# DIFFERENTIATION AMONG IMMUNOCOMPETENT CELLS

by

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# INTRODUCTION

## Statement of the Problem

This thesis is concerned with the *in vitro* initiation of a primary immune response to a chemically defined haptenic determinant, trinitrophenyl (TNP). It had been previously shown that coliphage can be used successfully as *in vitro* immunogens. Fishman (1961) obtained an *in vitro* antibody response against coliphage T2, Tao and Uhr (1966) obtained both IgM and IgG *in vitro* antibody responses to $\phi$X174, and Saunders and King (1966) obtained an *in vitro* antibody response to coliphage R17.

The first portion of this thesis involves characterizing the *in vitro* primary anti-hapten immune response to trinitrophenyl-bacteriophage T4 (TNP-T4). Evidence is presented indicating that the anti-TNP response to this immunogen is thymus (T-cell) independent.

Playfair and Purves (1971) proposed subclassification of antibody producing cells (B cells or bursa-equivalent cells) and coined the terms $B_1$ and $B_2$. They defined $B_1$ cells as those B cells not requiring T cells in order to mount an immune response, and $B_2$ as those B cells requiring interaction with T cells.

To date all the work dealing with $B_1$ and $B_2$ subpopulations has used sheep erythrocytes (SRBC) as the antigen. Since erythrocytes contain a myriad of antigenic determinants, the results may simply represent cells which recognize different antigenic determinants on the SRBC, some of which happen to be thymus dependent and some thymus independent.

The second portion of this thesis is to use this model system to study the anti-hapten response by *in vitro* challenge with various combinations of thymus independent and/or thymus dependent hapten-carrier complexes. By investigating the possibility of subpopulations of hapten-specific B cells, some of the interpretive difficulties associated with the more complex erythrocyte antigens which do not permit elucidation of helper dependent and helper independent determinants have been overcome.

## Lymphocytes and the Immune Response

The involvement of lymphocytes in immunological phenomena has long been suspected (Murphy, 1926). However, conclusive evidence for their role in immune responses has only existed since the 1950's (Hayes and Dougherty, 1954). Medawar (1958) reviewed the field of the homograft reaction and concluded that homograft rejections were due to immunologically competent lymphocytes.

Recently, evidence has accumulated which suggests that within hematopoietic tissue there is a class of multipotential stem cells which can differentiate into any of the mature blood cell types, both lymphoid and myeloid (Wu, Till, Siminovitch and McCulloch, 1968). Stem cells are first detected in blood islets of the yolk sac and later found in, or migrate to, fetal liver and fetal and adult bone marrow (Metcalf and Moore, 1971). As these multipotential stem cells seed other organs they give rise to stem cells which are restricted in their differentiating capacity and eventually undergo progressive differentiation, terminating as "end" cells. The factors determining

whether multipotential stem cells undergo self-renewal or differentiate to "end" cells are largely unknown. It is believed that these factors include local environmental stimuli, interaction with other cells (Metcalf and Moore, 1971), hormonal stimulation (Pierpaoli, Fabris and Sorkin, 1970) and interaction with "factors" derived from other lymphocytes (Gery, Gershon, and Waksman, 1971).

In view of the variables associated with an *in vivo* immune response it is difficult to dissect the interaction of specific immune cells with any given antigenic determinant *in vivo*. The combination of an *in vitro* model and the use of a chemically defined hapten offers two main advantages for detecting the induction of an immune response: a) an *in vitro* model insures exposure of the entire immunocompetent population to a known concentration of antigen, permitting a comparison of dose effects; b) detecting only cells synthesizing an anti-hapten antibody avoids variability resulting from additive responses to multiple, uncharacterized determinants on complex immunogens. However, even *in vitro* model systems are influenced by factors released by cultured cells. Gery, Gershon, and Waksman (1971) found that the addition of small numbers of human leukocytes to cultures of thymus cells from CBA/H or CBA/J mice greatly enhanced the incorporation of tritiated thymidine. The enhancement could not be attributed to simple addition of the response to cultures of just thymus cells or human leukocytes. Doria, Agarossi, and DiPetro (1972) found that cell-free medium from cultures of thymocytes from C57BL/10 X DBA/2 $F_1$ hybrid mice could enhance the anti-sheep erythrocyte (SRBC) response

of spleen cell cultures derived from neonatally thymectomized mice. Schimpl and Wecker (1972) found that culture supernates from allogeneic mixtures of spleen cells contained a factor which could replace the need for T cells in an *in vitro* anti-SRBC response by either T cell-depleted spleen cell cultures or spleen cell cultures derived from athymic nude mice. They termed this factor T-cell replacing factor or TRF, and showed that TRF was released by T cells present in allogeneic mixtures of spleen cells.

Until the early 1960's very little was known concerning the cellular mechanisms involved in an immune response. Miller (1962) found that neonatal thymectomy of mice abrogated a graft rejection (cellular immunity) and, additionally, lowered the response to Salmonella A antigens (humoral immunity). Subsequently, Cooper, Peterson, South and Good (1966) found that the bursa of Fabricius of the chicken influenced antibody responses and the thymus influenced primarily cell-mediated reactions, although the thymus also had a variable effect on antibody production, depending on the particular antigen used. These experiments helped explain the previous work of Glick, Chang and Jaap (1956) who had investigated the effect of bursectomy in the chicken. They had found that bursectomy induced an agammaglobulinemic state, but did not affect the ability of the same chicken to reject skin grafts. Warner and Szenberg (1964) were the first to propose that a dichotomy existed in the immune response. One branch of the immune system (thymus derived) is involved in cellular immunity and a second bursa-associated branch which is involved with humoral immunity or antibody production.

Roitt, Greaves, Torrigiani, Brostoff and Playfair (1969), in an analysis of a "two-cell" concept of immunity, categorized the distinctive properties of two types of lymphocytes and suggested the terminology, "T-cell" for thymus derived (thymus-processed or thymus dependent) lymphocyte, and "B-cell" for bursa-equivalent derived (non-thymus processed or thymus independent) lymphocyte. It is now generally accepted that these two classes of lymphocytes can be distinguished by different surface antigenic markers (reviewed by Raff, 1971). T dependent cells possess the theta (θ) marker, as first described by Reif and Allen (1963). Raff and coworkers (Raff, 1971) were unable to find an analogous allo-antigenic marker for B lymphocytes in mice. However, Raff, Nase and Mitchison (1971) were able to obtain a rabbit anti-mouse lymphocyte serum which after absorption with mouse liver, erythrocytes and thymocytes was cytotoxic to mouse lymphocytes that were θ negative. It was further established that the antiserum was specific for mouse lymphocytes only. They defined the antigen(s) on the mouse lymphocytes to which the antiserum was directed as "mouse-specific B lymphocyte antigen" or MBLA. A similar marker was reported by Niederhuber, Moller and Makela (1972). However, the specificity of such antisera for B cells is still considered an open question.

An alternative means of distinguishing between murine T and B cells is through the presence of readily detectable surface immunoglobulin. In determining the presence of surface immunoglobulin on murine lymphocytes, either by immunofluorescence techniques (Rabellino,

Colon, Grey, and Unanue, 1971) or by radioiodination techniques (Vitetta, Bianco, Nussenzweig, and Uhr, 1972) it has been found that murine B lymphocytes have readily detectable amounts of surface immunoglobulin. At the present time the existence of surface immunoglobulins on murine T lymphocytes is an open question (Crone, Kock, and Simonsen, 1972; Playfair, 1974). Marchalonis, Cone, and Atwell (1972) and Cone, Sprent, and Marchalonis (1972) demonstrated IgM on thymus and thymus-derived cells, whereas Vitetta, Bianco, Nussenzweig, and Uhr (1972) were unable to demonstrate immunoglobulins on the surface of T cells.

Cell-Cell Interactions

The field of immune cell interaction started with the work of Claman, Chaperon and Tripplett (1966). Their experiments involved reconstituting irradiated $LAF_1$ or DBA/J mice with either syngeneic thymus cells, marrow cells, or mixtures of both cell types. Those animals receiving the cell mixtures, when challenged with sheep erythrocytes (SRBC), gave an antibody response much greater than that predicted by summing the individual immune response made by animals reconstituted with thymus or marrow cells alone. In these experiments they were unable to determine which cell type contained the antibody producing cells and which population contained the "auxiliary cells". Their work was substantiated by Celada (1967), who transferred primed spleen cells to irradiated and therefore immunoincompetent mice (adoptive transfer). In this system primed spleen cells from A.SW/KL or A X CBA $F_1$ mice were transferred to irradiated

syngeneic mice. From his experiments Celada plotted the antibody response to human serum albumin (HSA) against the number of cells transferred on a log-log scale; the resulting slope of the regression line was greater than 1.0. This indicated that the production of antibody required the interaction of more than one cell.

The determination of which cell population produced antibody was first determined by the experiments of Davies, Leuchars, Wallis, Marchant and Elliott (1967). Using an adoptive transfer of cells having chromosomal markers they found that antigenic stimulation caused cell division of thymus cells but no antibody production. However, antigenic stimulation of bone marrow cells resulted in a small amount of antibody production. When both cell types were present an increased antibody response was observed. Additional evidence that bone marrow cells are the source of antibody production came from a series of experiments by Mitchell and Miller (1968) and Nossal, Cunningham, Mitchell, and Miller (1968). Using both anti-H2 sera (anti-histocompatibility) and chromosomal marker analysis, they also found that bone marrow cells were the source of antibody producing cells. Thymus cells, or thoracic duct cells, were termed antigen reactive cells (ARC). These latter cells were believed to interact with antigen and initiate the differentiation of antibody-forming cell precursors to antibody-secreting cells, which were bone marrow derived.

The ability to dissect the immune system more fully was achieved by the methodology developed by Mishell and Dutton (1967) and by Marbrook (1967). These techniques allowed for *in vitro* initiation of an antibody response against erythrocyte antigens. Using the

method of Mishell and Dutton, Mosier and Coppleson (1968) separated DBA/2 mouse spleen cells by the ability of part of the population to adhere to plastic petri dishes. Serial dilutions of the adherent cell populations were cultured in the presence of an excess of the other non-adherent cell population and vice versa. The slope of the regression line resulting from plotting the antibody response against SRBC versus the number of each cell type used, on a log-log scale, was used to predict the number of cell interactions required to produce the antibody response. The results indicated that one adherent and two nonadherent cells interacted during the primary immune response *in vitro*. They therefore suggested that the response to a single antigen involves at least two and possibly three antigen specific cells.

## Hapten-Carrier Relationship

The experiments which demonstrated the existence of cell interaction in an immune response utilized erythrocytes as antigens. Since a myriad of antigenic determinants are involved in such experiments, they were unable to delineate the mechanism of interaction between cells. A method of overcoming this problem is the use of hapten-protein conjugates. Haptens are usually small, chemically defined substances such as trinitrophenyl (TNP), which can combine specifically with antibody, but cannot by themselves induce an immune response; that is, they are antigenic but not immunogenic.

The question arose as to the relationship between the haptenic portions and carrier portions of an immunogen. Does the carrier just

play the role of a "schlepper", acting passively and contributing only size to the immunogen, or is the carrier actively involved in cell interactions? Rajewsky and Rottlander (1967) and Rajewsky, Rottlander, Peltre, and Muller (1967) investigated this question using lactic dehydrogenase (LDH) isoenzymes. This system consists of five tetrameric enzyme-proteins which are composed of two types of subunits, A and B, in all possible combinations. In their experiments they used LDH-I, which is composed of only subunits of B ($B_4$), LDH-V, composed of only subunits of A ($A_4$) and LDH-III which is composed of equal amounts of each subunit ($B_2A_2$). The A and B subunits do not cross-react serologically. Therefore this system allows two sets of non-crossreacting antigenic determinants to be arranged either on the same molecule (LDH-III) or on separate molecules (LDH-I and LDH-V).

It was found that most rabbits produced very little anti-B upon immunization with LDH-I, whereas most rabbits produced anti-A upon immunization with LDH-V. However, if rabbits were immunized with LDH-III they produced comparable amounts of both anti-A and anti-B. If rabbits were primed with LDH-III and boosted with LDH-I there was a slight increase in the production of anti-B, but such primed rabbits produced large amounts of anti-A when immunized with LDH-V. They also found that newborn rabbits injected with LDH-V became hyporesponsive (tolerant) to LDH-III in that both anti-A and anti-B titers were equally depressed. However, such hyporesponsiveness could not be induced by injection of LDH-I. Therefore in this system the A subunit can act as a carrier for the B subunit and the data were interpreted to suggest that a cell can be stimulated to respond to a hapten determinant

only if an unrelated carrier determinant, distinct from the haptenic site, is likewise recognized.

