20 minutes at 4°C and the supernatant filtered through a millipore membrane (0.22μ pore size). Method II consisted of plating an amount of PFU (T_2-phage) in a double layer assay (Adams, 1959), sufficient to cause confluent lysis (all bacteria lysed). To each plate (Falcon petri dish, #1029) was added 5 ml. of nutrient broth (Difco Laboratories, Bacto 0003-1); the plate was then left to incubate at room temperature for 20 minutes. The harvest was collected and centrifuged for two hours at 18,000 rpm at 4°C. The pellet of T_2-phage was carefully resuspended in nutrient broth. The results obtained, using both methods, are described in the results section (Page 50). After the T_2-phage were harvested, the concentration, as PFU/ml., was determined. If the T_2-phage titre was greater than 10^{11} PFU/ml., the harvest was used for coupling with DNP-hapten, otherwise it was stored as a stock solution of T_2-phage.

B. Determination of bacteriophage concentration (the phage assay): The concentration of T_2-phage in a solution was determined by diluting serially the solution in 10 fold steps using nutrient broth. 0.1 ml. portions of each dilution were plated by the agar layer method (Adams, 1959). The dilution range from 10^{-1} to 10^{-11} was found to be adequate for all preparations of phage assayed. The PFU/plate were then used to estimate the concentration of PFU in the solution under study.

C. Method of coupling: The DNP hapten was then coupled covalently to the coliphage by a modification of the method originally described by Carter et al (1968). 1 gm. of 2,4,-dinitrobenzene sulfonic acid (Eastman Organic Chemicals, #4309) was dissolved in 10 ml. of 0.3 M Na_2CO_3-$NaHCO_3$ buffer, pH 9.5. To this solution was added 1 ml. of a concentrated T_2-bacteriophage suspension (1×10^{11} PFU/ml., or greater). Coupling was allowed to take place at 37°C with continuous stirring for at least three hours. This reaction was shielded from light because the DNP reactant is photosensitive. The mixture was finally diluted five-fold with ice cold saline containing gelatin at a concentration of 25µg/ml. and then dialysed exhaustively against saline to remove free hapten. Tests were next performed to establish the extent of coupling and the concentration of the viable conjugated phage (PFU). The concentration was determined according to the method described above and the extent of coupling by establishing the susceptibility to neutralization, by an anti-DNP antibody, of the conjugated phage.

D. The neutralization assay: 0.9 ml. of the serum or a dilution thereof was incubated for 45 minutes with 0.1 ml. of a DNP-T_2phage solution. An aliquot of this was then plated, using the double layer assay described by Adams (1959) and the plate left overnight to develop. 0.1 ml. portions of the same DNP-T_2phage preparation were also incubated separately in (1) nutrient broth, to serve as a negative control and (2) a reference anti-DNP antiserum to be the positive control. Plaques were counted, using a colony

counter (Darkfield Quebec colony counter) and the % Neutralization calculated. The versatility of this assay was exploited to measure both IgG and IgM antibody, by taking advantage of the fact that the neutralizing capacity of IgM antibody, but not that of IgG antibody, can be eliminated by 2-mercaptoethanol (2-ME). An aliquot of each dilution of antiserum was therefore preincubated in 2-ME (0.1 M) for 30 minutes before the addition of 0.1 ml. of a DNP-T_2 phage solution. Under these conditions only IgG antibody will show a neutralization titre. By subtracting this titre from the titre derived from serum or dilutions thereof, not preincubated with 2-ME, an antibody titre was calculated which was attributed to IgM.

i) reference anti-DNP antisera: Antisera were raised in rabbits by immunizing them with DNP-BGG. The sera were titrated for anti-DNP antibody by means of a neutralization assay, using DNP-conjugated T_2-phage; no significant anti-T_2 activity was found in any of the reference antisera. Antisera were frozen at -20°C until used. When used as a standard in the neutralization assay, the antisera were diluted to the minimum concentration which would cause 100% neutralization. For example, the antiserum in Figure 3 was used at 1/120 dilution; between individual assays the serum was stored at this dilution at 4°C. The reference sera used in this thesis were #176 (at 1/120 dilution) and #173 (at 1/300 dilution).

ii) inhibition of neutralization: Antisera were pre-incubated with increasing concentrations of free hapten for one hour before the addition of phage. The residual neutralizing capacity of the antiserum-hapten mixture was then determined in

a neutralization assay. Controls were carried out to determine (1) the effect of the hapten on the phage and (2) the neutralizing capacity of the antiserum in the absence of free hapten.

E. Use of glutaraldehyde as a coupling reagent: Because of the rapidity with which glutaraldehyde reacts with amino acids, glutaraldehyde DNP-phage conjugates were synthesized for possible use in the above assay. 10λ of a 19.25 mM solution of glutaraldehyde was mixed with 30λ of a 5.7 mM solution of ε-DNP-lysine (K and K Chemicals, #10735, ε-DNP-lysHCl), and 30λ of T_2-phage (received from Dr. M. Sela, The Weizmann Institute of Science, Rehovot, Israel) at a concentration of 2×10^{13} PFU/ml. The reaction was allowed to proceed for 90 minutes at room temperature, the mixture was then diluted 10 fold in ice cold 0.5% NaCl, and dialysed exhaustively against 0.5% NaCl, at $4^{\circ}C$ to remove any uncoupled hapten.

F. Chromatographic techniques: Sephadex G-25 was used to separate DNP-coupled T_2-coliphage from free hapten. 1 ml. volumes were passed through a column (17 cm. X 1.34 cm.) of Sephadex G-25 equilibrated with 0.5% NaCl. The flow rate was approximately 100 ml./hr. 1 ml. fractions were collected and analysed for DNP-T_2 phage. Fractions containing phage were pooled to give a specific DNP-phage preparation.

II. LOCALIZED HEMOLYSIS IN A GEL

Introduction

Originally developed by Jerne et al (1963) and independently by Ingraham and Bussard (1964) the method relies upon the property

of erythrocytes (RBC) from certain species to lyse in the presence of homologous antibody and complement (C). Spleen cells (prospective antibody secreting cells) are surrounded by erythrocytes in a ratio of approximately 100 RBC to one spleen cell; a spatial arrangement which is stabilized by a gel. Any anti-RBC antibody secreted by the spleen cells combines with the surrounding RBC. After a suitable period of incubation (typically 60 minutes), during which the erythrocytes will have been adequately coated with antibody, C is added to the system to cause lysis of all susceptible Ab-RBC-C complexes. A circular area of lysis (plaque) becomes visible against the RBC-gel background. Each plaque is produced by one antibody-secreting cell or plaque-forming cell (PFC). The method's high sensitivity makes it a valuable assay for use in conjunction with tissue culture procedures.

Only IgM secreting cells (Direct Plaques) are enumerated by this method. The concentration and the molecular structure of IgG anti-RBC antibody secreted by single cells usually is not sufficient to cause lysis directly. In order to visualize the effect of these PFC, it is necessary to add a developing anti-serum (Sterzl and Riha, 1965; Dresser and Wortis, 1965). The developing serum is an anti-gammaglobulin (γG), specific to the species whose spleen cells are being assayed. The anti-γG antibody combines with the γG antibody which is already bound to the RBC, and so creates a larger complex. This complex has increased hemolytic activity and produces a plaque (Indirect Plaque). Similarly, IgA secreting cells require an anti-γA antibody to render them visible.

Experimental Procedures

Briefly, the Jerne plaque assay can be used to analyze quantitatively and qualitatively an antibody response to erythrocytes, and can be modified to describe antihapten responses when the erythrocyte is coated with the appropriate hapten (Strausbauch et al, 1970; Bullock and Rittenberg, 1970; Kettman and Dutton, 1970; Chiller and Weigle, 1970). The magnitude of the response is indicated by the number of PFC's in an arbitrary unit of spleen cells. The quality of the response is indicated by the use of appropriate developing antisera. The developing antiserum must be standardized before use (Chou et al, 1967). In order to decide on the appropriate concentration, the effect of various concentrations of developing antiserum on the IgM plaques visualized is measured. That dilution which affords maximal IgG plaque development and which also produces minimal inhibition of direct plaque formation is chosen.

There are two types of developing procedures which differ in their effect upon the direct plaques (Chou et al, 1967). The developing antiserum can either be incorporated into the top layer, or added on top of it after the cells have been incubating at $37^{o}C$ and have synthesized and secreted antibody. In the overlay method, adequate time must be provided to allow the antiserum to diffuse into the top layer before complement is added. With the overlay method, there is maximal development of indirect plaques, since the antiserum is not present to combine with newly synthesized antibody. Moreover, a lower concentration of antiserum is required. The anti-mouse IgG antiserum used in this study was standardized by an

incorporation assay, and was used at a final dilution of 1/220. Due to the relatively small size of the haemolytic plaques, a variety of procedures exist to count them. The most satisfactory method was found to be using a Nikon dissecting microscope (stereo-zoom) with a total magnification of 10X.

A. Materials: The sheep red blood cells were purchased from National Biological Co., Winnipeg, in 25.0 ml. lots in citrated dextrose solution. Sheep cells were used within four weeks of purchase. The guinea pig serum was purchased from North American Laboratory Supplies, Winnipeg, and stored at -20°C until used.

B. The hemolytic plaque assay: The hemolytic plaque assay used in this study is similar to that described by Jerne et al (1963). The modifications which were made are as follows:

i) agarose is used (agar being anticomplementary)

ii) 60 x 15 mm. petri dishes (Falcon, #1007) instead of 80 x 15 mm. dishes

iii) Hanks balanced salt solution (Difco Laboratories, 5775-23) was used instead of Eagles medium.

Briefly, 0.4 ml. of 0.83% agarose (L'Industrie Biologique Francaise, F. 6337) kept at 60°C, was added to 0.4 ml. of 2X Hanks, kept at 47°C. To this mixture was added immediately 0.1 ml. of a 25% SRBC suspension, and 0.1 ml. of developing antiserum (when used), followed by 0.1 ml. of the spleen cell suspension (kept at 0°C until used). The whole was then quickly mixed and poured into a petri dish already containing a base layer of 0.4% agarose and left to gel. After one hour incubation at 37°C, 2 ml. of 10%

freshly-thawed normal guinea pig serum (as a source of C), was layered over the gel. The plates could be read after 30 minutes of incubation at 37°C, or after further storage overnight at 4°C without any change in plaque count.

III. TISSUE CULTURE METHODS

Introduction

One of the aims of this research project was to stimulate an anti-DNP antibody response in an *in vitro* tissue culture system. Tissue culture has many advantages over *in vivo* investigation, e.g. the response is under more direct manipulation, free from the complexities of the intact animal, biochemicals may be added to, and subsequently removed from, the culture when required in accordance with the growing acceptance of tissue culture procedures; for example, there has been a clearly discernible shift from *in vivo* work to the development of immune responses in vitro (see Tables 1 and 2). The most widely used system is that developed by Mishell and Dutton (1967). It consists of a spleen cell suspension cultured in a nutrient, isotonic medium in the presence of antigen. The culture is kept continuously rocking, and fed regularly (every two days) with a nutrient cocktail. A response is defined in terms of PFC per 10^6 harvested cells, above the unimmunized cultures. The cells, during culture, are minimally disturbed and survive long enough to allow the kinetics of the response to be determined. Marbrook (1968) has modified the Mishell and Dutton culture system

TABLE 1

SUMMARY OF IN VITRO ANTI-SRBC RESPONSES WITH MOUSE TISSUE

Type of Response	Ag Dose	Type of Culture	Size of Tissue▼	Author *	Year
Primary	6×10^5	Organ	1-2 mm^3	Globerson and Auerbach	1965
Primary	2×10^5	Suspension	2×10^7	Mishell and Dutton	1966
Primary	3×10^6	Suspension	1×10^7	Mosier	1969
Primary	3×10^6	Suspension	1×10^7	Pierce et al	1969
Primary	2×10^6	Suspension	1.5×10^7	Marbrook	1968
Primary	2×10^6	Suspension	2×10^7	Ortiz-Ortiz et al	1970
Secondary	0.01 mg. to 0.5 mg. SRBC Stroma	Suspension	1.5×10^7	Adler et al	1970

* Only first publications are indicated.

▼ Unless stated otherwise, the size of the tissue is in cells/ml. of culture medium.

TABLE 2

SUMMARY OF IN VITRO ANTI-HAPTEN RESPONSES WITH MOUSE TISSUE

Type of Response	Ag Dose	Ag	Type of Culture	Size of Tissue †	Author *	Year
Primary	3×10^6	TNP-SRBC	Suspension	1.2×10^7	Kettman and Dutton	1970
Primary	3×10^6	NIP-SRBC	Suspension	1×10^7	Trowbridge et al	1970
Primary	3×10^6	Penn-SRBC	Suspension	1.2×10^7	Naor et al	1971
Primary	1×10^5	TNP-SRBC	Suspension	1.5×10^7	Katz et al	1971
Primary	5×10^5	TNP-BRBC	Suspension	1.5×10^7	Katz et al	1971
Primary	1×10^7-10^8	TNP-Ox174	Suspension	1.5×10^7	Katz et al	1971
Primary	5 µg	DNP-Hcy	Organ	1-2 mm^3	Segal et al	1970
Primary	50 µg	DNP-PLL				
Primary and Secondary	40-200 µg	TGAL HGAL	Leucocyte Suspension	1×10^6	Tynan and Ness	1971
Secondary	0.2-0.02 µg	TNP-KLH-B	Suspension	1.5×10^7	Bullock and Rittenberg	1970
Secondary	Various Concentrations	DNP-Hcy	Organ	1-1.2 mm^3	Klinman	1971

* Only first publications are indicated.

† Unless stated otherwise, the size of the tissue is in cells/ml. of culture medium.

by placing the cells on a dialysis membrane which forms the base of a glass tube immersed in tissue culture medium. The medium, being in abundance, does not need changing and the cells are continuously fed by diffusion, i.e., the cells are undisturbed until the day of assay. Strausbauch et al, (1970) have described a method in which the cells are suspended in an upright tissue culture tube which remains stationary. Cultures are fed daily in a manner similar to that described by Mishell et al. All three types of cultures are kept at 37°C under a gas mixture of defined composition. The other main type of in vitro tissue culture used in immunological studies is the organ culture, used notably by Segal et al (1969), Klinman (1971), and Tao (1968) as well as by others. In this type of culture, the organ fragments must not be totally submerged in tissue culture medium as the oxygen gradient is a critical factor (Trowell, 1959). The tissue pieces are, therefore, handled in one of two ways: they are either placed on a membrane filter which rests at the medium-air interface, so that capillary action causes the pieces to be wetted and so bathes the tissue with nutrients; or placed in a roller tube, wherein the tissue is kept continually moist as medium washes over the fragments at a predetermined rate. The medium is changed at regular intervals; cultures are kept at 37°C in a fixed atmosphere.

Organ cultures represent conditions which are closer to physiological than suspension cultures, since the microenvironment of the component cells is kept intact. Organ cultures can be kept alive for longer periods of time than suspension cultures (Strausbauch, 1971, personal communication). However, one feature restricts the

use of organ cultures: the fragments attach themselves to the support and cells start to migrate from the culture; i.e., the fragments cannot be removed intact. Unless the tissue is first fixed and then sliced for histological investigation, the individual cells cannot be studied. It is clear, then, that analysis of the medium affords the only satisfactory method for investigative work with organ cultures. A suspension culture is more versatile. Both the cells and the cell products in the medium can be investigated. The ease of handling of a suspension culture, and its flexibility, led to the method being used in this study for the production of an anti-DNP antibody response in vitro.

Experimental Procedures

A. Biological reagents: Fetal bovine serum (FCS) was obtained from two suppliers (Microbiological Associates, Bethesda, Maryland - cytopathogenic virus-free, IPT; and Grand Island Biological Company, Grand Island, New York) but the serum of choice proved to be that obtained from Microbiological Associates (see results section, page 71, for details). Only one bottle of FCS was thawed at a time, and then only immediately prior to use. The serum was heat-inactivated at 56°C for 30 minutes and sterilized through prewashed millipore filters (0.22μ). Frozen sera were kept at -20°C and fresh-thawed serum was kept at 4°C in sterile vials.

B. Chemical reagents: Balanced salt solution (BSS) was prepared as described by Mishell and Dutton (1967), with the following modifications: $MgSO_4.7H_2O$, 200 mg. was changed to $MgSO_4$, 97.5 mg.;

$Na_2HPO_4 \cdot 7H_2O$, 358 mg. was changed to Na_2HPO_4, 190 mg. Eagles minimal essential medium was purchased from Difco Laboratories (#5675) in powdered form and made up to 1000 ml. in 3x distilled H_2O after the addition of the following reagents: 10 ml. of Eagles non-essential amino acids (100x), 22 ml. of a 10% bicarbonate solution, 10 ml. of sodium pyruvate (100x), 6 ml. of a 5% glutamine solution, 1 ml. of a 1% phenol red solution, 10 ml. streptomycin-penicillin-fungizone mixture and 1 ml. fungizone (25 mg./ml.). Both the medium and BSS were sterilized by millipore filtration (0.22μ) and stored at $4^{o}C$ in 100 ml. sterile vials. Before use, each tissue culture medium vial was supplemented with 10% FCS.

C. Glassware, instruments and millipore filters: Instruments and glassware were washed in Microsolv (Microbiological Associates) and then thoroughly rinsed in tap water and then in distilled water. Millipore filters were washed in near-boiling water to remove toxic detergents from their surfaces (Andrews, 1970; Cahn, 1967). All reusable tissue culture equipment was rinsed thoroughly in 2x distilled water before sterilization.

D. Preparation of cell suspension cultures: All manipulations to extract spleens were done using instruments which had been soaked in 70% alcohol. The animal's hide was wetted down with 70% alcohol and the skin cut so that it could be peeled back, leaving an exposed, sterile peritoneum. The spleen, then extracted, was placed in ice cold BSS. Spleens were teased in BSS with sterile stainless steel rakes and the resulting cell suspension left in a tube for five

minutes to allow the clumps to settle. The cells were always kept at $0^{o}C$, unless otherwise stated. The cells still in suspension were removed, centrifuged at 275 x g for 10 minutes at $4^{o}C$ and the supernatant replaced with tissue culture medium. The cells were counted and extra medium added to adjust the cell concentration to 2×10^{7} cells/ml. Two millilitre aliquots were then distributed into tissue culture tubes (Falcon Plastic, #3033). To some tubes only medium was added. The antigen added was 70λ of a coupled (see method, page 46) or uncoupled 1% SRBC suspension; Sheep erythrocytes were always washed three times in BSS before use. Each tube was gassed with a 5% CO_2-95% air mixture, capped and placed in a rotator in a $37^{o}C$ incubator. Every two days, the medium was replaced and tubes regassed. The old medium was either kept in sterile tubes for assay, or discarded. Cultures to be examined were kept in an ice-water bath until assayed.

IV. THE ISOLATION OF GAMMAGLOBULIN FROM MOUSE AND RABBIT SERA

Gammaglobulins were prepared by precipitation in 18% Na_2SO_4, initially using the method as described by Kekwick, but subsequently using a modified form of this procedure. Instead of adding Na_2SO_4 crystals to produce an 18% solution, Na_2SO_4 in the form of a 36% solution was added dropwise to an equal volume of serum, and left to stir overnight. The precipitate was washed twice in 18% Na_2SO_4, each wash taking 24 hours. The precipitated globulins were dissolved in normal saline and dialysed for four days. A comparison was made

of the relative precipitability of mouse and rabbit gammaglobulins using this procedure and the results are described in the results section (page 77).

V. THE PREPARATION OF DNP-CONJUGATES

DNP-Proteins

All DNP-BGG conjugates were prepared by the method of Eisen (1964). Briefly, equal weights of protein, K_2CO_3, and sodium 2,4-dinitrobenzene sulfonate (SDNBS) in water were incubated at pH 11.0. The solution was protected from light and continually stirred at room temperature overnight. Between 35 and 44 DNP-groups were attached per molecule of BGG after this time period. After coupling, the conjugates were exhaustively dialysed (seven days) against saline and either lyophilized or stored as a sterile solution in vials at 4^oC. The DNP-conjugates of mouse and rabbit anti-SRBC antibodies were prepared as follows: equal weights of gammaglobulin, Na_2CO_3, and SDNBS were incubated at pH 9.5. The solution was protected from light and stirred continuously at room temperature overnight. Under these conditions only 2-3 DNP-groups are coupled to the Fc portion of the gammaglobulin (Strausbauch et al, 1970), and the antigen binding site is not altered, i.e., there is no change in antibody titre after the reaction. These conjugates were also exhaustively dialysed and stored sterile at -20^oC.

DNP-SRBC

Two methods are used in this investigation. The first method, using bisdiazotized benzidine (BDB), was described by Pressman et

al (1942). The exact conditions were to add 0.1 ml. of a 50% SRBC suspension to 3 ml. of a 0.1% solution of DNP-BGG; to this mixture was then added 1 ml. of a 0.44mM solution of BDB and the reaction left at room temperature for fifteen minutes. The cells were washed three times with 1% NRS in PBS and were then ready for use. The second method was described by Strausbauch et al (1970). DNP-conjugated immunoglobulins which were reactive to sheep erythrocytes, were incubated with SRBC, for 3 hours at 37°C and then washed three times in PBS to remove uncoupled gammaglobulin. Coupling in both cases was checked by use of an anti-DNP antiserum (or gammaglobulin preparation) in a hemagglutination assay. Evidence for coupling was provided if the cells were agglutinated in the presence of the anti-DNP antiserum. In controls--tubes which contained PBS instead of the antiserum--the coupled cells were not agglutinated.

VI. HEMAGGLUTINATION ASSAY

The antiserum to be titrated was serially diluted in 2-fold steps in PBS. To 0.5 ml. of each dilution was added 0.05 ml. of a 2.5% suspension of SRBC. The cells were left to settle overnight at 4°C, after an initial 1 hour incubation at 37°C. The titre of the antiserum was defined as the reciprocal of the greatest dilution which caused agglutination of the SRBC. This assay was also used to determine whether a haptenic residue had been successfully coupled to the sheep erythrocyte. The 'coupled' SRBC was used in place of uncoupled SRBC in the above assay; the antiserum being a known anti-hapten antiserum.

VII. ANIMAL HANDLING

Mice

The majority of Adult Balb/c mice and adult CBA mice used in this study were obtained from North America Laboratory Supplies; some mice were also obtained from Jackson Laboratories. No difference in responsiveness was observed between the different groups of animals. Mice were first immunized when two to six months old. Exsanguination was accomplished by cutting the jugular vein under light anesthesia, a technique commonly used before removal of spleens for tissue culture, in an attempt to deplete the erythrocyte content. Test bleedings were done by retro-orbital sinus puncture with light ether anesthesia, using heparinized blood collecting capillary tubes (Natelson, # B3092-2). Once filled with blood, the tubes were capped, and centrifuged at 2,500 rpm for 20 minutes to separate the cells from the serum, by breaking the tube at the cell-serum interface. The serum was then selectively removed to be tested or frozen immediately. For maximal stimulation of the spleen, most injections were made intravenously (i.v.). Intraperitoneal (i.p.) injections were used only when the antigen was in a physical state (clumps or agglutinates) which might have caused blocking of the blood capillaries and so seriously impair normal circulation of the blood. For i.v. injections, mice were placed under an infra-red light source until dilation of the superficial lateral tail veins was most pronounced. The immunizing dose was injected through a 27 g. bore needle. Animals were restrained mechanically for i.v. injections and manually for i.p. injections.

Rabbits

New Zealand white albino short ears were injected subcutaneously with a 1:1 mixture of antigen in complete freunds adjuvant (CFA). Test bleedings were made from the marginal ear vein, using a Natelson blood collecting capillary tube. Larger quantities of blood were also obtained from the marginal ear vein by direct bleeding into 40 ml. centrifuge tubes. Serum was withdrawn 12 days and 14 days after the challenging injection. Rabbits were rested for two-week intervals before reinjection.

RESULTS

Preparation of a DNP-T_2-Coliphage Conjugate

Fulfilment of the major objectives of this thesis was, to a large extent, dependent on the availability of a sensitive reagent capable of detecting the low concentrations of antibody encountered in *in vitro* investigations. The initial aim, therefore, was to prepare a DNP-conjugated bacteriophage stock solution, which was not only completely neutralizable by an anti-DNP antiserum, but which could be readily prepared in high concentration and in a reproducible manner.

Two methods were used to prepare high titred T_2-phage stocks for this purpose (Adams, 1959). The first method consisted of infecting a broth culture of E. Coli B, in the log phase of growth with T_2-phage to give a final concentration of 1×10^9 PFU/ml., and leaving it to incubate overnight at 37^oC. The progeny phage were then separated from the bacterial debris by low speed centrifugation and filtration of the supernatant using a membrane filter of 0.22μ pore size. The supernatant was then assayed for phage content. In the second method, enough phage were plated (1×10^5 PFU/ml.), using the double layer technique, to lyse completely the bacterial lawn. Nutrient broth was then added to each plate and the phage progeny collected. After millipore filtration (0.22μ pore size) and centrifugation at high speed (39,000 x g) for two hours to sediment the phage, the pellet was resuspended in fresh nutrient broth and assayed. The second method was found to be the

most efficient, yielding a final concentration of 1.8×10^{11} PFU/ml., as compared to 1×10^{10} PFU/ml. in the first method. The reason for the difference in yield between the two methods probably lies in the fact that bacteriophage adapt to different conditions of growth. Adams (1959) reported that a phage stock which was initially isolated and harvested by the agar layer method would not grow well in broth culture. The method of isolation, in fact, selects for phage variants. The same result can be found in the above experiment. The broth culture harvest technique was inefficient, yielding only a 10-fold increase in PFU/ml. over the added phage, while the agar layer method of harvest yielded a 10^6-fold increase in PFU/ml.

The method used initially to conjugate the DNP-determinant to T_2-coliphage was that described by Carter et al (1968), with one modification: a 0.37M solution of sodium 2,4,-dinitrobenzene sulfonate (SDNBS) was used instead of the originally described 44mM solution. Briefly, the method was as follows: the phage and SDNBS were incubated together at room temperature at pH 9.5 for 7.5 hours and then the reaction mixture was passed through a Sephadex G-25 column to separate the conjugated coliphage from free hapten. The results obtained by this method were erratic and only occasionally was a conjugate produced which was capable of being neutralized to more than 90% by a high titred anti-DNP antiserum.

The reaction conditions were changed therefore to obtain a more uniform result. A constant temperature of 37°C was used and the conjugated phage were freed of uncoupled hapten by exhaustive dialysis.