Supportive evidence for this idea came from the experiments of Rajewsky, Schirrmacher, Nase, and Jerne (1969) who used the hapten protein conjugates, sulfanilic acid-bovine serum albumin (Sulf-BSA) and sulfanilic acid-human gamma globulin (Sulf-HGG). They found that rabbits primed with Sulf-BSA gave a good secondary anti-hapten response to Sulf-HGG only if the animals were additionally pre-immunized with free HGG. They concluded that the immune stimulus involved the recognition of carrier determinants unrelated to the hapten.

Mitchison, Rajewsky and Taylor (1970) investigated the relationship between hapten and carrier using an adoptive transfer system. Transfer of spleen cells primed with 4-hydroxy-3-iodo-5-nitrophenylacetic acid-chicken gamma globulin (NIP-CGG) and challenged with the hapten on a heterologous carrier, NIP-BSA (NIP-bovine serum albumin) failed to produce a significant anti-NIP response. However, if spleen cells primed to the heterologous carrier BSA were also transferred to the irradiated mouse, the challenge of NIP-BSA resulted in an anti-NIP response. The carrier reactive cells, which did not themselves make the antibody, were referred to as "helper cells". It was later shown by Raff (1970) that helper cells are T lymphocytes in that they were inhibited by anti-$\theta$ serum and complement treatment while hapten primed cells were not affected.

Thymus Independent Antibody Responses

There are some antigens which are able to stimulate some B cell clones to secrete antibody without the help of T cells. These antigens are referred to as thymus independent antigens. Depletion of T cells, either by *in vivo* or *in vitro* techniques, has no significant effect on the subsequent antibody response to such antigens. There are two general properties associated with these T independent antigens. First, the structure usually consists of identical units arranged in a linear repetitive sequence. Also, the normal antibody response to these antigens is class restricted in that the antibody is predominantly of the IgM class. It was found by Feldmann and Basten (1971) that the antibody response to the hapten DNP was either T independent or T dependent contingent on whether the carrier used was T independent or T dependent.

Baker, Reed, Stashak, Amsbaugh, and Prescott (1973) found that the antibody response to the T independent antigen type III pneumococcal polysaccharide (SSS-III) was enhanced by pretreating normal mice with antilymphocyte serum (ALS) which caused a depletion of T cells (Leuchars, Wallis, and Davies, 1968). ALS treatment of athymic nude mice did not enhance the response to SSS-III. Their results suggest that thymus cells can play a "suppressor" role in modulating the antibody response to T independent antigens. However, similar results have not been observed with other T independent antigens (see review by Basten and Howard, 1973).

## Mechanisms of T-B Cell Cooperation

It is now generally accepted that collaboration between T cells and B cells is necessary for an antibody response to most antigens and several reviews have been written on this subject (Miller, Basten, Sprent, and Cheers, 1971; Playfair, 1971; Katz and Benacerraf, 1972; Greaves, Owen, and Raff, 1973). However, the mechanisms involved are not fully understood. From the work of Mitchison, Rajewsky, and Taylor (1970) it appears that the hapten and carrier must be physically linked. This suggests that the antigen forms a bridge between B and T cells. Using actinomycin D and antimycin A as inhibitors of RNA synthesis or protein synthesis respectively, Feldmann and Basten (1972) suggested that T cells must be able to synthesize RNA and protein in order to cooperate with B cells *in vitro*. Therefore it is unlikely that T cells play an entirely passive role in cell collaboration.

Further insight into the mechanism of immune cell cooperation came from experiments by Feldmann (1972a), who used a culture chamber with two compartments separated by a cell impermeable nucleopore membrane. He found that specific cell interactions occurred efficiently across such a membrane and that contact of T and B lymphocytes was not essential for cooperation during antibody production. Using different lymphoid cell populations, he found that macrophages were required for interaction with B cells, but not with T cells. He also provided evidence that an antigen specific T cell factor which crosses the membrane is monomeric IgM complexed with antigen. This complex was shown to be cytophilic for macrophages.

Therefore, at present the most likely mechanism for cell collaboration is as follows: antigen reacts with T cells by carrier specific receptors which are then shed as antigen-receptor complexes and attach to macrophages. This complex on the surface of the macrophage then either directly interacts with and triggers the specific B cells, or the complex is altered and/or rearranged on the surface of the macrophage and then stimulates the specific B cell.

It is apparent that not all antibody responses require cell interaction. For example, T independent antigens (see above) are apparently able to trigger B cells to antibody production in the absence of T cells. Additionally, evidence has been found that non-antigen-specific T cell factor(s) can bypass the need for cell interaction. Katz, Paul, Goidl, and Benacerraf (1971) coined the term "allogeneic effect". They found that transfer of allogeneic guinea pig lymphoid cells into dinitrophenyl-ovalbumin (DNP-OVA) primed guinea pigs stimulated the synthesis of both anti-DNP and anti-OVA antibodies. This allogeneic effect occurred spontaneously in the absence of any further antigenic challenge. They also found that this effect only occurred in a graft-versus-host (GVH) and not in a host-versus-graft (HVG) response. However, McCullagh (1970) was able to terminate tolerance to SRBC in rats by either a GVH or an HVG reaction.

It is therefore suggested (see review by Katz, 1972) that in the allogeneic effect, those T cells involved in the GVH release some factor(s), which are non-antigen-specific, which can trigger B cells that have had prior antigenic stimulation to antibody

production. However, it is not clear how the allogeneic effect relates to antibody production in a normal animal or individual, but this effect does indicate the complexities involved in triggering antigen specific B cells to antibody production.

Subpopulations of B Cells

Within the last few years the question has arisen as to the possible existence of more than one class or subpopulation of B cells. Playfair and Purves (1971) were the first to propose subclassification of B cells and coined the terms $B_1$ and $B_2$. They defined $B_1$ cells as those B cells not requiring T cells in order to mount an immune response. $B_2$ cells are the subclass which requires interaction with T cells. In their experiments they used an adoptive transfer system in which irradiated mice were reconstituted with various doses of either spleen cells or bone marrow cells with or without additional thymus cells. The mice were challenged with SRBC and the subsequent plaque-forming cell response determined. When the responses in the groups of mice with added thymus cells was higher than that in the groups without added thymus cells, this was taken to indicate synergism between thymus and spleen or marrow cells. They found that the amount of synergism between spleen cells and thymus cells was much greater than that found between bone marrow cells and thymus cells. The ability of thymus cells to increase the plaque response of spleen or marrow cells was not proportional to the reactivity of those cells in the absence of thymus cells. From their results they not only postulated

the existence of two subclasses of B cells, but also suggested that antigen reactive cells in the bone marrow were predominantly of $B_1$ type. They also felt that roughly equal numbers of $B_1$ and $B_2$ cell types were present in the spleen.

Additional evidence for the existence of a population of B cells that can react with antigen independent of T cells comes from the work of Gershon and Kondo (1970). Their experiments involved depleting mice of T cells ("B-mice"), reconstituting some with T cells ("T-B-mice"), and then injecting tolerigenic doses of SRBC in both groups of mice. After resting for 30 days all mice received a dose of T cells and were then challenged with another tolerigenic dose of SRBC. After an additional resting period, the mice were then challenged with an immunogenic dose of SRBC. All mice were then tested for serum levels of anti-SRBC antibody for up to 98 days after treatment. It was found that the IgG response in "B-mice" was unaffected by pretreating with tolerigenic doses of SRBC prior to reconstitution with T cells. The late appearing IgM response was likewise unaffected. However, the early appearing IgM response was significantly depressed. In the group of mice fully reconstituted prior to tolerization, "T-B-mice", the entire antibody response was significantly depressed. They concluded that a clone or class of B cells exists which is responsible for an early IgM response, which was made unresponsive by antigen pretreatment in the absence of T cells. They also suggested that the second class of B cells could only be tolerized in the presence of T cells. It has also been suggested that the second cell type, $B_2$, initially

secretes IgM and is the precursor of the cell which undergoes the switch to IgG (Gershon, 1973).

Haskill, Marbrook, and Elliott (1971) and Haskill and Marbrook (1971) obtained spleen cells by fractionation with equilibrium density gradient centrifugation. They found that the subsequent *in vitro* anti-SRBC response in one of the fractions was not only resistant to treatment with anti-θ serum and complement, but was actually enhanced by such treatment. They therefore suggested that for this one population of spleen cells, interaction with a thymus derived cell was no longer necessary. They gave no explanation for the increased response in the fractionated spleen cells treated with anti-θ serum and complement.

Other investigators, including Chan, Mishell, and Mitchell (1970) and Schimpl and Wecker (1970), have also found that after T cell depletion, either by neonatal thymectomy or anti-θ serum and complement treatment of spleen cells, there is a small residual antibody response to SRBC.

## Subpopulations of T Cells

The most compelling evidence for functionally distinct subclasses of T lymphocytes has been obtained by Cantor and Asofsky (1972) in studies of graft versus host (GVH) reactions in mice. They found that two different parental lymphoid cells can act synergistically in the $F_1$ recipient. Cantor (1972) found that the parental cells involved in the GVH reaction were depleted after neonatal thymectomy and were sensitive to treatment with anti-θ serum plus complement.

Stobo, Rosenthal, and Paul (1972) and Stobo and Paul (1973) have recently distinguished at least two subpopulations of θ positive cells on the basis of their mitogenic response to phytohemagglutinin (PHA) and concanavalin A. One population responds equally well to both mitogens, recirculates and is in higher concentration in lymph nodes, while the other responds mainly to concanavalin A, is relatively sessile and found mainly in spleen and bone marrow.

At the present time it is not known if there is a correlation between subpopulations of T cells and the ability of one population to act as helper cells in T cell-B cell collaboration and the other population of T cells being involved in cell-mediated immune reactions (Liew and Parish, 1974).

# MATERIALS AND METHODS

## Animals

Adult Balb/c female mice were purchased from Simonsen Laboratories, Inc., Gilroy, California. They were caged in groups of 6 with free access to food and water, and were used when 2 to 6 months of age. Hypothymic nude mice (nu/nu) and heterozygote littermates (nu/+), 5 to 6 weeks of age were purchased from Olac Group, Olac House, Blackthorn, Bicester, Oxon, England. They were used within 2 weeks of arrival.

## Sheep Erythrocytes

Sheep red blood cells were obtained in Alsever's solution at weekly intervals from Prepared Media Laboratory and Sheep Blood Supply, Tualatin, Oregon. Red blood cells were aged at least 2 weeks after bleeding before use (Rittenberg and Pratt, 1969).

## Spleen Cell Preparation and Assay Medium (Mishell and Dutton, 1967)

Eagle's powdered minimal essential medium (MEM-P) for suspension cultures, without L-glutamine, catalogue #F-14, from Grand Island Biological Co., Grand Island, New York, was dissolved in double distilled water. Solutions, 2X or 1X, were prepared with 2.14 or 1.07 gm/100 ml respectively. The pH was adjusted to 7.2 with 1.0 N sodium hydroxide.

Culture Medium (Mishell and Dutton, 1967)

Ingredients for 50 ml of culture medium (the first five ingredients were obtained from Microbiological Associates, Albany, California):

1. Minimal Essential Medium (Eagle) for suspension cultures, without L-glutamine, catalogue #12-126, stored at 4° C, 43.0 ml.
2. L-glutamine 200 mM solution, catalogue #17-605, stored at -20° C, 0.5 ml.
3. Sodium pyruvate 100 mM solution, catalogue #17-605, stored at -20° C, 0.5 ml.
4. Non-essential amino acids mixture-supplement for minimal essential medium (Eagle) 100X concentrate, catalogue #13-114, stored at 4° C (0.5 ml).
5. Penicillin-streptomycin mixture (5,000 units/ml), catalogue #17-603, stored at -20° C, 0.5 ml.
6. 2-mercaptoethanol, Eastman Organic Chemical Co., Rochester, New York, made to 0.1 M and stored at 4° C. Just prior to use, dilute 1:20 with MEM-P, 0.5 ml (Click, Benck and Alter, 1972).
7. It was observed that different batches of fetal calf serum varied in their ability to sustain an *in vitro* anti-hapten response. We therefore pre-tested different batches of fetal calf serum from

various suppliers. Whichever batch gave the best *in vitro* anti-hapten response was then obtained in a quantity sufficient to last approximately one year. Fetal calf serum was obtained in 500 ml volumes and aseptically aliquanted in 100 ml volumes and stored at -20° C. Fetal calf serum was obtained from either Colorado Serum Co., Denver, Colorado, catalogue #CS-1190, Reheis Chemical Co., Chicago, Illinois, catalogue #268, or Gray Industries, Inc., Fort Lauderdale, Florida, catalogue #2001, 5.0 ml.

Nutritional Cocktail

Ingredients for 227 ml of nutritional cocktail:

1. Autoclaved MEM-P, 1X solution, 140 ml.
2. Essential amino acids--amino acid mixture for minimal essential medium (Eagle), 50X concentrate, catalogue #13-606, Microbiological Associates, Albany, California, 20 ml.
3. Non-essential amino acids, as used in culture medium, 10 ml.
4. Dextrose, reagent grade, catalogue #72623, Merck and Co., Rahway, New Jersey. Two hundred mg/ml solution was prepared in double-distilled water, sterilized by filtration through an 0.45 μ Millipore membrane and stored at -20° C, 10 ml.

5. L-glutamine, as used in culture medium, 10 ml.

6. Penicillin-streptomycin mixture, as used in culture medium, 3.6 ml.

The above cocktail was adjusted to pH 7.2 with sterile 1.0 N sodium hydroxide.

7. Sodium bicarbonate solution, 7.5%, catalogue #17-603, Microbiological Associates, Albany, California, was added to the pH-adjusted cocktail, 30 ml.

The completed cocktail was aliquanted and frozen to -20° C to be thawed on the day used.

Hapten Reagent (Rittenberg and Amkraut, 1966)

Picryl-sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid (TNBS) from Nutritional Biochemicals Corp., Cleveland, Ohio, was recrystallized once from 1.0 N hydrochloric acid after dissolving 10 gm TNBS in 20 ml of hot hydrochloric acid.

Hemolytic Plaque Assay Reagents

Difco-Bacto Agar, catalogue #0140-01 was purchased from Difco Laboratories, Detroit, Michigan. DEAE-Dextran, catalogue #D-2000 was purchased from Sigma Chemical Co., St. Louis, Missouri, dissolved in 0.15 M sodium chloride to 30 mg/ml and adjusted to pH 7.2 with 1N sodium hydroxide. Sodium chloride, catalogue #74073, biological grade, was purchased from Merck and Co., Inc., Rahway, New Jersey.

Buffers

1. Modified barbital buffer (MBB), 0.012 M, pH 7.3 to 7.4, was stored as a 5X concentrate at 4° C (Campbell, Garvey, Cremer, and Sussdorf, 1963).
2. Cacodylate buffer, 0.28 M, pH 6.9, was stored at room temperature (Rittenberg and Amkraut, 1966). Cacodylate saline, the same buffer diluted 1:10 in 0.15 M sodium chloride.
3. Phosphate buffer, 0.1 M, pH 7.1 (Gomori, 1955). Phosphate saline, the same buffer diluted 1:10 in 0.15 M sodium chloride.
4. Borate buffer (Campbell et al., 1963), 0.08 M, pH 8.8. Borate saline, the same buffer diluted 1:20 in 0.15 M sodium chloride.
5. Phosphate buffer, 0.066 M, pH 7.45 (Barber and Rittenberg, 1969).
6. Phosphate buffer, 0.071 M, pH 7.1, containing 0.068 M sodium chloride (Barber and Rittenberg, 1969).

Hemocyanin

Hemocyanin (KLH) was obtained from the keyhole limpet, *Megathura crenulata*, purchased from Pacific Bio-Marine Supply Co., Venice, California. The animals were bled and the hemocyanin prepared by the method of Campbell et al., 1963. After dialysis KLH was concentrated by ultracentrifugation and stored at 4° C under 0.15 M sodium

chloride in a tightly stoppered tube or centrifuged 1 hr at 8,000 rpm and sterilized by filtration through an 0.45 μ Millipore membrane and stored at 4° C.

Bovine Albumin

Crystallized bovine plasma albumin (BSA) was purchased from Pentex, Inc., Kankakee, Illinois.

Protein Determination

Protein concentrations were determined either by Nesslerization according to the method of Campbell et al. (1963) or by the Lowry method as modified by Lichstein and Oginsky (1965).

Trinitrophenyl Hemocyanin (TNP-KLH)

General procedure: 450 mg of KLH were brought to 10 ml with cacodylate buffer, pH 6.9, and placed in a foil-covered 50 ml flask at 37° C. TNBS, 346 mg in 10 ml of cacodylate buffer was added dropwise to the slowly stirred 37° C KLH solution. Stirring was continued for 60 min, at which time the solution was chilled and then concentrated by centrifugation for 90 min at 36,000 rpm in a refrigerated Beckman model L2-65 centrifuge. The supernatant was discarded and the amber gelatinous pellets were dissolved in 10 ml of cacodylate buffer. The small amount of insoluble residue of denatured KLH was discarded by centrifuging at 1500 rpm for 10 min. The clear amber supernatant fluid was freed of unconjugated TNBS by passage through

a G-50 Sephadex column (2.5 cm x 42 cm) equilibrated with cacodylate saline. Throughout the procedure foil was used to protect the preparation from photodecomposition (Okuyama and Satake, 1960). O.D. readings at 350 and 280 nm were taken and the TNP-KLH recycled through the G-50 column. If the 350/280 O.D. ratio remained unchanged it was assumed that the conjugate was free of TNBS. TNP-KLH preparations used *in vitro* were dialyzed with three changes of saline (12 hr each) to remove the arsenic in cacodylate buffer and residual unbound hapten. The degree of hapten conjugation was estimated by spectrophotometric measurement according to the method of Rittenberg and Amkraut (1966). This procedure usually produces 850 to 1000 moles of TNP groups per mole of KLH, assuming a molecular weight of $8 \times 10^6$ for KLH.

Bentonite (Gallily and Garvey, 1968)

A suspension was prepared of 0.5 g bentonite (Fisher Scientific) in 500 ml distilled water. The sediment obtained by centrifugation at 150 X G for 10 min was discarded and the supernatant was recentrifuged at 1400 X G for 10 min. The sediment from the second centrifugation was resuspended in 100 ml of Hank's solution. This represented the stock solution which was autoclaved and refrigerated at 4° C until used.

KLH-Bentonite (K-B) and TNP-KLH-Bentonite (T-K-B) (Rittenberg and Pratt, 1969)

The KLH and TNP-KLH were adsorbed onto bentonite (Fisher Scientific Co., St. Louis, Missouri) using the method of Gallily and Garvey (1968). The bentonite pellet obtained after centrifugation of 40 ml of stock bentonite solution was resuspended in 2 ml of 0.28 M cacodylate buffer, pH 6.9, containing 5 mg of KLH or TNP-KLH/ml. The suspension was allowed to stand at room temperature for 1 hr with occasional shaking. After centrifugation, particles were washed 3 times in cacodylate saline. The amount of protein coated on the particles was determined by nesslerization (Campbell et al., 1963).

TNP-Sheep Red Blood Cells (TNP-SRBC) (Rittenberg and Pratt, 1969)

Sixty mg of TNBS were dissolved in 21 ml of cacodylate buffer in a foil-covered 50 ml flask. Three ml of wet-packed SRBC, washed 3 times with 0.15 M sodium chloride, were added dropwise with stirring. The mixture was stirred slowly at room temperature for 10 min. Reacted cells were brought to 40 ml with cold MBB and centrifuged for 10 min at 1230 X g. The resulting TNP-SRBC pellet was resuspended in 35 ml of cold MBB containing 22 mg of glycyl-glycine (Nutritional Biochemicals Corp., Cleveland, Ohio). Glycyl-glycine reacts with residual TNBS. This suspension of TNP-SRBC was recentrifuged. The supernatant was bright yellow due to the formation of TNP-glycylglycine. The TNP-SRBC pellet was washed twice more with cold MBB and stored at 4° C until

used. Haptenation and subsequent handling were carried out with foil-wrapped containers to prevent photodecomposition (Okuyama and Satake, 1960).

Phage Preparations

Coliphage T4 d+ were obtained from R. H. Epstein and R. S. Edgar. Preparations of high titered phage were prepared by the method of Yanagida and Ahmad-Zadeh (1970). 200 ml of an *E. coli* B culture were infected with T4 when bacterial concentrations attained 2 X $10^8$ cells/ml at a multiplicity of 4 phage/cell. Five minutes later the cells were super-infected with T4 at the same multiplicity. Forty-five minutes after the initial infection the culture was centrifuged at 5,000 X g for 10 min. Pellets of the infected cells were lysed in 4 ml phosphate buffer containing 4 drops of chloroform and DNase (10 μg/ml). This concentrated lysate was centrifuged at 5,000 X g for 10 minutes in order to remove input phage particles associated with cell debris. The titer of such preparations was 2 to 4 X $10^{12}$ plaque-forming units (PFU) per ml. The phage were partially purified by centrifugation through a discontinuous cesium chloride gradient according to the method of Barber and Rittenberg (1969). The visible phage bands were collected in drops from the bottom of the tubes and dialyzed against phosphate buffer, 0.071 M, pH 7.1.

Haptenation of T4

Haptenated phage were prepared according to the method of Barber and Rittenberg (1969) by adding 5.0 ml of phage at $10^{10}$ PFU/ml to 45 ml of 0.067 M phosphate buffer, pH 7.4, containing 9.0 mg TNBS. The mixture was stirred slowly in the dark for 5 hr at 30° C. The reaction was stopped by adding 68 mg glycylglycine in 1.0 ml phosphate buffer and stirring an additional 15 minutes. Haptenated phage were dialyzed for 4 days against several changes of 6 L each of phosphate buffer containing 0.01% gelatin (Baker Chemical Co., catalogue #2124). Total viable phage recovery varied from less than 5% to 80% of the starting material. Attempts were made to determine the amount of hapten conjugated to the phage by the spectrophotometric method of Rittenberg and Amkraut (1966); however, there was no detectable absorbance at 350 nm. Therefore the number of TNP groups per phage particle in the different preparations of TNP-T4 was not calculated. Even though the phage are haptenated under standard conditions there may be a difference in the degree of haptenation of phage from one preparation to the next.

*In Vitro* Titration of Immunogens

In view of the variations in recovery of viable phage in different preparations of TNP-T4, each preparation was titered to determine the optimal *in vitro* immunizing dose as follows: half-$\log_{10}$ dilutions, through a 100-fold range, were prepared in MEM-P and 0.1 ml of each dilution was mixed with 0.9 ml of dissociated spleen cells. Assuming

the number of phage particles remained constant through the haptenation procedure, the maximum number of haptenated phage particles per culture was $10^8$ PFU.

Additional Haptenated Antigens

Dinitrophenylated polymeric flagellin (DNP-POL) prepared from *Salmonella adelaide* was a gift of Dr. M. Feldmann, University College, London, England. The degree of hapten conjugation was 2.7 DNP groups per molecule of monomeric flagellin (Feldmann, 1971).

Dinitrophenylated chicken gamma globulin (DNP-CGG) was a gift of Dr. N. A. Mitchison, University College, London, England. The degree of hapten conjugation was 6.6 DNP groups per molecule of CGG.

Treatment With Anti-θ Serum

Rabbit anti-mouse brain-associated anti-θ (Br-anti-θ), prepared according to the method of Golub (1971), was a gift of Dr. R. Gorczynski of University College, London, England. Cytotoxicity activity against mouse thymus and spleen cells was assayed by M. Baltz according to the method of Golub (1971).

For experimental purposes, equal volumes of spleen cells, at $10^7$ cells/ml, and Br-anti-θ serum, diluted 1:10 in MEM-P, were incubated at 37° C for 30 minutes. The spleen cells were washed twice, resuspended in agarose absorbed guinea pig serum and incubated at 37° C for an additional 30 minutes. Residual cells were washed twice, resuspended

in tissue culture medium and placed in culture. Control cells were mixed with normal rabbit serum; further treatment was identical to Br-anti-θ serum-treated cells.

Phage Assay

Phage titrations were made by the soft agar overlay method of Adams (1959). Hershey agar was used for plating and Hershey broth with 0.1% gelatin added was used as dilutent for both phage and tissue culture supernates (Steinberg and Edgar, 1962).

Phage Neutralization Assay

Neutralization of native or haptenated phage was carried out according to Adams (1959). Phage were added to serum or tissue culture supernates and incubated at 37° C for an appropriate length of time (see Results). The phage-antibody reaction was stopped by 100-fold dilution in broth immediately prior to plating. The concentration of phage in the reaction mixture was 1 to 2 X $10^6$ PFU/ml. Reported results are averages from triplicate plating.

Immunizations (Rittenberg and Pratt, 1969)

Mice were given intraperitoneal injections of KLH-bentonite every week for a total of 3 injections. For some experiments, mice were given TNP-KLH-bentonite every 2 weeks for a total of 3 injections. In all cases each injection contained 100 μg protein in 0.5 ml saline.