Since the use of columns required that each sample occupy a column for approximately 30 minutes (the recycling time of the column), the change was made from gel filtration to dialysis, to facilitate the purification of many samples simultaneously. The first experiment in which the above changes were incorporated was a time course study of the conjugation reaction. The objectives were to study the effect of SDNBS on phage viability, and further to determine the susceptibility of these phage to inactivation by an anti-DNP antiserum. At 15 minute intervals, beginning three hours after the addition of the phage, an aliquot of the reaction mixture was removed, diluted five-fold in 0.5% NaCl, and dialysed against 0.5% NaCl. Then, the contents of each dialysis bag were assayed for bacteriophage. Those samples containing phage were further tested for their susceptibility to neutralization in the presence of reference antiserum #173. In Figure 4 is illustrated the effect of time on the viability of phage in the presence of SDNBS. It was found that an inverse relationship existed between the logarithm of the concentration of the surviving phage and the time of coupling, i.e., the longer the duration of the reaction, the lower the yield of viable phage. The DNP-phage conjugate produced in this coupling reaction was capable of being neutralized to 100% for each time interval studied. The specificity of the neutralization reaction was demonstrated by inhibition with ε-DNP-lysine. As illustrated in Figure 5, it was found that a concentration of 1mM ε-DNP-lysine would inhibit almost completely the neutralization of the DNP-T_2-phage conjugate by a

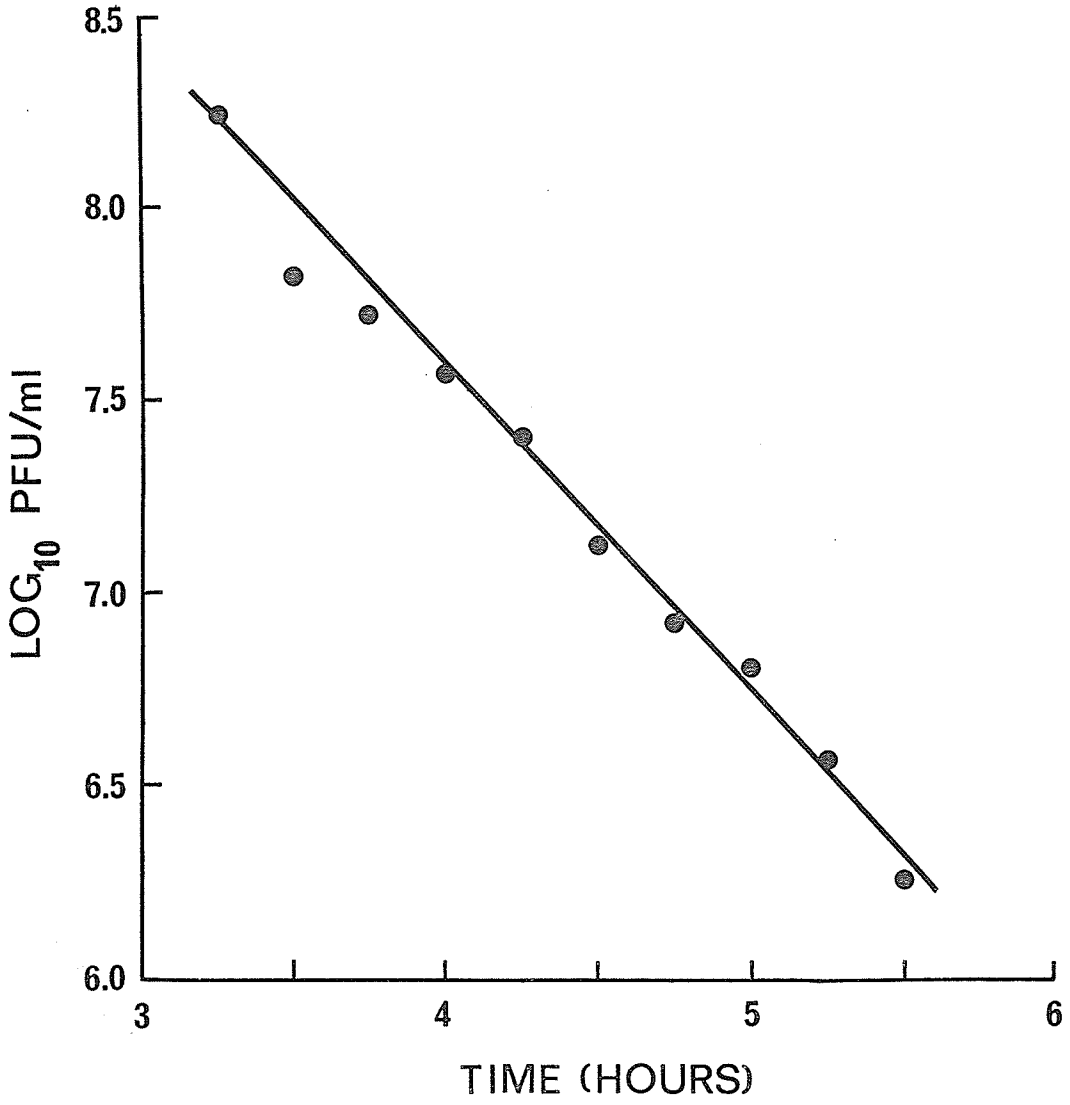


FIGURE 4. THE REACTION OF SDNBS WITH T_2-COLIPHAGE--THE EFFECT OF REACTION TIME ON THE VIABILITY OF THE COLIPHAGE. Aliquots of the reaction mixture were withdrawn and then dialysed. The contents of each dialysis bag were titrated by the phage assay to determine the concentration of viable phage at each time interval.

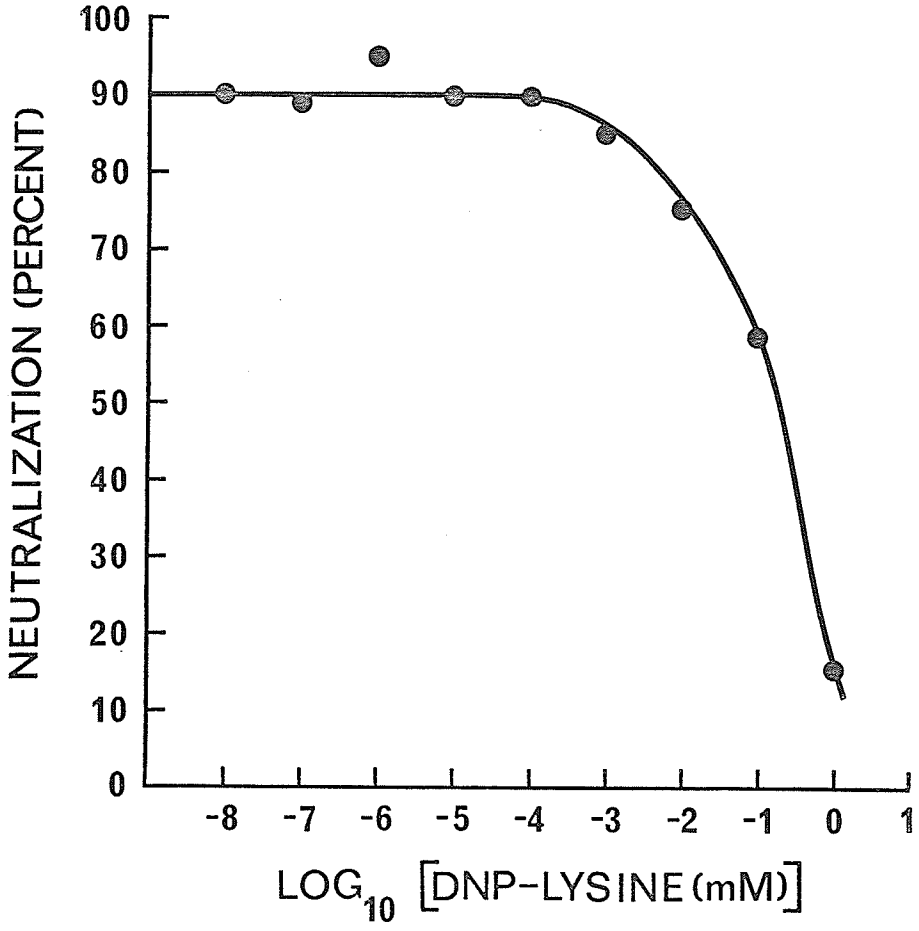


FIGURE 5. INHIBITION BY ε-DNP-LYSINE OF THE NEUTRALIZATION OF DNP-T_2-PHAGE BY A STANDARD ANTI-DNP ANTISERUM. The anti-DNP antiserum was pre-incubated with graded concentrations of ε-DNP-lysine and then incubated with DNP-T_2-phage in a neutralization assay.

standard rabbit anti-DNP antiserum. This batch of conjugated phage was therefore used for all of the relevant experiments described in this thesis.

In addition to the method just described, an attempt was made to use glutaraldehyde to couple the DNP-determinant to T_2-phage. This method was investigated because of the short reaction times involved. Glutaraldehyde is a bifunctional reagent which in principle can be used to couple DNP-amino acids to the protein coat of bacteriophage. Accordingly, hapten-conjugated phage was prepared by incubating T_2-coliphage with DNP-lys [5.7 mM] and with varying concentrations of glutaraldehyde, for various time intervals. It was found that a 19.25 mM solution of glutaraldehyde incubated for 90 minutes with T_2-phage at a concentration of 1×10^{11} PFU/ml. and 5.7 mM of DNP-lysine yielded a product which was capable of being neutralized to 90% by an anti-DNP antiserum. This DNP-phage conjugate was not used for the work described in this thesis.

Standardization of the Neutralization Assay

In order for the neutralization assay to be reproducible and compatible with any anti-DNP antibody preparation, it was necessary to standardize the conditions of the assay. One of the variables which was examined was the time required for the reaction between DNP-T_2-phage and anti-DNP antibody to reach equilibrium. To study this aspect, duplicate 0.1 ml. aliquots of the mixture of DNP-T_2-phage and the reference antiserum #173 (1/300 dilution) were withdrawn at 5 minute intervals and plated. To correct for non-specific reduction in the number of PFU with time, duplicate 0.1 ml. portions of DNP-

T_2-phage in nutrient broth were also plated at five minute intervals. It was found that the DNP-T_2-phage was completely neutralized within 5 minutes in the presence of the reference anti-DNP antiserum. It was important, next, to determine whether lower concentrations of antibody would require a longer incubation time for complete neutralization of the conjugated phage. Various dilutions of anti-DNP antiserum (#173) were incubated for 45 minutes, 3 hours, and 4.5 hours with two different concentrations of DNP-T_2-phage. In Table 3 are summarized the results of this study. The titre of this antiserum was calculated for each reaction time and for each antigen concentration. It was found that there was no difference in the degree of neutralization between 45 minutes and 4.5 hours of incubation, over a four-fold range of DNP-T_2-phage concentration. On the basis of this result, 45 minutes was selected as the standard period of incubation for the neutralization assays described in this thesis.

It is known that E. Coli are temperature sensitive and that the initial temperature of the assay mixture ($47^{\circ}C$) will cause cell death (Adams, 1959). An experiment was designed therefore to study the effect of incubation at $47^{\circ}C$ on the reproducibility of the neutralization assay. 0.1 ml. of a standard dilution of DNP-T_2-phage was added to each of a series of tubes containing 2.5 ml. of a 0.5% noble agar solution (the upper layer) in a $47^{\circ}C$ water bath. Two drops of an E. Coli B suspension were then added to each tube and at one minute intervals thereafter three tubes were removed and the contents plated. The results are summarized in Table 4. As can be seen, the plaque count decreases with increasing time of incubation and, after only one minute,

TABLE 3

TIME STUDY OF THE NEUTRALIZATION OF DNP-T_2 PHAGE BY ANTI-DNP ANTIBODY

$(\text{antibody dilution})^{-1}$	% neutralization at two antigen concentrations					
	50 PFU/ml			200 PFU/ml		
	45 min	3 hr	4.5 hr	45 min	3 hr	4.5 hr
40	100	100	100	100	100	100
160	100	100	100	100	100	100
640	100	95	100	86	90	90
2560	20	50	54	28	20	15
10240	10	0	1	7	11	0
40960	0	0	0	0	0	0
163840	0	0	0	0	0	0
$\log_{10}$antibody titre	3.301	3.400	3.400	3.301	3.346	3.326

DNP-T_2 phage was incubated at two different concentrations with anti-DNP antibody. The effect of antibody concentration and time of reaction on the degree of neutralization was determined.

TABLE 4

THE EFFECT OF INCUBATING E. COLI B AT 47°C ON ITS ABILITY TO SUPPORT PHAGE REPLICATION

time of incubation (minutes)	average plaques per plate	% reduction in plaques
0	167	0
1	142	12
2	130	20
3	131	20
4	123	24
5	111	31
9	107	34

TABLE 5

THE SECONDARY ANTIBODY RESPONSE TO DNP-BGG: A COMPARISON OF STRAINS BALB/C AND CBA

Days after secondary injection*	anti-DNP antibody titres**	
	CBA	BALB/C
5	10	40
6	160	5,000
7	640	40,960

* 100 μg DNP_{44}-BGG was injected i.p. on day 0.

** antisera were assayed by DNP-phage neutralization.

there was a 12% decrease in plaque count. This indicates that no more than one minute should elapse between the addition of E. Coli B and plating, and that for reproducibility of results a constant plating procedure must be used.

The stability of DNP-T_2-Phage at -20°C

In the Methodology section (p. 32), reference has been made to the spontaneous gradual fall in titre which occurs when DNP-T_2-phage is stored over chloroform at 4°C. A study was made therefore of the possibility of storing the phage at -20°C. An experiment was constructed to examine the effect of freezing and thawing on phage viability. Various concentrations of conjugated phage (from 10^5 PFU/ml. to 10^{10} PFU/ml.) were subjected to short term (1-2 days) freezing at -20°C and were then thawed. This procedure was repeated 5 times and the viability of the phage (PFU/ml.) was determined after each thawing. In Figure 6 are illustrated the results of this experiment. There was an initial drop in titre of 20%. The phage titre however became stable after 90% of the phage had been killed. Below 1 x 10^5 PFU/ml., there seemed to be a more drastic reduction in viability and only 5% of the phage remained after only a single cycle of freezing and thawing. Three preparations of phage (see below) were incubated at -20°C for a longer period of time (two months) and then thawed, to determine the long term effects of freezing on phage stability. In this instance, there was a drop in phage titre greater than 99.99% as exemplified in the following table.

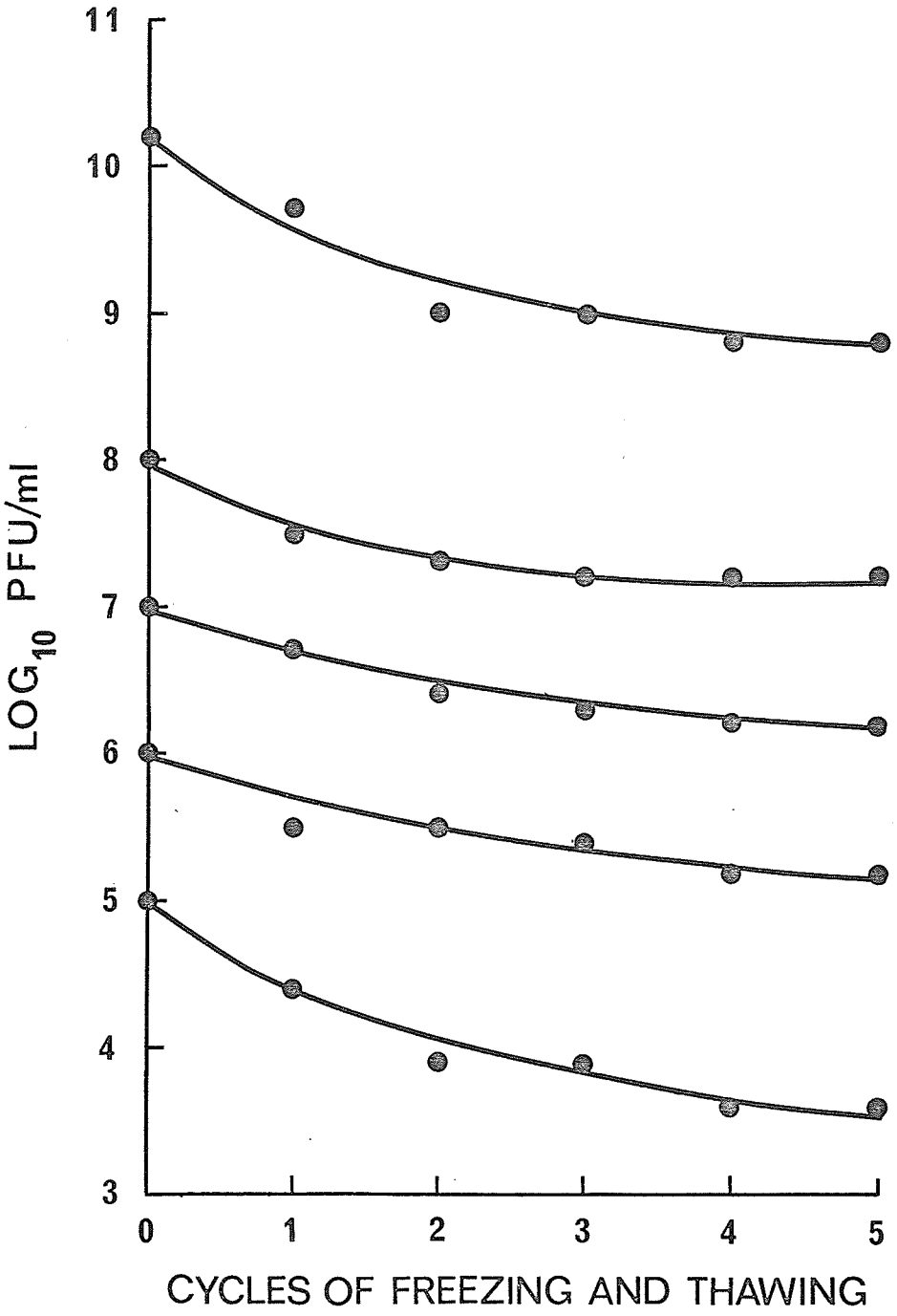


FIGURE 6. THE EFFECT OF FREEZING AT -20°C ON THE STABILITY OF A DNP-T_2-PHAGE SOLUTION. DNP-T_2-phage preparations at several concentrations were subjected to cycles of freezing and thawing and the concentration of phage (PFU/ml) established after each cycle. The duration of each cycle was 24-48 hours.

INITIAL PFU/ml.	PFU/ml. AFTER 2 MO. AT -20°C
1.7×10^6	5.0×10^1
3.8×10^6	8.0×10^2
5.2×10^6	1.0×10^3

These tests indicated that conjugated DNP-T_2-phage could not be stored at -20°C with retention of activity and therefore all of the DNP-T_2-phage used in this thesis was stored at 4°C over chloroform.

The Responsiveness of Balb/c and CBA Mice to DNP-BGG

Both CBA and Balb/c mice have been shown to be good responders to a variety of antigens *in vivo* (Havas, 1969; and Mitchison, 1967), and *in vitro* (Segal et al, 1970; and Feldmann and Basten, 1971). A choice between the two strains was difficult to make, since their relative responsiveness depended on the antigen used as well as on the laboratory in which the comparison was made (Davidsohn et al, 1954; and Gerottini et al, 1969). Balb/c and CBA mice were therefore immunized with DNP_{44}-BGG to determine which was the best responder to this antigen. The strain which responded with the highest antibody titres *in vivo* could be expected also to respond more vigorously *in vitro*. Both strains were immunized i.p. with 800 μg of lyophilized DNP_{44}-BGG suspended in saline and then reinjected 21 days later with 2 mg. of the same antigen. Serum samples were obtained from mice of each strain on days 10, 13 and 15 during the primary response and on days 5, 6 and 7 during the secondary response, and were assayed for anti-DNP activity by DNP-T_2-phage neutralization. The average serum titres of these two strains on successive days after the secondary injection are listed in Table 5. It was found that Balb/c mice responded more

vigorously than CBA. The serum titres measured during the primary response of both strains were essentially equal. On the basis of these results, a detailed study of the primary and secondary responses of Balb/c mice was undertaken therefore to establish the kinetics of the *in vivo* anti-DNP antibody response. 100 µg of DNP-BGG was injected i.p. into adult Balb/c mice and 33 days later, a second injection of 100 µg was given. The serum anti-DNP antibody was titrated by means of phage neutralization. The primary and secondary responses are depicted in Figure 7. The anti-DNP antibody response to 100 µg of DNP-BGG reached a maximum titre 6 days after the primary injection of antigen and returned to the background level after 28 days. Reinjection of 100 µg of DNP-BGG at this time resulted in a maximum titre, appearing 4 days after the injection. This titre was 3 times higher than the maximum titre elicited during the primary response.

Establishment of Tissue Culture Conditions

Armed with a sensitive reagent (DNP-phage) to detect anti-DNP antibody and a strain of mice responding well *in vivo* to the DNP-hapten, organ cultures were set up to study the *in vitro* response to the DNP-determinant. Mice stimulated to undergo a secondary anti-DNP response 2 days previously were killed and their spleens removed. Fragments of the spleens were then placed into organ culture dishes (Falcon plastic, #3010). The first objective of this experiment was to establish *in vitro* whether Balb/c spleens would indeed respond better than CBA mice since it was possible that the responsiveness of the two

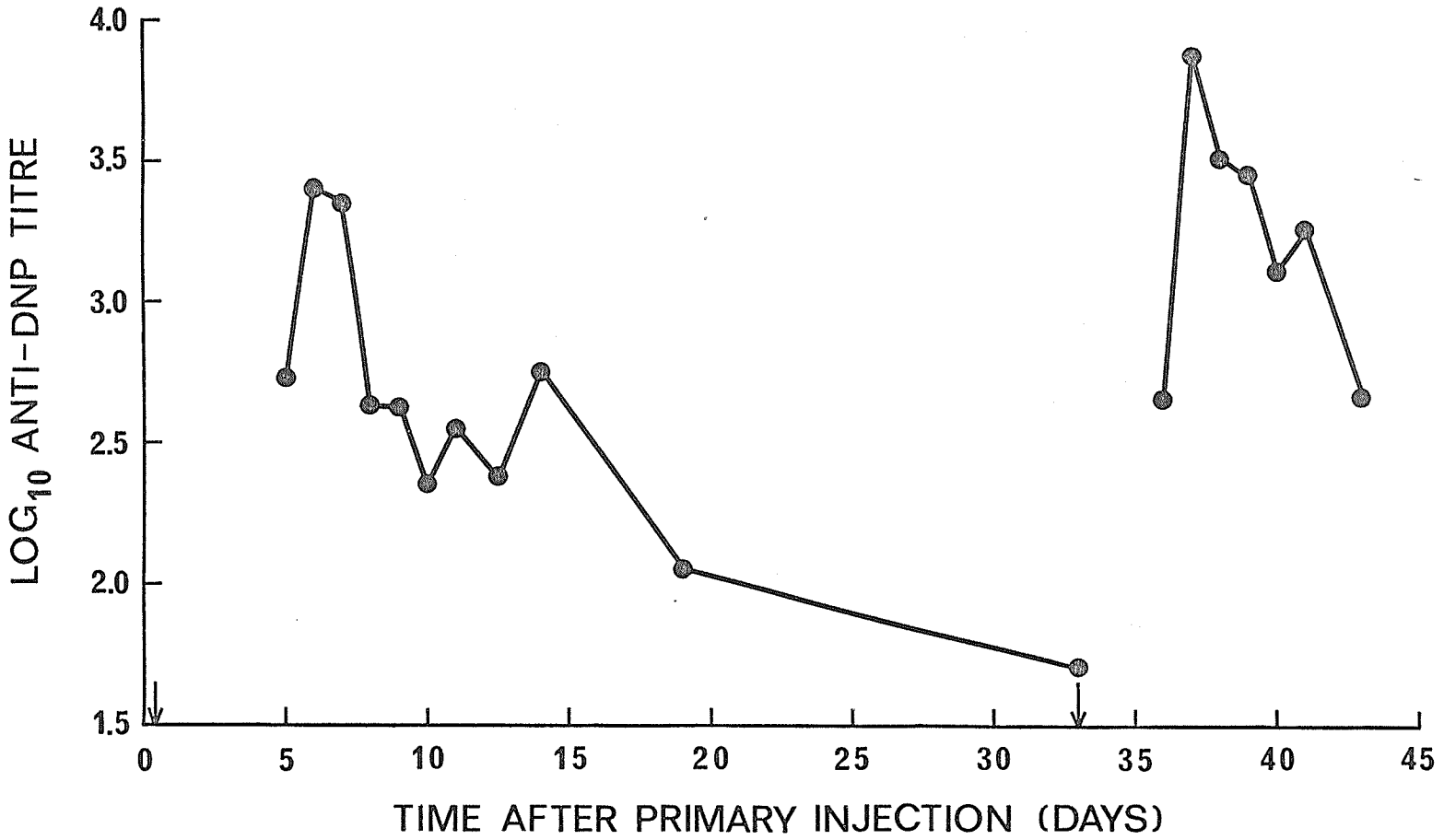


FIGURE 7. THE PRIMARY AND SECONDARY RESPONSE OF BALB/C MICE TO DNP-BGG. 100 µg DNP-BGG were injected i.p. on day 0 and day 33 (indicated by the arrows). The antisera obtained from each group of mice were pooled for any given day and titrated by DNP-T_2-phage neutralization.

strains might change under *in vitro* conditions. For example, C57Bl/6 does not respond in tissue culture unless l-asparagine is added to the tissue culture medium, and this appears to be a requirement for this strain unique to the *in vitro* response. The second objective of this experiment was to determine whether spleen fragments could be sustained *in vitro*, and for what period of time. The results of this experiment are shown in Table 6. The Balb/c strain produced the highest titres; CBA responded only slightly above background. Thus it was clear that Balb/c was indeed the strain to use for *in vitro* experiments on the anti-DNP antibody response.

Further analysis of Table 6 will show that tissue culture medium interfered with the production of plaque formation (10-40% reduction in PFU). An experiment was designed therefore to test the hypothesis that the E. coli B used in the assay were sensitive to the concentrations of antibiotics which were present in the tissue culture media being tested. To create conditions in which the E. coli would be highly sensitive to antibiotics, a bacterial suspension in the log phase of growth was stored at $4^{o}C$ for 12 hours before use in the neutralization assay. As the E. coli warmed up to room temperature, they would begin metabolizing and dividing at an increasing rate (Adams, 1959). Under these conditions E. coli would be more susceptible to antibiotics than under the usual conditions of the assay. Tissue culture media (with and without antibiotics), nutrient broth and an anti-DNP antiserum (with and without antibiotics) were all tested with this E. coli preparation. In Table 7 are summarized the results obtained from this experiment. It can be

TABLE 6

THE SECONDARY ANTI-DNP ANTIBODY RESPONSE MAINTAINED IN VITRO: A COMPARISON OF BALB/C AND CBA ORGAN CULTURES

Days in culture	% neutralization of DNP-phage			
	Balb/c cultures	CBA cultures	medium only	N.B.*
2	90	40	40	0
4	79	33	10	0
6	71	25	16	0
8	62	17	17	0

* N.B. = Nutrient Broth (Negative Control)

TABLE 7

THE EFFECT OF ANTIBIOTICS ON THE NEUTRALIZATION ASSAY AS MEASURED BY THE PLATING EFFICIENCY OF DNP-PHAGE AND E. COLI GROWTH

Solution tested	E. coli growth		% plating efficiency
tissue culture medium (neat)	normal	(+++)	56
tissue culture medium + 20% FCS	normal	(+++)	63
nutrient broth	normal	(+++)	100
tissue culture medium + 20% FCS + antibiotics	sparse	(+)	N.C.
tissue culture medium + 20% FCS + antibiotics (*)	sub-normal	(++)	86
nutrient broth + antibiotics	no growth	(-)	N.C.