## Cell Cultures

Mice were killed by cervical dislocation. Spleens were removed aseptically and placed into a 60 mm petri dish with 5 to 8 ml of 1X MEM-P. The spleens were gently disrupted with sterile forceps and the cells dispersed with a capillary pipette. The cells were transferred to a tube and placed into an icebath for 3 to 5 minutes to allow particles to settle. Cells remaining in suspension were transferred to a conical tube and centrifuged at 630 X g for 15 minutes. After centrifugation the supernatant fluid was discarded and the pellet resuspended in culture medium to a concentration of 1.0 to 1.2 X $10^7$ nucleated spleen cells/ml. Cells were counted on a Coulter counter, model F. They were cultured in 1.0 ml volumes in 35 mm culture dishes with the appropriate antigens as shown in the results. Cultures were incubated in plastic boxes at 37° C in an atmosphere of 7% oxygen, 83% nitrogen and 10% carbon dioxide. The cultures were rocked 7 to 8 cycles per minute. Cells were cultured for a maximum of 6 days.

## Feeding

Every day each culture was fed 3 drops from a capillary pipette of a 2:1 mixture of nutritional cocktail and fetal calf serum.

## TNP-Plaque Assay

A localized hemolysis in gel plaque assay (Jerne and Nordin, 1963) was modified to detect cells synthesizing anti-TNP antibody

(Rittenberg and Pratt, 1969). Guinea pig serum was diluted 1:25 with MEM-P as a source of complement. On the day of assay, cultured cells were scraped from the culture dishes, placed in plastic tubes and packed by centrifugation at 630 X g for 15 minutes; culture supernatant fluids were stored at -20° C for separate analysis. Cells were washed by resuspending in 2.0 ml of MEM-P per culture and recentrifuged. The second supernatant fluid was discarded and the cells were resuspended in 1 to 2 ml of cold MEM-P, counted and plated in quadruplicate with TNP-SRBC or non-haptenated SRBC. The assay plates consisted of base layers of agar, 1.4% in 0.85% sodium chloride. A 0.47% suspension of red blood cells and an aliquot of spleen cells were added as thin 1.0 ml 0.62% agar layers on top of the base layers. This upper 1.0 ml layer contained 0.022 ml of DEAE-dextran, 30 mg/ml in saline, to prevent the anti-complementary action of agar. The plates were incubated for 3 hr at 37° C with complement to detect plaque forming cells (PFC) due to cells producing IgM antibody. Indirect plaques were determined by incubating the plates 1.5 hr with complement alone and then replacing the complement with anti-mouse IgG diluted with complement and re-incubating for an additional 1.5 hr. Guinea pig serum and anti-globulin serum were absorbed with SRBC prior to use (Kabat and Mayer, 1963). The anti-globulin serum was heated at 56° C for 30 minutes prior to use in order to inactivate complement (Bullock and Rittenberg, 1970). After incubation the plates were stored overnight at 4° C, stained the following day with benzidine stain and the PFC's counted on a New Brunswick Scientific colony counter with an electronic probe.

## Benzidine Stain for Assay Plates

Benzidine base 0.2 gm, reagent grade (Hartman Leddon Co., Philadelphia, Pennxylvania) was mixed with 10 ml glacial acetic acid and dissolved with low heat. Double distilled water, 90 ml, was added to the benzidine acid solution and mixed. To this mixture was added 1.5 ml of 30% hydrogen peroxide. The final mixture was mixed well and used within a few hours after preparation (Jerne, Nordin, and Henry, 1963).

## Supernatant Titration

The lytic spot test of Bullock and Rittenberg (1970) was used to titer small amounts of anti-TNP antibody in culture supernatant fluid. Soft agar layers containing 0.47% TNP-SRBC or SRBC were used. Drops of diluted supernatant fluid were placed on these layers and allowed to soak in by incubating for 1 hr at 37° C, after which the plates were developed as in the anti-TNP plaque assay. Lytic spot testing was used to determine the presence of 19S and 7S antibody in the supernates. Supernatant fluid samples were treated with 0.1 M 2-mercaptoethanol for 30 minutes at 37° C to inactivate IgM antibody and dialyzed against 0.01 M iodoacetamide and then against 0.15 M sodium chloride (Pearlman, 1967). Supernatant fluids so treated were then compared with comparable untreated samples.

# RESULTS

## Purification of Bacteriophage T4

When bacteriophage T4 was partially purified by cesium chloride density centrifugation two visible bands were consistently observed. The density gradients were calibrated using gradient range markers (Cal-Spheres). One band of phage was seen at a density of 1.40 and a second band was seen at a density of 1.30. These bands will be referred to as bottom and top band respectively. Similar double bands have been observed in bacteriophage T2 by Cummings and Kozloff (1960), who found that variations in both pH and temperature altered the head protein of intact particles. This accounted for dual sedimentation of either T2 ghosts or intact T2. Kellenberger, Bolle, Boy de la Tour, Epstein, Franklin, Jerne, Reale-Scafati, Sechaud, Bendet, Goldstein and Lauffer (1965) correlated dual sedimentation of T4 with the extension or retraction of tail fibers. Kozloff[1] feels that the observed dual sedimentation is a result of variations in the porosity of the head protein and that others have found similar results when purifying T4 in cesium chloride. He feels that there is no significance in this phenomenon. A limited electronmicroscopic examination of samples of both top band and bottom band T4 was performed by Dr. R. Brooks, Department of Pathology. As seen in Figure 1, no significant differences were observed in either extension or retraction of tail fibers or size of head particles.

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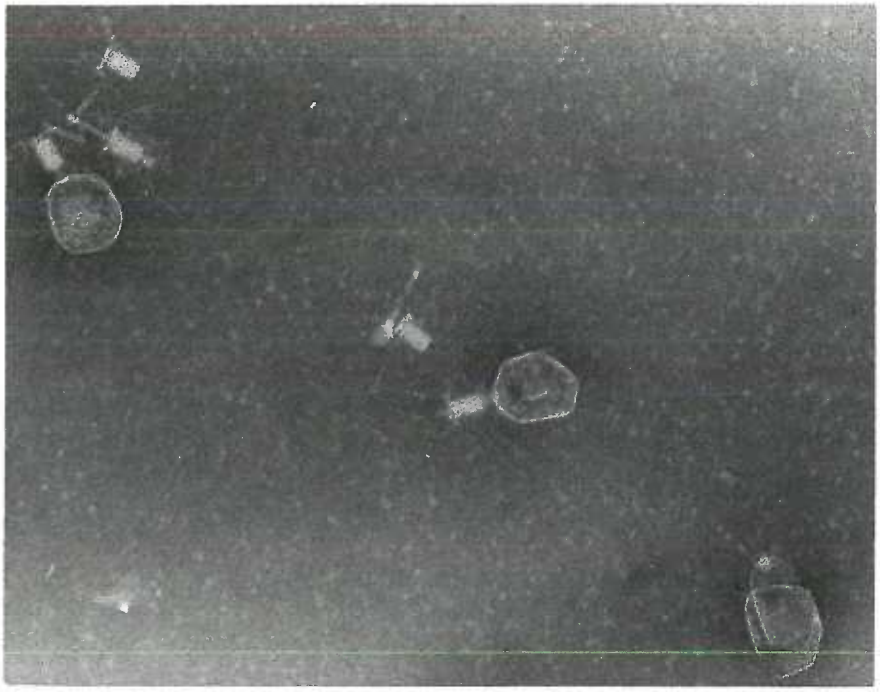
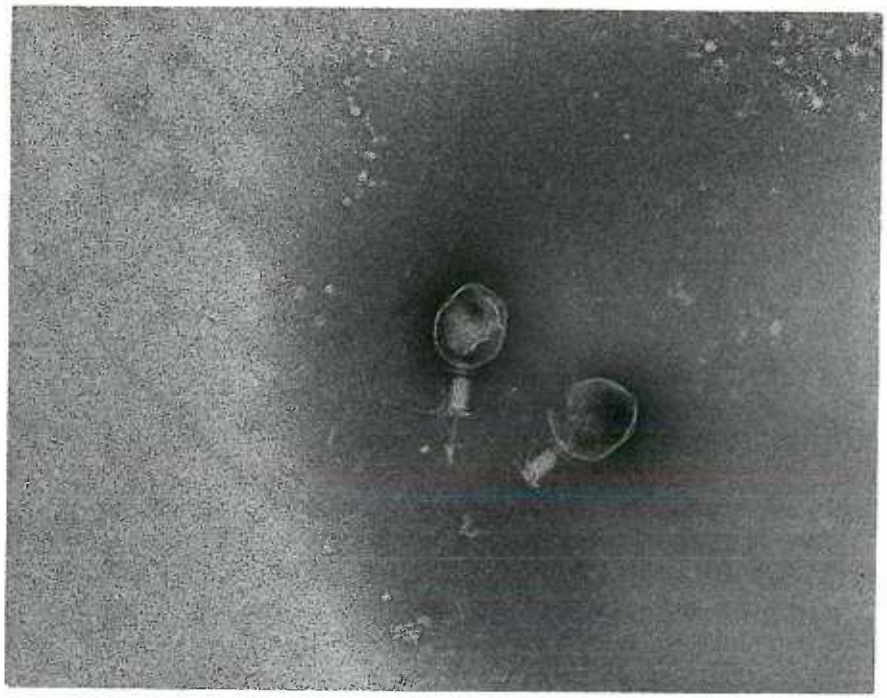
1. Lloyd Kozloff, Personal Communication.

Figure 1

Electronmicrographs of top band (a) and bottom band (b) cesium chloride purified T4. Final magnification = 119,000 X.



1A



1B

*In Vitro* Immunogenicity of Haptenated Top Band Versus Bottom Band T4

Both bands of T4 were adjusted to the same concentration, based on PFU/ml. They were then haptenated under standard conditions and used to immunize spleen cell cultures *in vitro*. The results of a single experiment are seen in Table I. In this experiment the top band of TNP-T4 was found to be about 7-fold more immunogenic than the bottom band of TNP-T4. The reason for the observed difference in immunogenicity is not understood. It is possible that some bacterial cellular debris co-sedimented with the top band of T4 and was subsequently haptenated. This could possibly explain the difference in immunogenicity between the haptenated top band versus bottom band T4. However, this would not alter the interpretation of the subsequent experiments. Except where noted in the following experiments, the top band of TNP-T4 was used as the *in vitro* immunogen.

Is TNP-T4 Pyrogenic?

Bacterial lipopolysaccharide (LPS) or endotoxin has been shown to stimulate an anti-hapten response to a hapten carrier complex in which the carrier was nonimmunogenic (Schmidtke and Dixon, 1972; Watson, Trenkner, and Cohn, 1973). Therefore it was necessary to test for possible contamination of TNP-T4 by bacterial endotoxin, since the phage had been grown and isolated from *E. coli* which produces endotoxin. The method used was the determination of a pyrogenic effect in the rabbit as recommended in the *Pharmacopia of the United States*, 18th

Table I

This table compares the immunogenicity of haptenated top band versus bottom band T4. Three cultures at each point were pooled on day 5 of culture and plated in quadruplicate. The protein concentration of top band TNP-T4 was 20 µg/ml; the concentration of bottom band TNP-T4 was < 5 µg/ml.