(*) - Previously incubated at 37°C for 4 days

N.C. - not calculable

seen that antibiotics caused the upper layer of coli to achieve only sparse growth. It was also found that fresh media had a more pronounced effect on the growth of the E. coli than media which had been already in tissue culture for a period of time. This can be accounted for since the antibiotics used are known to have a short half-life at 37°C. The effect was attributed therefore to antibiotics. On the basis of these results, it was decided that the medium must be diluted before assay in order to lower the concentration of antibiotics. A five-fold dilution of the tissue culture medium in nutrient broth was found to eliminate the effect of antibiotics; the negative control consisted of tissue culture medium diluted to the same degree.

Since Balb/c organ cultures could be maintained successfully in tissue culture, the next step was to immunize the culture in vitro. Balb/c mice which had gone through a primary immune response five months previously, were killed and their spleen fragments put into organ culture. Five μg of DNP_{44}-BGG were then added to the tissue culture medium and the fragments incubated for 48 hours with antigen. After this time, the antigen containing medium was replaced with fresh medium (no added antigen). Anti-DNP antibody activity was not detected after 7 days of culture. At this time the cultures had to be terminated because of a persistent yeast infection -- identified as a candida species by laboratory analysis.[1] A sensitivity test to Fungizone was prepared to determine the concentration of this anti-fungal antibiotic required to inhibit yeast growth. A small

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[1]The yeast was identified by the Medical Microbiology department associated with this university.

number of spores were inoculated into medium containing graded doses of Fungizone. Yeast growth was determined microscopically after 4 days of incubation at 37°C. 3.125 µg of Fungizone per millilitre of tissue culture medium was found to prevent fungal growth. Prior to this test, medium had contained only 0.25 µg/ml. of Fungizone. All subsequent tissue culture media were fortified, therefore, with an additional 3 µg/ml. of Fungizone.

Renewed attempts to elicit a secondary anti-DNP antibody response in vitro failed and so the following possible causes were investigated: (1) the tissue culture technique was faulty; (2) the tissue culture medium was not suitable for Balb/c cells; or (3) the antigen was not immunogenic. Use was made of a viability stain-thiazyl blue (Black et al, 1953) - to gain information directly from the organ cultures, but tests comparing various tissue culture media and their effect on the organ culture's viability were equivocal. Emphasis was placed, therefore, on the propagation of a Balb/c polyoma cell line (donated by Dr. E. Berczi) as an indicator of the adequacy of the tissue culture techniques. Since it was found possible, employing the previously used techniques to sub-culture the polyoma cells at least five times over a period of five weeks, it was concluded that the tissue culture apparatus was compatible with cell growth, i.e., it was free from heavy metal ion and micro-organism contamination. The viability of the polyoma cells was used as an index for selecting the most suitable buffer system for use in tissue culture (see Table 8). Since a growing culture produces acid as a by-product of its metabolism, it is necessary to buffer the medium with an agent capable of maintaining a constant pH. Only strong buffers, such as HEPES (Shipman, 1969), and bicarbonate

TABLE 8

A COMPARISON OF SEVERAL TISSUE CULTURE MEDIA BASED ON THEIR ABILITY TO SUPPORT THE GROWTH OF A BALB/C POLYOMA CELL LINE

Medium	Buffer	Salt base	Gas ratio	Polyoma growth
Eagle L	bicarbonate	EARLES	95%Air:5%CO_2	+
Eagle L	HEPES	EARLES	100% Air	+
199	bicarbonate	HANKS	95%Air:5%CO_2	-
RPMI	phosphate	-	100% Air	-

TABLE 9

THE ANTI-SRBC RESPONSE IN VITRO USING BALB/C SPLEEN CELLS IN A MISHELL-DUTTON TISSUE CULTURE SYSTEM.

Time after in vitro stimulation (days)	PFC/10^7 cells	
	normal cultures	immunized cultures
3	20	107
4	70	81
5	4	6

(2.2 gm/litre)--as found in an Earles base--were found able to sustain the Balb/c polyoma cell line. A fundamental difference exists between HEPES and bicarbonate in terms of their buffering mechanism. HEPES irreversibly accepts hydrogen ions until it becomes saturated, whereas the bicarbonate buffer produces a dynamic equilibrium about pH 7.2, maintained by the CO_2 in the gas and liquid phase. Only media containing 2.2 gm/l of bicarbonate were used for all succeeding tissue culture work.

Because of the success achieved in growing the Balb/c polyoma cell line, and because this was done with a suspension culture, the possibility was explored of stimulating an anti-DNP response from a Balb/c spleen cell suspension culture instead of continuing with the organ cultures. An attempt was therefore made to reproduce Mishell and Dutton's suspension culture system: using spleen cells from normal Balb/c mice and sheep erythrocytes (2×10^7 SRBC per culture) as the antigen. The cultures were tested for PFC by the Jerne plaque assay on days 3, 4 and 5 after establishing the cultures. The results are shown in Table 9. All of the immunized cultures responded higher than did the unimmunized cultures. These results indicated that the Mishell-Dutton culture system could indeed be reproduced with Balb/c mouse tissue, and so an <u>in vitro</u> anti-DNP response was attempted. Immune Balb/c spleen cells were incubated with 5 µg of DNP_{44}-BGG. Every two days the culture media were assayed for anti-DNP activity by the phage neutralization assay. Each sample of tissue culture medium assayed, however, was found to contain four-times as many PFU as was originally added to the

medium. It was therefore not possible to show any neutralization since these PFU appeared to be unaffected by anti-DNP antibody. Since the nutrient broth used as a diluent did not contain any PFU above that added to the test system, it was concluded that the tissue culture medium must have contained coliphage. Subsequently, it was found that unused, sterile, tissue culture medium contained large numbers of PFU which were not neutralized by anti-DNP antibody. Of all the constituents in the tissue culture medium only the fetal calf serum (FCS) was considered as a possible source of coliphage. Accordingly, all batches of FCS in current use were examined. Bottles representing different lot numbers were freshly thawed and compared to bottles of the same lot number already in use, to establish whether the contamination originated either from the supplier, or as a result of use. One ml. from each bottle was plated in a phage assay. In Table 10 are listed the results of this investigation. It was found that several batches of FCS from one supplier were already contaminated with coliphage at the time of receipt. Therefore, as a precaution, all new batches of FCS were analysed, for coliphage before use as a tissue culture supplement.

In an effort to increase the magnitude of the anti-SRBC response, the method of culture was changed and the anti-SRBC response itself was used as the indicator of culture conditions. It was assumed that changes which would allow an increase in the level of the anti-SRBC response would also allow an increase in the anti-DNP response. Cultures were established using tissue culture tubes instead of dishes. The tubes were gassed, tightly capped and then,

TABLE 10

ANALYSIS OF FCS FOR BACTERIOPHAGE*

Lot number	Supplier	Description	PFU/ml
A2074E	GIBCO	IPT; sterile	1×10^5
A3034X	GIBCO	IPT; sterile	nil
A6112G	GIBCO	IPT; sterile	nil
C2121Q	GIBCO	IPT; sterile	7×10^4
76363	Microbiological Associates	Cytopathogenic virus free; IPT; sterile	nil
77543	Microbiological Associates	Cytopathogenic virus free; IPT; sterile	nil

IPT = Immunoprecipitin tested

* Bacteriophage typed only as a coliphage; further identification was not done.

TABLE 11

THE ANTI-SRBC RESPONSE IN VITRO OF SPLEEN CELLS FROM BALB/C MICE USING TISSUE CULTURE TUBES: THE TUBES WERE ROTATED AT 0.2 RPM DURING CULTURE.

Time after in vitro stimulation (days)	PFC/10^7 cells	
	normal cultures	immunized cultures
2	630	1643
4	2550	5700

instead of being rocked, were rotated at a rate of 1/5 rpm in a 37°C incubator. In Table 11 is described the anti-SRBC response obtained under these new conditions. There was about an 80-fold increase in the response as compared to that obtained using the Mishell-Dutton culture conditions (Table 9). Since the anti-SRBC response had been increased in magnitude, additional cultures were established using the new conditions to elicit an anti-DNP antibody response. Alum precipitated DNP_{44}-BGG was used to stimulate the cultures (30 µg per culture). Tissue culture media were assayed every two days for the presence of anti-DNP antibody. Using the same tissue culture conditions, other cultures were stimulated with 3×10^6 SRBC to serve as a reference response, and these cultures were assayed for PFC on day 4 by using the Jerne plaque assay. A response to the DNP-residue could not be detected despite a measurable response to SRBC: 250 PFC/10^7 spleen cells were measured in the cultures which had been stimulated with SRBC, and only 120 PFC/10^7 spleen cells in the unimmunized cultures.

Stimulation of an Anti-DNP Antibody Response In Vitro

As a result of the success achieved with the SRBC antigen, the DNP-residue was attached to the sheep erythrocyte in an effort to elicit a measurable anti-DNP response. The *in vitro* response could then be monitored for both anti-hapten and anti-carrier responsiveness by studying both the cells, using a haemolytic plaque assay (anti-carrier response) and the medium, using a neutralization assay (anti-hapten response). DNP-BGG was coupled to the sheep erythrocyte using BDB. The resulting antigen--DNP-BGG-SRBC--was first tested

in vivo to determine its immunogenicity with respect to the DNP-determinant. 5×10^8 conjugated erythrocytes were injected i.v. into Balb/c mice. On days 4, 6 and 8 after the primary injection, animals were bled and the serum assayed for anti-DNP antibody. In Table 12 are recorded the average anti-DNP titres on each day. Unlike the anti-hapten response obtained following stimulation with DNP-BGG (Figure 7) in which the maximum response was given on day 6, the anti-hapten response to DNP-BGG-SRBC showed a maximum on day 4. Having shown that the DNP-BGG-SRBC conjugate was indeed immunogenic, the next experiment was to stimulate a primary *in vitro* anti-DNP antibody response using the same conjugated erythrocytes. 70λ of a DNP-BGG-SRBC suspension were therefore added to a series of Balb/c spleen cell suspension cultures. Control cultures were incubated without antigen. Every second day, a sample of each culture medium was tested for DNP-T_2-phage neutralizing ability; medium from cultures incubated with antigen was in each case compared to that from cultures without antigen. The results depicted in Figure 8 show that media from cultures incubated with antigen neutralized DNP-T_2-phage to a greater extent than did media from unimmunized cultures. Figure 9 demonstrates the number of immunized cultures which responded above the range of the unimmunized cultures. Despite the fact that an anti-DNP response had been elicited, further work was dependent upon this response being augmented. Therefore the *in vivo* work was continued in order to develop an antigen, whose properties were known *in vivo*, and which could then be used to elicit a utilizable *in vitro* anti-DNP response.

TABLE 12

THE RESPONSE OF BALB/C MICE TO DNP-BGG-SRBC.

Time after injection (days)	Anti-DNP titre
0	28
4	648
6	186
8	92

Each mouse was injected with 5×10^8 conjugated SRBC. Serum anti-DNP antibody levels were determined by use of the DNP-T_2-phage neutralization assay on days 4, 6 and 8 after the injection of antigen.

TABLE 13

THE PRECIPITATION OF ANTIBODIES FROM RABBIT AND MOUSE ANTISERA USING 18% Na_2SO_4: A COMPARISON OF ANTIBODY YIELD.

Salting-out fractions	adjusted* Hemagglutination titre	
	rabbit Ig	mouse Ig
Supernatant after 18% ppt'ation	2	128
Supernatant after first wash in 18% Na_2SO_4	2	64
Supernatant after second wash in 18% Na_2SO_4	2	4
Dissolved precipitate	800	1024
Original serum	800	1024

* all titres were adjusted to the original serum volume

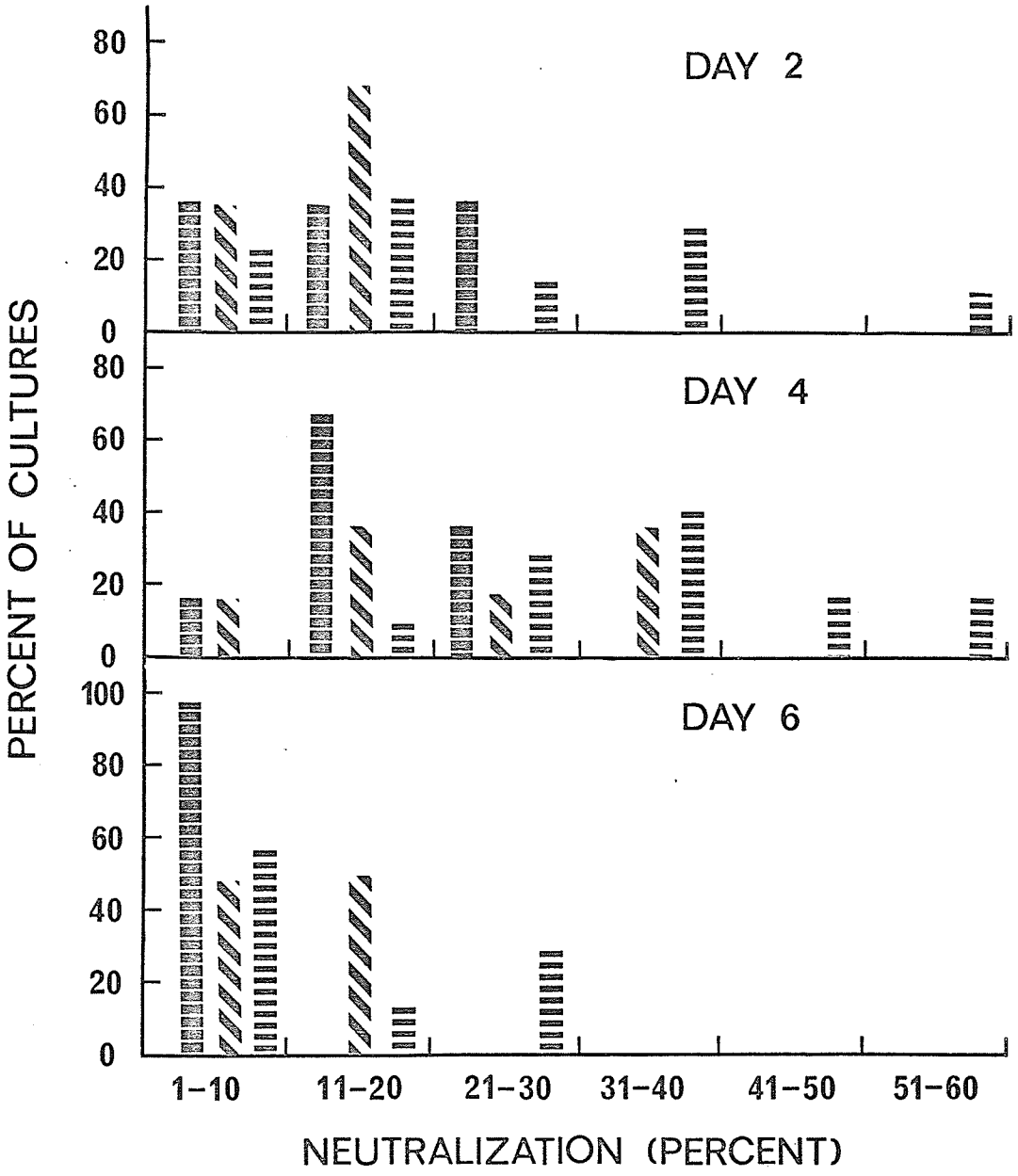


FIGURE 8. THE IN VITRO ANTIBODY RESPONSE TO DNP-BGG-SRBC CONJUGATES ELICITED FROM BALB/C SPLEEN CELLS. Test cultures were established and immunized in vitro (▤). Controls consisted of cultures containing spleen cells and no antigen (▨), and culture vessels containing only tissue culture medium (▤). Media were assayed for anti-DNP antibody by the DNP-T_2-phage neutralization assay.

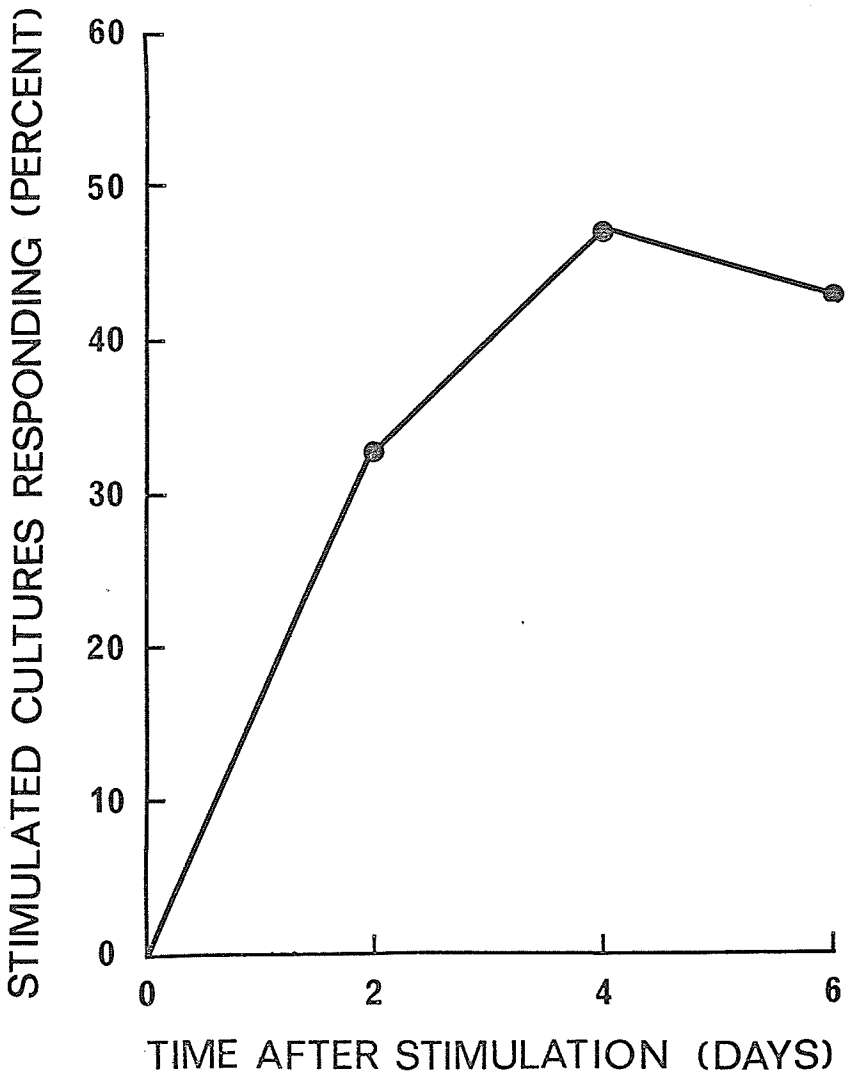


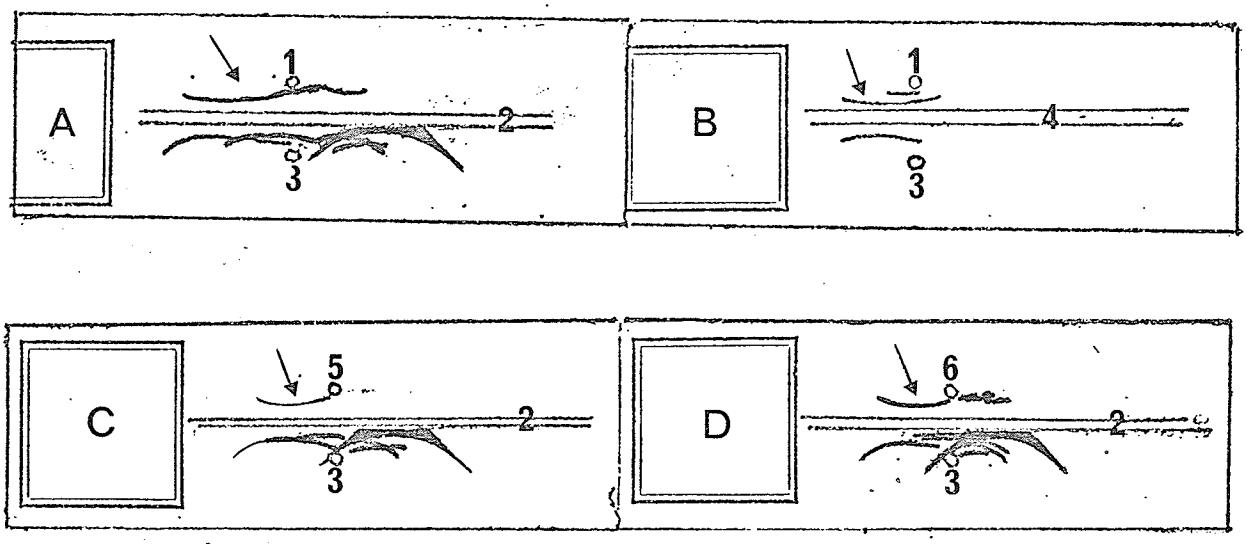
FIGURE 9. THE IN VITRO ANTI-DNP ANTIBODY RESPONSE TO DNP-BGG-SRBC BY BALB/C SPLEEN CELLS IN SUSPENSION. The culture media were titrated by the DNP-T_2 neutralization assay. Each point represents the percent of stimulated cultures which neutralized at least 5% greater than the unstimulated cultures.

IN VIVO INVESTIGATION OF THE CARRIER EFFECT

Use was made of anti-SRBC antibodies to bind the DNP-determinant to sheep erythrocytes, as described by Strausbauch et al (1970). Two species of anti-SRBC antibodies were employed: mouse anti-SRBC and rabbit anti-SRBC. After collecting the sera from mice and rabbits which had been hyper-immunized with SRBC, the gammaglobulins were isolated by a salting-out procedure using 18% Na_2SO_4, as described in Methodology, section IV. A comparison of the recovery of gammaglobulin suggested that MGG behaved differently than RGG at 18% Na_2SO_4. Therefore, the supernatant fraction from each step of the procedure was analysed for anti-SRBC activity in a hemagglutination (HA) assay. Agglutination of SRBC by any of the supernatant fractions would indicate the presence of anti-SRBC antibody. The magnitude of the titre would provide a quantitative estimate of the immunoglobulins not precipitated by 18% Na_2SO_4. The dissolved precipitate was also titrated using the same technique. It was found that approximately 10% of the mouse anti-SRBC gammaglobulin was not precipitated by 18% Na_2SO_4, whereas less than 0.1% of the rabbit anti-SRBC gammaglobulin remained unprecipitated under the same conditions. These results are shown in Table 13. The gammaglobulin which had been isolated by salting-out was electrophoresed and allowed to diffuse against a rabbit anti-(normal mouse serum) antiserum (Figure 10-A) and rabbit anti-MGG (Figure 10-B) to show the purity of the product: normal mouse serum was also immunoelectrophoresed to provide reference precipitin lines. It was found that further purification of mouse immunoglobulins, with the

FIGURE 10

IMMUNOELECTROPHERETIC ANALYSIS OF FRACTIONS FROM 18% and 12% Na_2SO_4 PRECIPITATION OF MOUSE SERUM



1 MGG isolated by 18% Na_2SO_4 precipitation

2 Rabbit anti-(normal mouse serum) antiserum

3 Normal mouse serum

4 Rabbit anti-(MGG) antiserum

5 Supernatant from 12% precipitation of MGG isolated by 18% Na_2SO_4

6 Supernatant from 12% wash of 12% precipitation

⟶ Indicates γG immunoglobulin

Each fraction was electrophoresed and then left to diffuse in the presence of either rabbit anti-normal mouse serum or rabbit anti-MGG. In each case, normal mouse serum was immunoelectrophoresed as a reference.

objective of obtaining an IgG rich fraction using 12% Na_2SO_4 (Kekwick, 1940), was not feasible. Figure 10-C and 10-D show that ɣG remains in solution when the product of an 18% precipitation is further precipitated in the presence of 12% Na_2SO_4. For this reason both MGG and RGG were isolated using 18% Na_2SO_4 only.

Each conjugated gammaglobulin was incubated with SDNBS at pH 9.5, as described in Methodology, section V. After the unreacted SDNBS had been removed from the conjugate by dialysis, it was established by spectrophotometric analysis[2] that the DNP-MGG and DNP-RGG products had on the average 2-3 DNP-groups per molecule. The concentration of the DNP-gammaglobulin preparations was then adjusted until both had the same HA titre, i.e., they were equal in terms of their ability to agglutinate SRBC, subsequently each preparation was incubated separately with 5×10^8 SRBC. The purpose of this procedure was to prepare DNP-coupled SRBC in which the concentration of DNP-residues per SRBC was the same and the coupled erythrocytes would only differ in the type--not the number--of gammaglobulin molecules bound. After washing, the coupled SRBC's were injected into two groups of Balb/c mice, one received 5×10^8 DNP-MGG coupled SRBC (DNP-MGG-SRBC) i.p., and the other received 5×10^8 DNP-RGG coupled SRBC (DNP-RGG-SRBC) i.p. Half of

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[2]Each DNP-gammaglobulin preparation was read at 280 mμ and 363 mμ. The concentration of protein and the molar ratio's of DNP per gammaglobulin were calculated using the following parameters:

O.D. of 1mM ε-DNP-lysine at 363 mμ = 18

O.D. of a 1% solution of gammaglobulin at 280 mμ = 14

$\frac{\text{O.D. at 363 m}\mu}{2.89}$ = O.D. of DNP-lysine at 280 mμ

each group of animals were exsanguinated on day four, and the other half on day eight. The serum and spleen from each mouse were tested individually. The serum was assayed for anti-DNP antibody by determining its ability to neutralize DNP-T_2-phage in the presence and absence of 2-ME: the 2-ME sensitive titre was attributed to IgM; and the 2-ME resistant titre was attributed to IgG. The anti-SRBC response was assayed by determining the PFC content of each spleen in a hemolytic plaque assay designed to visualize both IgM (direct PFC) and IgG (indirect PFC) producing cells. In other words, both anti-DNP and anti-SRBC responses were analysed for IgM and IgG content. The objective of this experiment was to establish the individual roles played by the sheep erythrocyte and the DNP-residue, and further, the role played by the gammaglobulin moiety in the complex antigens which were used. The results are displayed in Figures 11 and 12, and in Table 14. Table 14 is a summary of the results. Figure 11 represents the anti-SRBC antibody response elicited by the DNP-MGG-SRBC and the DNP-RGG-SRBC complexes broken down into IgM and IgG antibody responses. The response to 5×10^8 unconjugated SRBC is included as a standard. Figure 12 presents the anti-DNP antibody responses to DNP-MGG-SRBC and DNP-RGG-SRBC immunogens, which is also divided into IgM and IgG antibody responses. The background anti-DNP antibody level is included to represent a negative response. Statistical analysis (Student's t-test for 95% significance) indicated that both antigenic complexes stimulated responses which were not significantly different. Neither was there any difference found when the responses were compared in terms of antibody class (IgG vs IgG; and IgM vs IgM). The total anti-DNP