Table I

CARRIER POTENTIAL OF CESIUM CHLORIDE BANDED T4 PHAGE

| TNP-T4 Dilution[1] per Culture | Anti-TNP PFC/$10^6$ Cells Assayed |
|---|---|
| Top Band | |
| 1:10 | ND[2] |
| 1:100 | 1257 |
| 1:1000 | 305 |
| 1:10,000 | 96 |
| Bottom Band | |
| 1:10 | 192 |
| 1:100 | 186 |
| 1:1000 | 10 |
| 1:10,000 | 66 |

1. Final dilution of TNP-T4 in spleen cell culture.

2. ND = not determined.

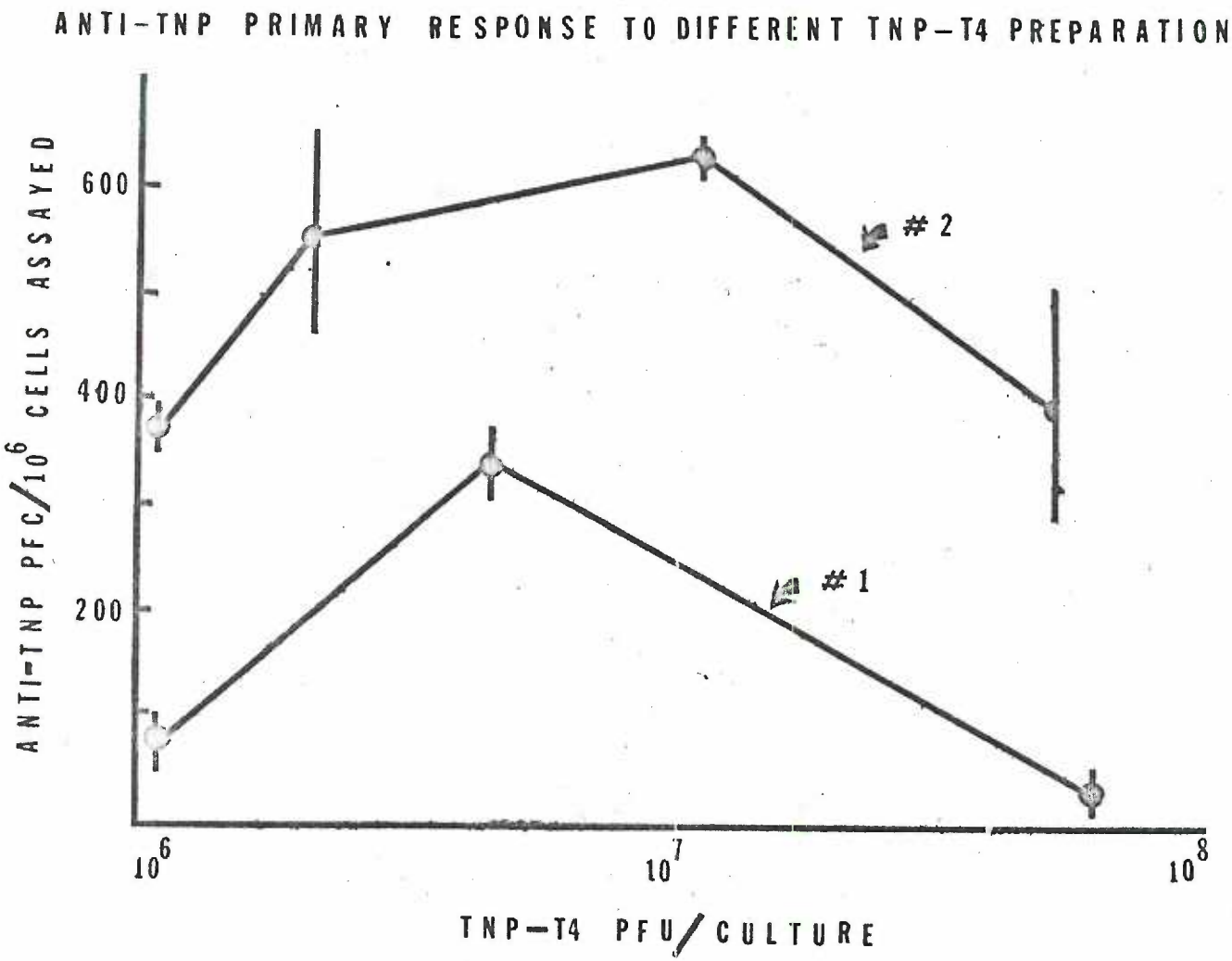
Revision (1970). Rabbits were injected intravenously with 1.0 ml amounts of either undiluted top band or bottom band TNP-T4 and the subsequent change in body temperature determined. Six rabbits were used for each of the preparations tested. The total temperature change observed when the top band of TNP-T4 was tested was 4.2° C. The bottom band of TNP-T4 caused a total temperature increase of 4.4° C. Therefore, according to these results, both bands were pyrogenic. However, it should be noted that these results do not correlate with the differences seen in Table I in the *in vitro* immunogenicity of the two preparations of haptenated phage. The pyrogenicity test indicates the same degree of LPS contamination and would suggest both top and bottom bands to be equally immunogenic.

Dose Response to TNP-T4

Different preparations of TNP-T4 varied as to their *in vitro* immunogenicity. Figure 2 shows the anti-TNP response to two different preparations of TNP-T4. In all cases high doses of antigen suppressed the *in vitro* response and low doses of antigen were suboptimal in their ability to stimulate an anti-TNP response. Invariably the optimum *in vitro* dose of TNP-T4 was about $10^7$ PFU/ml or a final dilution of 1:100 in culture. As seen in Figure 2 there was a considerable difference in the maximum anti-TNP response between the two different preparations of TNP-T4. The degree of hapten substitution was determined spectrophotometrically by the method of Rittenberg and Amkraut (1966), but no detectable absorbance at 350 nm was seen. Therefore, the number

Figure 2

This graph compares the immunogenicity of two different preparations of trinitrophenylated top band T4 (TNP-T4). Each point shown is the mean of triplicate cultures plated in quadruplicate. The bars refer to 95% confidence limits. Spleen cell cultures were harvested on day 4 of culture.


ANTI-TNP PRIMARY RESPONSE TO DIFFERENT TNP-T4 PREPARATIONS
ANTI-TNP PFC/$10^6$ CELLS ASSAYED
600
400
200
# 2
# 1
$10^6$
$10^7$
$10^8$
TNP-T4 PFU/CULTURE

of TNP groups per phage particle in the different preparations of TNP-T4 was not calculated. Even though the phage were haptenated under standard conditions there may well be a difference in the degree of haptenation of phage from one preparation to the next. This might explain the differences seen in the maximum response to different preparations of TNP-T4.

In order to demonstrate the specificity of the anti-TNP plaques, either TNP-BSA or BSA was incorporated into the agar-spleen-cell plating mixture. If the observed plaques were due to anti-TNP antibody as opposed to anti-SRBC antibody the presence of free TNP-BSA in the plating mixture would inhibit the plaques due to anti-TNP antibody, but should have no effect on anti-SRBC antibody. The results of a single experiment are seen in Table II. It was found that 0.1 mg/ml of TNP-BSA severely inhibited the plaques resulting from antigenic stimulation, whereas the same concentration of BSA had no effect. The same results were found when 1.0 mg/ml of either TNP-BSA or BSA was used to inhibit the antigen stimulated plaque response. However, the spontaneous, non-antigen stimulated plaques required the higher concentration of TNP-BSA in order to be inhibited significantly. This is consistent with the idea that these plaques are of low anti-TNP avidity (Claflin and Merchant, 1972; Siskind and Benacerraf, 1969), and thus bind the inhibitor less well than antigen-stimulated plaque-forming cells. It is possible also that a portion of the "background" plaques are not TNP specific since they also were inhibited slightly by BSA alone.

Table II

This experiment demonstrates the specificity of the plaques resulting from *in vitro* stimulation with TNP-T4. Cultures were harvested at day 4 of culture. Final dilution of TNP-T4 in culture was 1:100.

TABLE II

SPECIFICITY OF ANTI-TNP PLAQUES:

PERCENT INHIBITION OF PFC'S

| Concentration of Inhibitor | Antigen Stimulation | |
|---|---|---|
| | TNP-T4 | None |
| TNP-BSA | | |
| 0.1 mg/ml | 95 | 69 |
| 1.0 mg/ml | 99 | 85 |
| BSA | | |
| 0.1 mg/ml | 7 | 16 |
| 1.0 mg/ml | 22 | 63 |

*In Vitro* Response to TNP-T4 With Time

Figure 3 shows the anti-TNP response obtained through six days of culture. During the first two days of culture there were few anti-TNP plaques produced. On day 3 of culture there was a sharp rise in development of anti-TNP producing cells. The peak response occurred on day 4, and in these experiments there was a dramatic fall in the number of anti-TNP producing cells on day 5. By day 6 the number of anti-TNP plaque forming cells was the same as background. In other experiments the peak response tended to plateau at days 4 and 5, but was still down to background levels by day 6. As also seen in Figure 3, those cultures either not stimulated with antigen, or challenged with carrier alone, produced a small anti-TNP response usually about 100 PFC/$10^6$ on days 4 and 5. Such a nonspecific background response to TNP was reported previously by Kettman and Dutton (1970), and background plaques to 2,4-dinitrophenyl (DNP) were reported by Chiller and Weigle (1970).

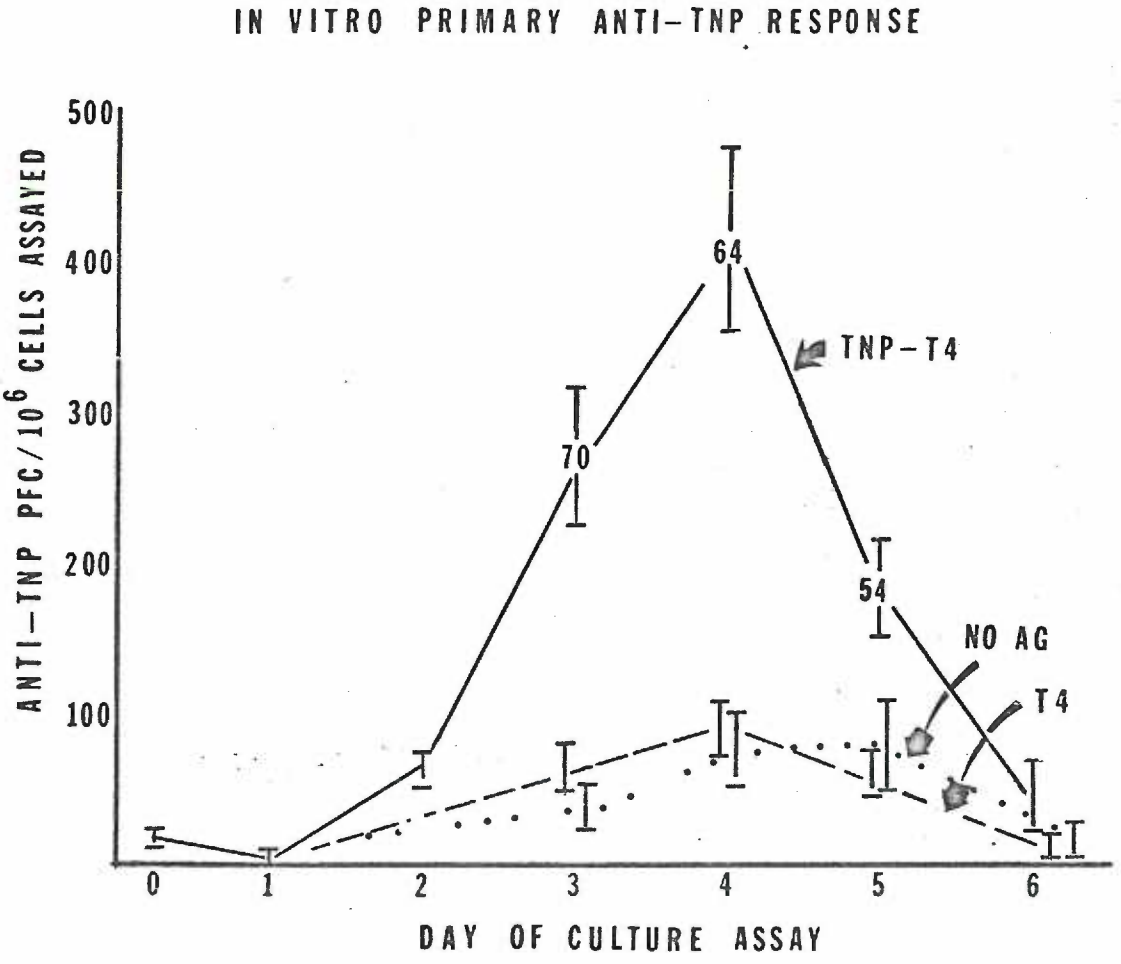
The fact that the response seen in cultures challenged with T4 alone, at the same concentration as TNP-T4, was the same as non-challenged cultures suggests that the response to the haptenated phage is not due to a nonspecific effect of contaminating lipopolysaccharide (see Discussion).

Lack of an *In Vitro* Anti-Carrier (T4) Response

Primary *in vitro* antibody responses to various coliphage have been reported. Tao and Uhr (1966) obtained an *in vitro* response to

Figure 3

This graph shows the development of anti-TNP plaques through 6 days of culture. Final dilution of both TNP-T4 and T4 in culture was 1:100. The numbers in parentheses indicate the number of cultures assayed at that point. The bars refer to 95% confidence limits.