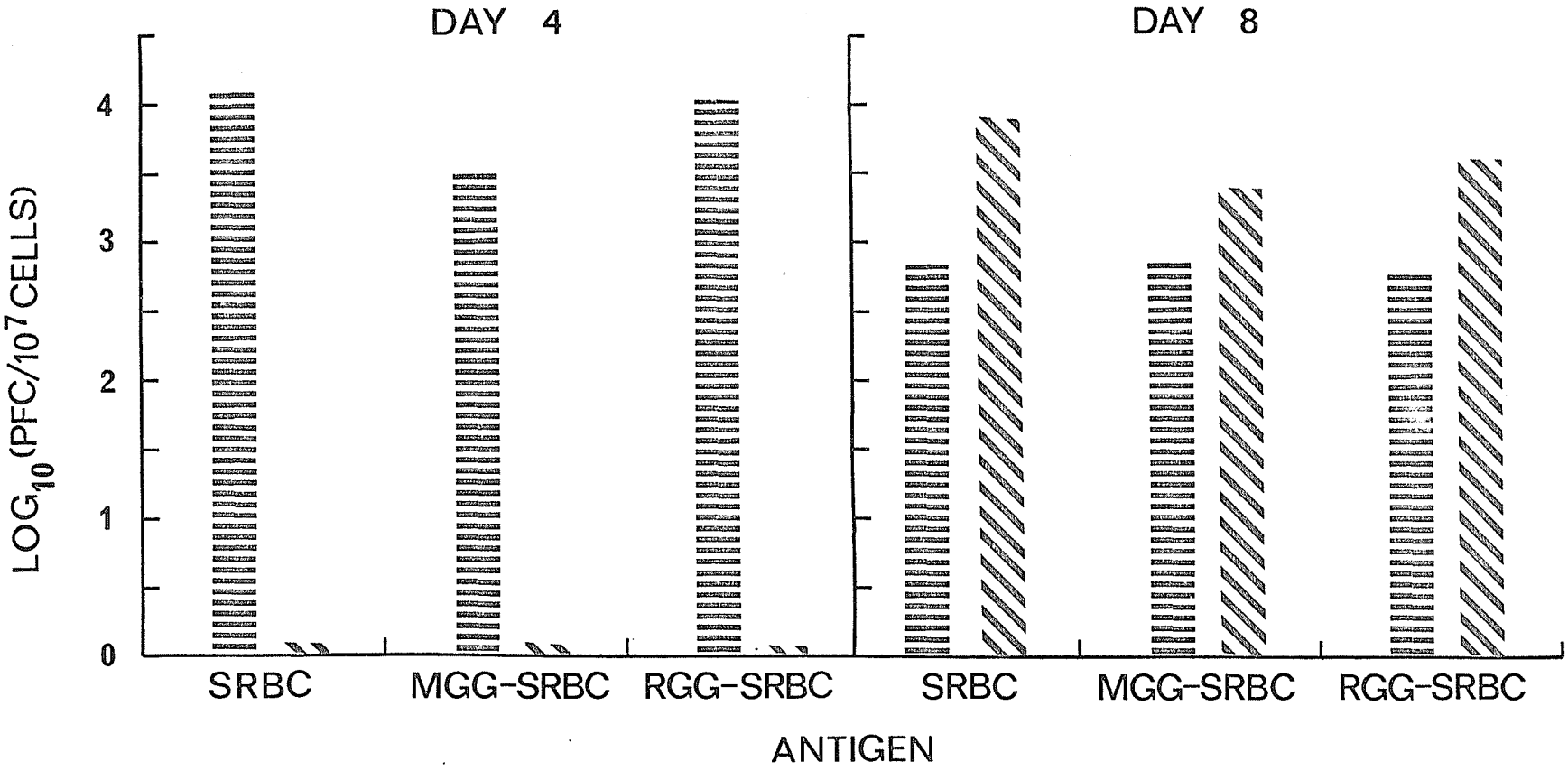

FIGURE 11. A COMPARISON OF ANTI-SRBC RESPONSES ELICITED IN BALB/C MICE BY DNP-MGG-SRBC, DNP-RGG-SRBC, AND SRBC. Mice were injected i.p. with 5 x 10^8 normal or conjugated erythrocytes and assayed for IgG and IgM antibody on days 4 and 8 after injection. The anti-SRBC response was assayed by the Jerne plaque assay; direct plaques represented an IgM response (▬), and indirect plaques represented an IgG response (▨).

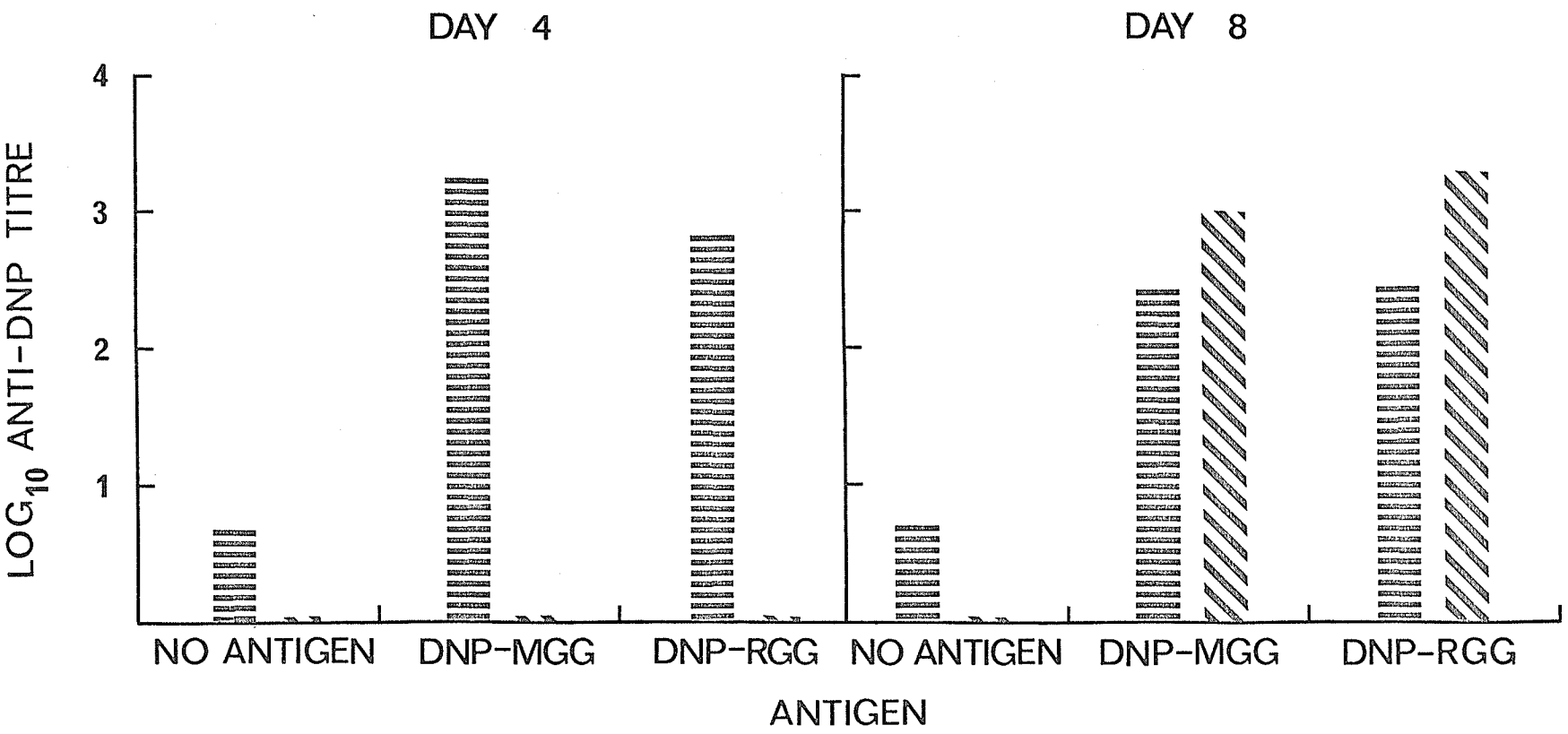

FIGURE 12. A COMPARISON OF ANTI-DNP ANTIBODY ELICITED BY DNP-MGG-SRBC AND BY DNP-RGG-SRBC ANTIGENS. Mice were injected i.p. with 5×10^8 conjugated erythrocytes and assayed for IgG and IgM antibody of days 4 and 8 after injection. The anti-DNP antibody response was assayed by the DNP-T_2-phage neutralization assay: antibody activity in the presence of 2-ME represented IgG anti-DNP antibody (▨); and antibody activity sensitive to 2-ME represented IgM anti-DNP antibody (▤).

TABLE 14

THE RESPONSE OF BALB/C MICE TO DNP-MGG-SRBC, DNP-RGG-SRBC, OR SRBC

Time after immunization (days)	Species origin of gammaglobulin	PFC/10^7 spleen cells		% Neutralization	
		direct PFC (IgM) x 10^{-3}	indirect PFC (IgG) x 10^{-3}	2ME sensitive (IgM) x 10^{-3}	2ME resistant (IgG) x 10^{-3}
4	no gammaglobulin	28.9	nil	0.01	nil
4	mouse	3.32	nil	1.4	nil
4	rabbit	9.1	nil	0.49	nil
8	no gammaglobulin	0.60	7.8	nil	nil
8	mouse	0.49	3.9	0.27	1.3
8	rabbit	0.56	4.0	0.27	2.0

The anti-SRBC antibody response is expressed as PFC/10^7 spleen cells, and the anti-DNP antibody response is expressed as the antibody titre at 50% neutralization of DNP-T_2-phage.

response expressed as the $\log_{10}$ of the anti-DNP titre, and the total anti-SRBC response, expressed as the $\log_{10}$ of the total PFC (direct and indirect) per 10^7 spleen cells, elicited in each individual mouse were compared to determine if there was any relationship between the two. The correlation analysis is described in Figure 13. The two responses were found to be very significantly related ($p \geq 0.001$). When the analysis was broken down into DNP-MGG-SRBC and DNP-RGG-SRBC stimulated populations, and the two populations were compared by an analysis of residuals (a t-test for regression data), it was found that no significant difference existed between the two responses of the groups.

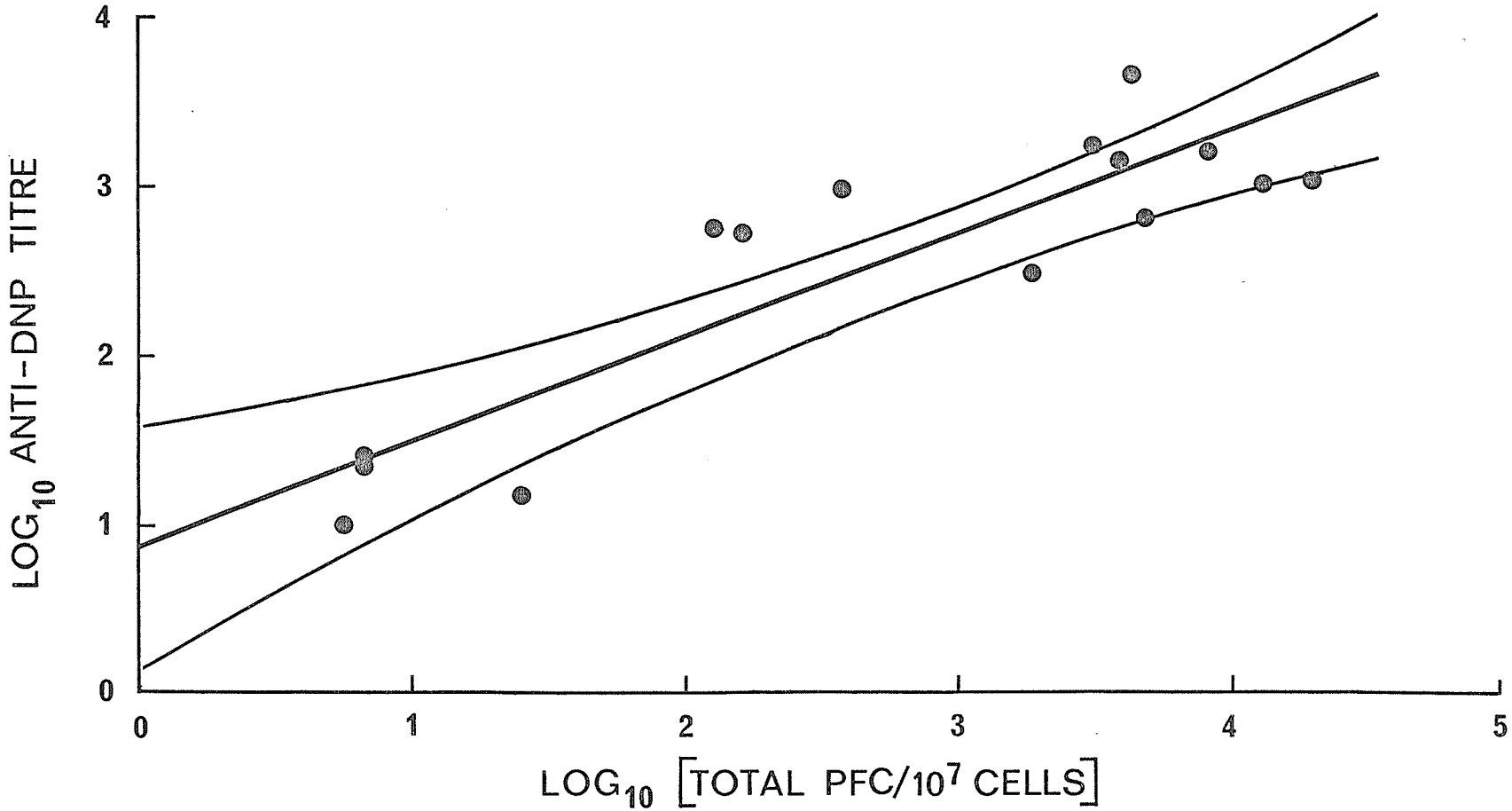

FIGURE 13. THE CORRELATION BETWEEN THE ANTI-DNP AND ANTI-SRBC ANTIBODY RESPONSES STIMULATED BY DNP-CONJUGATED ERYTHROCYTES. Balb/c mice were divided into two groups: one group was injected i.p. with 5×10^8 DNP-MGG-SRBC conjugates and the other group was injected i.p. with 5×10^8 DNP-RGG-SRBC conjugates. The total (IgM and IgG) anti-SRBC antibody response and the total anti-DNP antibody response of each mouse was plotted and a regression analysis performed. Included are the 95% confidence limits of the data.