IN VITRO PRIMARY ANTI-TNP RESPONSE
ANTI-TNP PFC/10^6 CELLS ASSAYED
500
400
300
200
100
0
1
2
3
4
5
6
DAY OF CULTURE ASSAY
64
70
54
TNP-T4
NO AG
T4

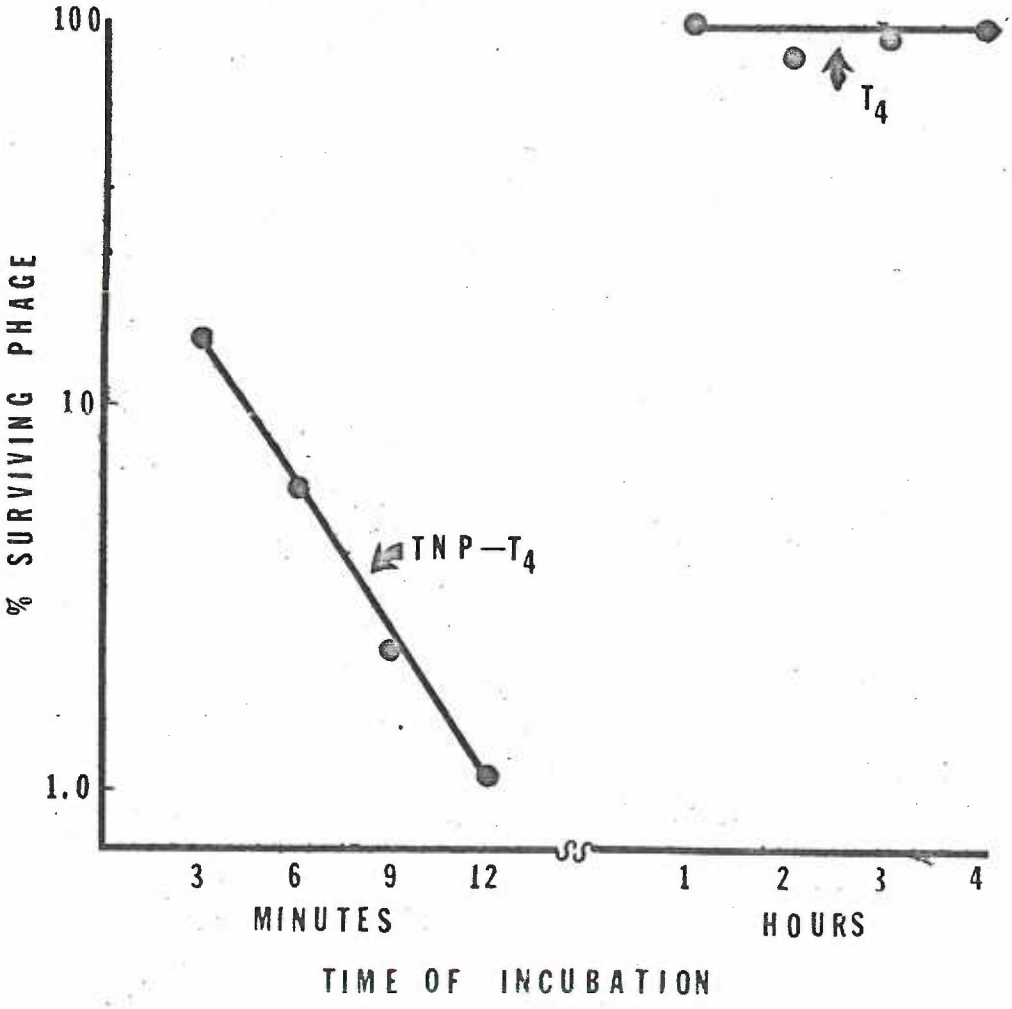
$\phi$X174 and Saunders and King (1966) reported an *in vitro* response to R17. Therefore it was of interest to determine the response made against the carrier T4 in this system. Tissue culture supernates were tested for their ability to neutralize either TNP-T4 or T4. The results are shown in Figure 4. Supernatant fluids from cultures challenged with TNP-T4 were able to neutralize TNP-T4. In this experiment about 90% of the haptenated phage were neutralized in 3 minutes and 99% of the phage were neutralized in 12 minutes. However, these same culture supernates showed no ability to neutralize native T4, even during 4 hours of incubation. Not shown in Figure 4 are the results of incubating either native T4 or TNP-T4 with supernates from cultures either not challenged with antigen or challenged with native T4 alone. As in cultures challenged with TNP-T4 there was no detectable neutralization of either TNP-T4 or native T4.

The lack of a detectable response to T4 could be explained by at least three possibilities. First, phage determinants could be masked by TNP groups and thereby rendered unable to stimulate anti-T4 antibody-producing cells. Second, it is possible that antigenic competition between trinitrophenyl groups and phage determinants resulted in the lack of an anti-T4 response. Neither of these two possibilities would account for the lack of detectable anti-phage antibody in supernates of cultures challenged with T4 alone. Additionally, Barber and Rittenberg (1969) found that the addition of TNP groups to coliphage T4 did not greatly affect the sensitivity of haptenated phage to inactivation by anti-T4 antibody. This suggests that a major alteration

Figure 4

This graph shows the ability of supernatants from cultures stimulated with TNP-T4, at a final dilution of 1:100, to neutralize TNP-T4 but unable to neutralize native T4. Initial concentration of phage in the reaction mixture was 1 to 2 X $10^6$ PFU/ml. Reported results are averages from triplicate plating.

PHAGE NEUTRALIZATION BY SUPERNATANTS FROM CULTURES STIMULATED IN VITRO WITH TNP—T4 5 DAYS EARLIER



of phage protein does not occur by the haptenation procedure. A third possibility was that the cultures were not challenged with an optimum dose of phage. To test for this, cultures were challenged with doses of native T4 from $10^2$ to $10^8$ PFU/culture, at $\log_{10}$ increments. After 5 days incubation, culture supernatant fluids were tested for their ability to neutralize native T4. None of the culture supernatant fluids were found to neutralize T4. Apparently in this system the antibody response is directed only against the hapten trinitrophenyl.

Is the Response to TNP-T4 a Primary Anti-Hapten Response?

In all of the above experiments the results are expressed as direct (IgM) plaques. I have never been able to detect indirect (facilitated or IgG) plaques in this primary anti-TNP culture system. However, it was of interest to determine the class of the anti-TNP antibody released into the culture medium. Therefore, supernates from cultures challenged with TNP-T4 were titered using the lytic spot test of Bullock and Rittenberg (1970a) subsequent to reduction and alkylation by the method of Pearlman (1967). The supernatant culture fluids were incubated for 30 minutes at 56° C with 0.1 M 2-mercaptoethanol and dialyzed against 0.1 M iodoacetamide and then against 0.15 M sodium chloride. This treatment entirely eliminated detectable anti-TNP antibody. These results correlate with the detection of direct plaque forming cells only and indicate that the anti-hapten response to TNP-T4 is a primary IgM antibody response. The anti-TNP titers found in

culture supernates ranged from 1:8 to 1:16. This is significantly lower than that found in the secondary *in vitro* anti-hapten response to TNP-KLH (Bullock, 1971).

Effect of *In Vivo* Priming With Carrier T4

The above results indicated that anti-TNP responses occurred in this system without carrier priming. Furthermore, no evidence of an antibody response against the carrier portion of the immunogen was seen. Therefore, experiments were done to determine whether the carrier effect was operative in this system. If the carrier effect were operative, the immune response to the hapten would be increased by pre-immunizing the spleen donors with carrier. Mice were primed with native T4 at either $10^8$ or $10^9$ PFU's. At various times after priming, spleens were removed, placed in culture and challenged with doses of TNP-T4 over a 100-fold range. The results of these experiments are shown in Table III. As can be seen, at no time during the 21 days studied did *in vivo* carrier priming enhance the *in vitro* anti-TNP response. Furthermore, in cultures initiated 4 days after *in vivo* priming the subsequent *in vitro* anti-TNP response was inhibited regardless of the dose of phage used to prime the mice. By 9 days after priming with $10^8$ PFU's, *in vitro* suppression was no longer evident. However, suppression of the *in vitro* response in the group primed with $10^9$ PFU persisted through 14 days after priming. In one case, at 9 days after *in vivo* priming with $10^9$ PFU's, 10-fold higher *in vitro* concentration of TNP-T4 overcame the suppressive effect of

Table III

This table shows the effect of *in vivo* priming with T4 on the subsequent *in vitro* anti-TNP response to three different doses of TNP-T4. Cultures were harvested on day 4 of culture.

Table III

EFFECT OF *IN VIVO* PRIMING WITH CARRIER T4

PRIOR TO CULTURE OF SPLEEN CELLS WITH TNP-T4

| Primed ____ Days Before Culture | Priming Dose of T4[1] | Anti-TNP PFC/$10^6$ *In Vitro* Dilution of TNP-T4 | | |
|---|---|---|---|---|
| | | 1:1,000 | 1:100 | 1:10 |
| 4 | $10^9$ | 41 ± 41[2] | 128 ± 34 | 178 ± 107 |
| 4 | $10^8$ | 38 ± 51 | 69 ± 20 | 4 ± 4 |
| Control | | 380 ± 362 | 833 ± 139 | 248 ± 154 |
| 9 | $10^9$ | 133 ± 95 | 263 ± 109 | 596 ± 201 |
| 9 | $10^8$ | 390 ± 183 | 567 ± 139 | 397 ± 61 |
| Control | | 487 ± 229 | 544 ± 82 | 243 ± 101 |
| 14 | $10^9$ | 129 ± 40 | 391 ± 177 | 567 ± 66 |
| 14 | $10^8$ | 214[3] | 851 ± 100 | 244 ± 52 |
| Control | | 440 ± 30 | 988 ± 196 | 460 ± 60 |
| 21 | $10^9$ | 24 ± 28 | 210 ± 97 | 471 ± 58 |
| 21 | $10^8$ | 73 ± 38 | 239 ± 97 | 444 ± 169 |
| Control | | 190 ± 34 | 457 ± 179 | 299 ± 176 |

1. PFU/mouse given I.P.

2. 95% confidence limit.

3. Average of only 2 cultures.

*in vivo* priming. Even in this case the anti-hapten response was not enhanced beyond control cultures challenged with an optimum dose of TNP-T4. The significant points are that in none of the spleen cell cultures from primed mice was there a shift in the optimum dose of TNP-T4 required to obtain a maximum anti-TNP response, nor did *in vivo* priming lead to an increased response at any dose tested. These results are in sharp contrast to other systems used to investigate the same anti-hapten response. Dutton, Campbell, Chan, Hirst, Hoffmann, Kettman, Lesly, McCarthy, Mishell, Raidt, and Vann (1971) showed that the response to TNP-SRBC was significantly enhanced by priming the mice with SRBC 3 days prior to culturing the spleen cells with TNP-SRBC. Bullock (1971) found that it was necessary to prime mice with the carrier KLH in order to obtain a primary anti-hapten response to TNP-KLH. Katz, Bluestein, Rouques, and Pierce (1971) and Bluestein and Pierce (1973a) investigated the *in vitro* anti-TNP response using TNP-$\phi$X174 as an *in vitro* antigen. They not only found that priming with the phage 7 days prior to culture augmented the *in vitro* response, but also phage priming caused a shift in the *in vitro* dose of TNP-$\phi$X174 required for a maximum anti-hapten response. Spleen cell cultures from primed mice showed the greatest response to a dose of TNP-$\phi$X174 one-tenth of that required by non-primed spleen cell cultures.

An attempt to correlate the inhibition of the *in vitro* response shortly after carrier priming with levels of serum anti-T4 antibody from the primed mice at the time spleens were removed for culture

was made. The results are shown in Table IV. It was found that in the group of mice primed with $10^9$ PFU the serum 50% neutralization titer increased through the 21 days of the experiment. However, the group of mice primed with $10^8$ PFU exhibited detectable anti-T4 serum antibody only 4 days after priming. No correlation was observed between suppression of the *in vitro* anti-hapten response and levels of serum T4 neutralizing capacity. These results indicate that inhibition of the *in vitro* anti-TNP response by carrier priming was independent of the humoral immune status of the spleen donor at the time of culture.

Effect of Free Carrier in the *In Vitro* Anti-TNP Response

In view of the failure to find evidence of a carrier effect by direct priming with carrier, an attempt was made to detect a carrier effect indirectly by showing that the *in vitro* response to TNP-T4 could be abrogated by high doses of the carrier T4 alone. Rittenberg and Bullock (1972) showed that high doses of either hapten or carrier alone could establish tolerance *in vitro* and thereby paralyze the immune response to TNP-KLH, the intact immunogen. Their work suggested that high doses of carrier caused paralysis of helper T cells and that high doses of hapten resulted in paralysis of hapten-specific B cells. It was possible that the suppressive effect of high doses of TNP-T4 shown in Figure 2 resulted from paralysis or tolerance of both carrier specific helper cells and hapten specific B cells. If this were true, and if the response depended on cooperation between helper cells and B cells, the addition of free carrier, T4, to spleen cell cultures

Table IV

This table shows the effect of *in vivo* priming of mice on the development of serum phage neutralizing activity. The titer was determined by plotting phage neutralization according to Von Krogh as described in Campbell et al. (1963). Initial concentration of T4 in the reaction mixture was 1 to 2 X $10^6$ PFU/ml. Reported results are averages from triplicate plating.

Table IV

EFFECT OF *IN VIVO* PRIMING ON SERUM PHAGE

NEUTRALIZATION ACTIVITY

| Prime ____ Days Prior to Culture | Priming Dose of T4 | Serum 50% Neutralization Titer[1] |
|---|---|---|
| 4 | $10^9$ | 8.3 |
| 4 | $10^8$ | 8.3 |
| Control | | < 2 |
| 9 | $10^9$ | 5.9 |
| 9 | $10^8$ | 2.0 |
| Control | | < 2 |
| 14 | $10^9$ | 11.1 |
| 14 | $10^8$ | < 2 |
| Control | | < 2 |
| 21 | $10^9$ | 20.0 |
| 21 | $10^8$ | < 2 |
| Control | | < 2 |

1. Reciprocal of serum dilution $\log_2$.

challenged with an optimum dose of TNP-T4 should also suppress the subsequent anti-hapten response.

Table V shows the effect of adding free carrier to cultures stimulated with an optimal dose of TNP-T4. For purpose of comparison, the effect of free carrier, KLH, on the *in vitro* anti-hapten response to TNP-KLH is also shown. The data on the primary anti-TNP response to TNP-KLH are taken from Bullock (1971). Whereas high doses of the intact antigen TNP-T4 were highly suppressive, the addition of carrier had no suppressive effect. By comparison, it is seen that in the KLH system, even a 10-fold excess of free carrier was highly suppressive. These results suggested that the anti-TNP response to TNP-T4 occurred independent of carrier directed helper cells.

Effect of T-Cell Depletion By Br-Anti-θ Serum Treatment

The above experiments suggest that TNP-T4 may trigger hapten specific B cells independent of interaction with T cells. Therefore, KLH primed spleens were depleted of T cells by treatment with Br-anti-θ serum and complement (C). Control KLH primed spleen cells were treated with normal rabbit serum and complement. Cultures were challenged with TNP-T4 or TNP-KLH. As a positive control, additional cultures were challenged with DNP-POL since it had been shown by Feldmann and Basten (1971) that the *in vitro* anti-hapten response to this immunogen was not affected by anti-θ treatment of spleen cells. Table VI shows the results of three such experiments. The anti-TNP response to both TNP-T4 and DNP-POL was reduced equally, but

Table V

This table shows the ability of free KLH to inhibit the anti-TNP response to TNP-KLH, and the inability of free T4 to inhibit the anti-TNP response to TNP-T4. Values are the mean of three cultures, assayed on day 5 of culture and plated in quadruplicate.

Table V

SUPPRESSION OF THE PRIMARY *IN VITRO* ANTI-TNP RESPONSE BY EXCESS FREE CARRIER

| Antigen | PFC/10 | % Suppression |
|---|---|---|
| TNP-KLH[1] | 932 (1083-781)[3] | |
| " + 10X free carrier | 163 ( 192-134) | 83% |
| " + 100X free carrier | 63 ( 82- 44) | 95% |
| TNP-T4[2] | 479 ( 329-582) | |
| " + 10X[4] free carrier | 668 ( 482-800) | 0% |
| " + 100X[5] free carrier | 632 ( 443-967) | 0% |

1. *In vitro* concentration of TNP-KLH = 0.002 µg/cult.
2. Final concentration of TNP-T4 = 1:100.
3. Range of 95% confidence limits.
4. Final concentration of T4 = $10^9$ PFU/cult.
5. Final concentration of T4 = $10^{10}$ PFU/cult.

Table VI

This table shows the effect of Br-anti-θ plus complement (C) treatment of KLH primed spleen cells on the subsequent *in vitro* anti-TNP response to TNP-T4 or TNP-KLH or DNP-POL. Control KLH primed spleen cells were treated with normal rabbit serum (NRS) plus complement. Unless noted, the values are the means of three cultures, assayed on day 5 of culture and plated in quadruplicate.

Table VI

EFFECT OF Br-ANTI-θ TREATMENT OF SPLEEN CELLS

| Antigen + Treatment | Anti-TNP PFC/$10^6$ | | |
|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 |
| TNP-T4 (top band)[1] | | | |
| Br-anti-θ + C | 468[4] | 249 ± 32[5] | 608 ± 211 |
| NRS + C | 692 ± 170 | 754 ± 258 | 2266 ± 1037 |
| TNP-T4 (bottom band)[1] | | | |
| Br-anti-θ + C | ND[6] | ND | 282 ± 145 |
| NRS + C | ND | ND | 111 ± 44 |
| TNP-KLH[2] | | | |
| Br-anti-θ + C | 17[4] | 0 | 21 ± 17 |
| NRS + C | 1736 ± 613 | 1555 ± 296 | 2088 ± 301 |
| DNP-POL[3] | | | |
| Br-anti-θ + C | 97[4] | 32 ± 11 | 0 |
| NRS + C | 137 ± 31 | 57 ± 42 | 44 ± 56 |

1. Final dilution in culture = 1:100.
2. Final concentration in culture = 0.2 μg/ml.
3. Final concentration in culture = 1.0 μg/ml.
4. Average of two cultures plated in quadruplicate.
5. 95% confidence limit.
6. ND = not determined.

"relatively" unaffected when compared to the response to TNP-KLH which was reduced by at least 99% after T cell depletion. By this additional criterion the response to TNP-T4 appears to be T cell independent.

Response of Spleen Cells From "Nude" Mice

Mice homozygous for the mutation "nude" (nu/nu) suffer from thymic hypoplasia and are believed to have few, if any, functional T cells (Pantelouris, 1971). Spleen cell cultures from such mice were used to establish further whether T cells are required for an *in vitro* anti-hapten response to TNP-T4. Littermates heterozygous for the mutation (nu/+) do have functional T cells and were used as controls. The results of four such experiments are shown in Table VII. As can be seen, both TNP-T4 and DNP-POL were capable of stimulating TNP specific B cells in the absence of functional T cells. These results are additional evidence that T cells are not required for an *in vitro* anti-hapten response to TNP-T4.

Effect of *In Vitro* Challenge With Both TNP-T4 and TNP-KLH

The above experiments suggest that TNP-T4 can stimulate hapten specific B cells independent of interaction with carrier-directed T cells. In view of the proposed existence of two subpopulations of B cells it was of interest to determine what effect the addition of a T dependent hapten-carrier complex (TNP-KLH) along with a T independent hapten-carrier complex (TNP-T4) would have on the *in vitro* anti-hapten

Table VII

This table compares the *in vitro* immune response of hypothymic "nude" mice versus the response of littermate controls against the following immunogens: TNP-T4 (top band), DNP-POL, and SRBC. In experiments 1-3, those results with 95% confidence limits are the means of three cultures plated in quadruplicate. The results without 95% confidence limits are the average of two cultures plated in quadruplicate. All cultures were assayed on day 5 of culture. In experiment 4, spleen cells were cultured in Micro-Test tissue culture plates (Falcon Plastics) in which all volumes were one-tenth those used in experiments 1-3.

Table VII

*IN VITRO* IMMUNE RESPONSE IN SPLEEN CELL CULTURES

FROM NUDE MICE

| Antigen | PFC/$10^6$ | | | PFC/Cult.[1] |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
| | | ANTI-TNP | | |
| TNP-T4[2] | | | | |
| nu/nu[3] | 68 ± 23[5] | 149 ± 21 | 11 ± 6 | 51 |
| nu/+[4] | 481 ± 142 | 233 ± 55 | 170 ± 31 | 120 |
| DNP-POL[6] | | | | |
| nu/nu | 0 | 110 ± 50 | 7 | 22 |
| nu/+ | 95 ± 59 | 112 ± 48 | 46 ± 32 | 38 |
| | | ANTI-SRBC | | |
| SRBC[7] | | | | |
| nu/nu | 0 | 2 | 4 | 0 |
| nu/+ | 204 ± 30 | 6 | 72 ± 20 | 26 |

1. Pool of eight micro-replicate cultures.
2. Final dilution in culture = 1:100.
3. Hypothymic mice homozygous for mutation "nude".
4. Heterozygous littermate controls.
5. 95% confidence limits.
6. Final concentration in culture = 1.0 µg/ml.
7. Final concentration in culture = 3 X $10^6$ cells/ml.

Table VIII

This table demonstrates the additive effect in cultures simultaneously challenged with an optimum dose of both TNP-KLH and TNP-T4. On the days of culture indicated, three cultures for each point were pooled and plated in quadruplicate.

Table VIII

ADDITIVE EFFECT OF HAPTEN (TNP)

ON DIFFERENT CARRIERS

| Culture Antigen | Anti-TNP PFC/$10^6$ | | |
|---|---|---|---|
| | Day 4 | Day 5 | Day 6 |
| TNP-KLH[1] | 831[2] | 920 | 322 |
| TNP-T4[3] | 539 | 574 | 410 |
| TNP-KLH + TNP-T4 | 1177 | 1643 | 1279 |

1. Final concentration in culture = 0.02 g/ml.
2. Pool of three cultures per point.
3. Final dilution in culture = 1:100.

response. Table VIII shows the results of a typical experiment. Spleen cell cultures were challenged with an optimal dose of either TNP-KLH or TNP-T4 or a mixture of TNP-KLH and TNP-T4. As can be seen, a substantial anti-hapten response was induced by either TNP-KLH or TNP-T4. In view of the proposed subpopulations of B cells the response to the mixture of these two immunogens is of importance. The response in these double-challenged spleen cell cultures is additive in relation to the response seen when either antigen was used alone. Control cultures in all such experiments were challenged with a 2-fold higher dose of either TNP-KLH or TNP-T4. This was done in order to determine if the increased response seen in the double-challenged culture was due to the single challenge cultures being stimulated with a slightly suboptimal dose of either antigen.

Effect of Suppressive Dose of One Hapten-Carrier Complex on a Stimulatory Dose of a Second Hapten-Carrier Complex

The previous experiments not only indicate that TNP-T4 is a T cell independent antigen and TNP-KLH is a T cell dependent antigen, but also suggest that these hapten-carrier complexes apparently stimulate separate subpopulations of hapten specific B cells. An additional means of investigating this apparent separable B cell stimulation was to determine what effect a suppressive dose of one immunogen had on the *in vitro* anti-hapten response to an optimal dose of the other immunogen. Five such experiments were performed and the

results of one experiment are shown in Table IX. Three points should be noted in the table. First, high doses of either of the immunogens were suppressive. Second, a suppressive dose of either immunogen was unable to suppress the response to an optimal dose of the other immunogen. In fact, an additive effect was observed. Third, when optimal doses of both immunogens were used to challenge the spleen cell cultures there was an additive effect as seen in Table VIII. These results are additional suggestive evidence that TNP-T4 and TNP-KLH trigger separate subpopulations of TNP-specific B cells.

Of the five experiments performed investigating the effect of a suppressive dose of one hapten-carrier complex on a stimulatory dose of a second hapten-carrier complex, three of the experiments suggested that each immunogen triggered separate subpopulations of hapten-specific cells. One of the other experiments was a technical failure in that *in vitro* challenge with a high dose of TNP-KLH was more stimulatory than what normally was the optimum dose of the immunogen. In the fifth experiment, the responses in those cultures simultaneously challenged with a high dose of one immunogen and an optimum dose of the other immunogen were suppressed when compared to cultures challenged with either immunogen alone. However, when cultures were simultaneously challenged with an optimum dose of both immunogens an additive effect was observed.

Table IX

This table shows the effect of a suppressive dose of either TNP-T4 or TNP-KLH on a stimulatory dose of the other immunogen. This table also shows that the anti-TNP response in cultures simultaneously challenged with an optimum dose of both immunogens is additive when compared to the response to either immunogen used alone. For each point shown, three cultures were pooled and plated in quadruplicate on the day of culture indicated.

Table IX

EFFECT OF A SUPPRESSIVE DOSE OF ONE HAPTEN-CARRIER COMPLEX ON A STIMULATORY DOSE OF A SECOND HAPTEN-CARRIER COMPLEX

| Antigen + Concentration | Anti-TNP PFC/$10^6$ Day 5 | Day 6 |
|---|---|---|
| Opt. TNP-T4[1] | 811 | 486 |
| 10X TNP-T4[2] | 190 | 54 |
| Opt. TNP-KLH[3] | 648 | 648 |
| 10X TNP-KLH[4] | 277 | 356 |
| Opt. TNP-T4 + 10X TNP-KLH | 1126 | 1085 |
| Opt. TNP-KLH + 10X TNP-T4 | 1089 | 1048 |
| Opt. TNP-T4 + Opt. TNP-KLH | 1838 | 1797 |

1. Final dilution in culture = 1:100.
2. Final dilution in culture = 1:10.
3. Final concentration in culture = 0.02 µg/ml.
4. Final concentration in culture = 0.2 µg/ml.