DISCUSSION

The *in vivo* and *in vitro* anti-DNP antibody responses studied in this thesis were detected by the use of DNP-coupled T_2-phage. This sensitive reagent was synthesized by two different methods: the first employing sodium 2,4-dinitrobenzene sulfonate and the second glutaraldehyde and ε-DNP-lysine. Both of the methods yielded a product which was neutralized by anti-DNP antibody, and which could be used therefore to quantitate the anti-DNP antibody response. When sodium 2,4-dinitrobenzene sulfonate is incubated with T_2-phage at pH 9.5, it will react with free amino groups in the phage protein and thereby attach the DNP-residue directly to the phage. The second method, using the bifunctional reagent glutaraldehyde, facilitated the binding of ε-DNP-lysine to T_2-phage. Since both aldehyde groups react with free amino groups, reactant concentrations were adjusted (low phage concentration and high ε-DNP-lysine concentration) so that phage-phage coupling was reduced to a minimum.

Initial experiments were designed to facilitate a choice of a mouse strain with which to carry out the *in vivo* and *in vitro* analyses of the anti-DNP antibody response. Reference to the literature indicated that two strains, CBA and Balb/c responded well *in vivo* to the DNP-hapten. Selection of the strain which would mount the most vigorous anti-DNP antibody response in tissue culture was therefore made from these two strains. Using the DNP-T_2-phage to titrate the anti-DNP antibody responses of the Balb/c and CBA mice to DNP-BGG, it was found that Balb/c mice

were better responders than CBA both *in vivo* and *in vitro*. Important strain differences have been shown to exist in the immune responsiveness of mice to other antigens (Fink and Quinn, 1953; Davidsohn and Stern, 1954; and Warner et al, 1968). In these reports the differences have been attributed to the inherent properties of the antigen. One example of a response which is clearly under genetic control has involved the synthetic antigens [(T,G)-A--L] and [(H,G)-A--L] (McDevitt and Sela, 1965). These antigens are composed of a poly-lysine backbone and side chains of poly-DL-alanine terminating in short random sequences of either tyrosine and glutamic acid, or histidine and glutamic acid. Some strains of mice that respond well to [(T,G)-A--L] respond poorly to [(H,G)-A--L] and vice versa, i.e., for both antigens there are clearly defined responders and non-responders. The differences are thought to reflect a genetic influence on responsiveness. The gene controlling the responsiveness to [(T,G)-A--L], termed IR-1, lies within the H-2 region, the major histocompatibility locus of the mouse. Fink and Quinn (1953) studied Balb/c and four other mouse strains (A/J, C57Bl/6, DBA, and C3H) to establish the relative responsiveness of each to egg albumin. Balb/c was found to be a poor responder to this antigen. Davidsohn et al (1954) comparing the responsiveness of eleven inbred strains, two of which were CBA and Balb/c, found that CBA responded consistently better than did Balb/c to both sheep and chicken erythrocytes. In contrast to the above is evidence obtained by Cerottini et al (1969). These investigators compared the immune responses of NZB and NZB/W mice to those of A/J, CBA/J, and Balb/cJ. The antigens used were SRBC, Hcy, HGG, and BSA. It was reported

that each of the five strains responded differently to each antigen. If the responses of only the Balb/c and CBA strains are compared, the following generalization can be made from the results obtained by Cerottini et al: Balb/c mice responded better than CBA to all of the antigens except HGG. In the experiments reported in this thesis, Balb/c spleen fragments were able to respond in vitro to DNP-BGG under conditions which failed to support a response by CBA mice to the same antigen. An additional complicating factor to the inherent differences between strains is the finding by Tan and Gordon (1971) that the pattern of in vitro responsiveness does not always parallel the in vivo results. They found that tissue culture conditions which were adequate to sustain an in vitro anti-SRBC response from CBA spleen cells, would not allow a response by C57Bl/6 spleen cells. The apparent tissue culture deficiency was overcome by incorporating an excess of L-asparagine into the tissue culture medium and under these conditions C57Bl/6 spleen cells then were able to mount a normal anti-SRBC response. The unresponsiveness exhibited by CBA spleen fragments in vitro in this thesis must therefore be attributed to an undefined deficiency in the tissue culture medium, since this strain did respond in vivo.

One of the objectives of the experiments described in this thesis was the development of an anti-DNP antibody response in vitro. Since an in vitro anti-SRBC response has already been well studied (Mishell and Dutton, 1966; Marbrook, 1968; and Mosier, 1969), an in vitro anti-SRBC response was established using Balb/c spleen cells to serve as a reference response for the optimization of

tissue culture conditions for the anti-DNP antibody response. It was assumed that changes in the tissue culture technique which would increase the response of Balb/c spleen cells to the sheep RBC, were changes which would serve also to optimize the conditions for an in vitro anti-DNP antibody response. This assumption proved to be correct; the tissue culture system which was found to support the optimum anti-SRBC response was in fact the only system which supported an anti-DNP antibody response. The tissue culture system consisted of rotating air-tight tissue culture tubes containing 1.0 ml. of medium and spleen cells under a gas mixture of 95% air and 5% CO_2 at 1/5 rpm at 37°C. Prior to incubation, the culture was stimulated by the addition of 3×10^6 coupled or uncoupled SRBC. Although it has been reported that organ cultures of mouse spleen fragments will respond to DNP-Hcy to produce anti-DNP antibody (Segal et al, 1970; and Klinman, 1971), the culture conditions used in the present study were not adequate to support an anti-DNP response by spleen fragments in organ culture. Several investigators have reported the use of hapten-coupled SRBC to elicit an anti-hapten antibody response in tissue culture from mouse spleen cells in suspension culture: for example, Kettman et al (1970) and Katz et al (1971) have used TNP-SRBC; Trowbridge et al (1970) have used NIP-SRBC; and Naor et al (1970) have used Penn-SRBC. In each of these latter three reports a suspension-type culture was used to support the anti-hapten response. Tarrab et al (1971), employing rabbit spleen cells in suspension were able to elicit a secondary anti-DNP response to DNP-BSA in vitro. Because a soluble antigen was used in this

latter study, Tarrab et al were able to remove residual free antigen from the cell suspension after an initial pulse of antigenic stimulation. This was done to reduce the concentration of free antigen in the tissue culture which might otherwise combine with the antibody produced and lead to spurious results in which the antibody levels detected would be either low or absent. The anti-DNP response described by Tarrab et al was higher than that reported in this thesis, eg., on day 6 the media from DNP-BSA stimulated tissue cultures in the former study had an average titre of 450, while in the response described in this thesis the titre on day 6 was only about 5. A possible reason for this discrepancy may lie in the type of antigen used to elicit the response. The coupled erythrocyte which was used in the present experiments as antigen was present throughout the duration of the experiment and was probably responsible for sequestering some of the antibody which was produced, thereby reducing the effective titre of the medium.

Because the anti-DNP antibody response which was elicited with DNP-BGG-SRBC from Balb/c spleen cells cultured <u>in vitro</u> was low, other DNP-antigens were studied in an attempt to augment this antibody response. The carriers used for this further study were DNP-coupled anti-SRBC gammaglobulins prepared from the serum of mice and rabbits which had been immunized with SRBC. The technique used for isolating the gammaglobulins from the two sets of antisera was precipitation with 18% Na_2SO_4. During the isolation, it was found repeatedly that although 99.99% of the RGG was precipitated, only 90% of the MGG could be isolated under the same conditions. It was clear, therefore, that the original procedure which had been

developed for the isolation of gammaglobulins from human serum was not directly applicable for the isolation of these proteins from the serum of other species, for example, the mouse.

The conjugated antibodies were then allowed to react with SRBC to yield DNP-MGG-SRBC and DNP-RGG-SRBC, respectively. The two preparations of conjugated erythrocytes were injected separately into two groups of Balb/c mice and the resultant primary anti-DNP and primary anti-SRBC antibody responses were analysed for IgM and IgG antibody content. It is evident from the data previously presented in the Results section that these complexes each elicited both classes of antibody, and at the concentrations used, there was no apparant selectivity for any one class of antibody. Also, irregardless of the source of the gammaglobulin moiety (isogenic or xenogenic animals) used to attach the DNP-residue to the sheep erythrocyte, the anti-DNP antibody responses were not significantly different when analysed by a Student's t-test.

There are several ways of interpreting this finding. One would be to assume that the mouse gammaglobulin component of the DNP-MGG-SRBC complex was as immunogenic as the corresponding rabbit gammaglobulin component. It was possible that the mouse gammaglobulin was rendered immunogenic in the mouse, as a result of structural changes induced by coupling DNP-residues to it. Havas (1969) used MGG as one of several DNP-conjugated carriers to study the effect of the carrier molecule on the immune response to the DNP-determinant. She coupled 35 DNP-residues per MGG molecule and immunized Balb/c mice with 0.1 mg. of DNP_{35}-MGG in CFA. The responses elicited were analysed for antibodies directed against DNP-determinants, MGG-determinants, and/or new determinants created by the coupling reaction. Havas found that only 3 out of 9 mice

responded to DNP-MGG. It was also found that the titres of these mice were lower than those obtained with carriers naturally foreign to the injected mice, and that all of the antibody was directed toward new determinants found only on the conjugated MGG, i.e., the antibodies produced failed to agglutinate DNP-SRBC or MGG-SRBC. The results of this study indicate that a heavily coupled gammaglobulin can be weakly immunogenic in the strain from which the gammaglobulin was isolated. Fronstin et al (1967) were able to demonstrate that DNP-MGG (30 DNP-groups per MGG molecule) could stimulate an anti-DNP response when they injected 1 mg. of protein with CFA into C3H mice. These authors were studying the relationship between immunogenicity of the carrier and the magnitude of the anti-hapten response. Although DNP-MGG was found to be immunogenic, the response was always lower than that elicited by the xenogenic proteins (RGG, RtGG; and BGG). Golan and Borel (1971), on the other hand, were not able to demonstrate an anti-DNP antibody response to DNP-MGG using a less heavily coupled conjugate (23 DNP-groups per molecule of MGG), despite the fact that the protein (0.2 mg.) was injected in CFA.

The conjugation and immunization procedures used in the experiments described in this thesis militate against the possibility that the DNP-MGG component of the DNP-MGG-SRBC complex was immunogenic in Balb/c mice for the following reasons: (1) the conditions of the conjugation reaction were designed so that a lightly substituted protein would be produced to avoid creating any new antigenic determinants in the MGG protein; in fact only 2-3 DNP-groups were actually attached to each molecule, (2) there was no detectable loss of anti-SRBC activity as a consequence of the conjugation reaction at least as judged by HA titration, (3) the concentration of DNP-MGG actually injected into any

one mouse was much less than any of the doses reported in the above studies (the amount was calculated to be no more than 7 μg)[3] and (4) all injections were made without the use of powerful adjuvants like CFA, as was used in the previously cited works. In regards to the last point, an anti-hapten antibody response, using carrier molecules from syngeneic animals, was elicited only when the antigen was injected in CFA (Havas, 1969; Golan and Borel, 1971; and Fronstin et al, 1967) or when the animal was skin-painted beforehand with the hapten (Iverson, 1970).

Thus, it is much more likely that the MGG component of the DNP-MGG-SRBC complex was not immunogenic, and that the anti-DNP response must have depended upon the SRBC component for carrier function, i.e., the MGG molecule was attached to the erythrocyte membrane at the time of stimulation and this erythrocyte membrane functioned as the carrier for the anti-DNP antibody response. The immunogenic entity must therefore have contained at least two sheep RBC antigenic determinants: one which would be bound by the carrier specific T-cell; and the other by the DNP-mouse anti-SRBC gammaglobulin--the DNP-determinant of which was bound by a DNP-specific B-cell.

With respect to the other conjugate (DNP-RGG-SRBC), it is highly possible that one of the states in which it functioned as an immunogen was analogous to that described for the DNP-MGG-SRBC immunogen. Since gammaglobulins from xenogenic sources have been shown to be immunogenic

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[3] 140 μg./ml. of DNP-MGG was incubated with sheep erythrocytes. After the incubation, the cells were washed and 0.5 ml. of coupled cells were injected into each mouse. If as much as 10% of the DNP-MGG was anti-SRBC antibody, no more than 7 μg of anti-SRBC was injected into each mouse.

in mice in this thesis (DNP-BGG) and elsewhere (Fronstin et al, 1967), the DNP-RGG anti-SRBC moiety of the DNP-RGG-SRBC complex is likely to have been immunogenic in the mouse, and could have provided a helper function to the anti-DNP antibody response by acting itself as a source of added carrier determinants. However, the relative roles of the RGG and the SRBC determinants have not yet been clearly established. Since no significant difference could be shown to exist between the responses elicited by the two conjugates (DNP-MGG-SRBC and DNP-RGG-SRBC), it is probable that the sheep erythrocyte functioned as the carrier moiety for the anti-DNP antibody response to DNP-RGG-SRBC as well.

It is possible to analyse the anti-DNP antibody response to describe further the properties of the DNP-MGG-SRBC immunogen. By comparing the average IgM anti-DNP titre to the average IgG anti-DNP titre elicited by the DNP-MGG-SRBC antigen complex, the ratio was found to be approximately equal to one (0.93). According to Makela's receptor hypothesis, which was reviewed on page 21 of the introduction, this requires that the distance between like determinants be on the average, greater than 120 $\mathring{A}$ (the maximal distance between receptor sites which can stimulate an IgG antibody response) but not greater than 350 $\mathring{A}$ (the maximal distance of receptor site molecules which can stimulate an IgM antibody response). On the basis of the results described in this thesis, it may be assumed that the distance between DNP-groups on the DNP-MGG-SRBC complex, effective in stimulating an IgM anti-DNP antibody response, was between 120 $\mathring{A}$ and 350 $\mathring{A}$.

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