## Effect of *In Vitro* Challenge With Combinations of T-Cell Dependent and T-Cell Independent Hapten Carrier Complexes

The results of the previous experiments are consistent with the theory of two subpopulations of B cells, distinguished by their susceptibility to being stimulated by T cell dependent or T cell independent antigens. However, at this point only TNP-T4 and TNP-KLH had been used as *in vitro* antigens. The question arose as to whether this relationship between T cell dependency and *in vitro* additive effects would hold when utilizing other combinations of hapten-carrier complexes. In the first experiments of this section mice were primed with TNP-KLH to allow for an increased response to DNP-POL[2]. Also, bottom band TNP-T4 was used as the immunogen since the *in vitro* response to this band of haptenated phage more closely approximates the response to DNP-POL than does the upper band of TNP-T4. If the upper band of TNP-T4 had been used, any additive effect of DNP-POL would most likely have been obscured by the magnitude of the response to TNP-T4.

Table X shows the results of one of four experiments in which TNP-T4 or DNP-POL, two thymus independent antigens in the mouse, or TNP-KLH, a thymus dependent antigen in the mouse, were used as immunogens. It is seen that the response to a mixture of the two T cell independent antigens showed no additive effect in relation to the response when either antigen was used alone. However, when either of the two T cell

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2. M. Rittenberg and M. Feldmann. Personal Communication.

Table X

This table shows the effect of *in vitro* simultaneous challenge of spleen cell cultures with various combinations of two T-cell independent immunogens, TNP-T4 and DNP-POL, and one T-cell dependent immunogen, TNP-KLH. The results are the means of three cultures, plated in quadruplicate, and assayed on day 4 of culture.

Table X

ADDITION EXPERIMENT USING COMBINATIONS OF

T-CELL DEPENDENT AND T-CELL INDEPENDENT HAPTEN-CARRIER COMPLEXES

| Antigen | Thymus Dependency | Anti-TNP PFC/$10^6$ |
|---|---|---|
| DNP-POL[1] | - | 519 ± 170[2] |
| TNP-T4[3] (bottom band) | - | 376 ± 244 |
| TNP-KLH[4] | + | 1804 ± 33 |
| DNP-POL + TNP-T4 | - - | 456 ± 217 |
| DNP-POL + TNP-KLH | - + | 4716 ± 728 |
| TNP-T4 + TNP-KLH | - + | 3660 ± 571 |

1. Final concentration in culture = 1.0 µg/ml.
2. 95% confidence interval.
3. Final dilution in culture = 1:100.
4. Final concentration in culture = 0.2 µg/ml.

independent antigens was used along with TNP-KLH, there was a definite additive effect. The results of one of two reciprocal experiments in which the two thymus dependent antigens and one thymus independent antigen were tested for their additive ability when combined in culture are shown in Table XI. In this experiment the presence or absence of an additive effect was determined using TNP-T4, a T independent antigen, and TNP-KLH or DNP-CGG, both T cell dependent antigens. The anti-TNP response to DNP-CGG is considered T cell dependent in that Feldmann (1972) showed that spleen cells from non-primed mice were unable to develop an *in vitro* anti-hapten response to DNP-CGG. Similar results have been obtained in our laboratory by M. Baltz. As seen in Table XI, the *in vitro* anti-hapten response to an immunizing mixture of either TNP-KLH or DNP-CGG along with TNP-T4 gave an additive effect. However, a mixture of both TNP-KLH and DNP-CGG was not additive.

These results are consistent with the theory of two populations of B cells, with these cells being distinguished by their ability to react with T cell dependent or T cell independent antigens. Immunizing mixtures of either two T cell dependent or two T cell independent hapten carrier complexes showed no additive effect in the *in vitro* anti-hapten response. However, immunizing mixtures of a T dependent and a T independent antigen did show an *in vitro* additive effect.

An interesting unexpected observation in these latter experiments was the "super-additive" response. In the previous experiments in which spleen cell cultures were challenged with multiple hapten-carrier complexes, Tables VIII and IX, the anti-hapten responses in multiple challenged cultures were very close to simple addition of the individual

Table XI

This table shows the effect of *in vitro* simultaneous challenge of spleen cell cultures with various combinations of two T-cell dependent immunogens, TNP-KLH and DNP-CGG, and one T-cell independent immunogen, TNP-T4. The results are the means of three cultures, plated in quadruplicate, and assayed on day 4 of culture.

Table XI

ADDITION EXPERIMENT USING COMBINATIONS OF

T-CELL INDEPENDENT AND T-CELL DEPENDENT HAPTEN-CARRIER COMPLEXES

| Antigen | Thymus Dependency | Anti-TNP PFC/$10^6$ |
|---|---|---|
| TNP-T4[1] | - | 157 ± 45[2] |
| TNP-KLH[3] | + | 138 ± 98 |
| DNP-CGG[4] | + | 24 ± 22 |
| TNP-T4<br>+ TNP-KLH | -<br>+ | 1007 ± 138 |
| TNP-T4<br>+ DNP-CGG | -<br>+ | 547 ± 203 |
| TNP-KLH<br>+ DNP-CGG | +<br>+ | 225 ± 123 |

1. Final dilution in culture = 1:100.
2. 95% confidence limit.
3. Final concentration in culture = 0.02 μg/ml.
4. Final concentration in culture = 2.0 μg/ml.

responses. However, in Tables X and XI the anti-hapten response in those cultures showing addition is considerably greater than additive. The reason for this super-additive effect is unknown. One possibility is that for some reason the antibody forming cells in these multiply challenged cultures underwent an additional cell replication cycle. However, if this were the situation an additive effect in those cultures challenged with either two T independent or two T dependent antigens would have been observed. Therefore, at the present time, the reason for a super-additive effect is unclear, but may be related to T cell regulatory factors (see Discussion).

# DISCUSSION

This thesis presents data obtained with an *in vitro* experimental model of the immune system, which permitted study of an anti-hapten (trinitrophenyl [TNP]) primary immune response. The principal findings obtained with this model were: a) the *in vitro* anti-hapten response to TNP-bacteriophage T4 (TNP-T4) is thymus (T cell) independent; b) *in vitro* simultaneous challenge with a thymus independent TNP-immunogen and a thymus dependent TNP-immunogen greatly enhanced the subsequent anti-TNP response; and, c) these data provide the first evidence, with a chemically defined determinant, that there may be two distinct subpopulations of B cells ($B_1$ and $B_2$) producing antibody to the same determinant, but differing in response to thymus independent or to thymus dependent antigens.

The anti-TNP response was measured as an increase in the number of cells synthesizing anti-TNP antibody and detected as plaque-forming cells (PFC). The response was shown to be specific for TNP by the ability of hapten to inhibit plaque formation (Table II).

Roseman, Leserman, Fitch and Rowley (1969) found that spleen cells from recently immunized rats showed a high frequency of "false" PFC, in that some PFC were not inhibited by metabolic inhibitors. These false plaques were attributed to either passive release of intracellular antibody or to release of antibody which had adhered to cell-platelet aggregates. This is not the case in immune responses generated *in vitro*, where it has been shown that the PFC result from antigen stimulation and that both cell division and *de novo* antibody synthesis are required.

The secondary anti-TNP response using this model system was studied previously by Bullock and Rittenberg (1970). Bullock (1971) showed that the anti-TNP response required actively metabolizing cells and that chloramphenicol, an inhibitor of mammalian mitochondrial protein synthesis, inhibited the secondary response to TNP-KLH. Edney and Rittenberg (unpublished observations) found that methotrexate, which inhibits cell division, also inhibited the secondary response to TNP-KLH. Bullock and Rittenberg (1970) also found that the *in vitro* anti-TNP antibody released in culture contained $^{14}C$ labelled amino acids if the label was added at the initiation of culture, indicating that the antibody was newly synthesized. These findings were in accord with earlier work of Dutton and Mishell (1967), who used the same *in vitro* system to study both primary and secondary responses to sheep erythrocytes. By means of a "hot pulse" of high specific activity tritiated thymidine, they showed that dividing cells were necessary for the anti-SRBC response.

Use of TNP-T4 Bacteriophage

A major concern in using TNP-T4 as an immunogen was whether the preparations of TNP-T4 were contaminated with lipopolysaccharide (LPS) derived from the host *E. coli* cells in which the phage were propagated. The reason for this concern was that LPS or endotoxin has been shown to stimulate an anti-hapten response to a hapten carrier complex in which the carrier was nonimmunogenic (Schmidtke and Dixon, 1972; Watson, Trenkner and Cohn, 1973) and to activate background

anti-hapten B cells (Coutinho, Möller, Andersson and Bullock, 1973). The results of the pyrogen tests, although weak according to the U. S. Pharmacopoeia standards, suggested that there might be LPS in the phage preparation.

Since LPS has been shown to be a B cell mitogen (Peavy, Adler, and Smith, 1970), such activity was tested for directly in a conventional lymphocyte transformation assay which was kindly performed by S. Fairchild, Oregon Regional Primate Center. She determined the ability of TNP-T4 and T4 to cause blast transformation, as measured by uptake of tritiated thymidine by cultured mouse spleen cells. Both TNP-T4 and T4 were slightly mitogenic; however, the doses of either preparation that showed any stimulatory activity were 1/20 to 1/40 the concentration that gave a maximum *in vitro* anti-TNP response. Consequently it appeared unlikely that the anti-hapten response to TNP-T4 occurring in cultures was due to the mitogenic effect of contaminating LPS. Moreover, the hallmark of LPS stimulation is the nonspecific activation of cells forming plaques to SRBC (Sjöberg, Andersson and Moller, 1972). Such background responses were tested for in more than 100 cultures challenged with various doses of TNP-T4 or T4. The background to SRBC never differed significantly from background responses in cultures receiving no antigenic stimulus. Furthermore, the use of T4 phage alone did not raise the background of anti-TNP producing cells (see Figure 3), another criterion of LPS stimulation (Coutinho et al., 1973). Thus by immune biologic criteria there was no evidence of significant LPS contamination.

Nature of the Response to TNP-T4

The anti-hapten responses reported in this thesis may be considered primary responses in the sense that they result from the first experimental exposure of the spleen cells to the hapten TNP. Additionally, only direct IgM plaque responses were detected and the antibody in the tissue culture supernates was sensitive to reduction with 2-mercaptoethanol.

If one accepts any form of the clonal selection theory (Jerne, 1955; Burnet, 1959) the differences between primary and secondary immune responses become semantic in terms of activation of B cells. Genetic studies of the immune response (McDevitt and Benacerraf, 1969; Benacerraf and McDevitt, 1972; McDevitt, 1973) as well as the genetic basis of immunoglobulin structures (Potter and Lieberman, 1967; Hood and Talmage, 1970), indicate that the ability to make all immune responses requires the appropriate genetic endowment. Furthermore, B cells must be expressing the appropriate gene products as immunoglobulin-like receptors on their surfaces in order to bind the appropriate antigen (Davie and Paul, 1973). In addition, secondary IgM responses have been well documented (Bullock and Rittenberg, 1970), and only the "switch" from IgM to IgG remains a question (Nossal, Warner, and Lewis, 1971; Gershon, 1973). Thus the basic difference between a primary and secondary response would appear to be a quantitative one, that is to say in a primary immune response one is dealing with a small population of antigen-specific B cells, whereas in a secondary response the population of antigen-specific cells has been increased by selective antigenic stimulation.

Lack of an Anti-Carrier Response

The inability to detect an antibody response against the carrier T4 was unexpected (Fig. 4). It had been shown previously that coliphage can be used successfully as *in vitro* immunogens. Fishman (1961) obtained an *in vitro* antibody response against coliphage T2, Tao and Uhr (1966) obtained both IgM and IgG *in vitro* antibody responses to $\phi$X174, and Saunders and King (1966) obtained an *in vitro* antibody response to coliphage R17. Therefore the lack of a detectable antibody response to the carrier T4 in cultures challenged *in vitro* with TNP-T4 (Fig. 4) made it essential to test this observation more rigorously.

Spleen cell cultures were challenged with native T4 at doses from $10^2$ to $10^8$ PFU/culture, at $\log_{10}$ increments, but still failed to demonstrate an antibody response to T4 (data not shown). These experiments indicated that the failure to obtain a detectable anti-T4 response in cultures challenged with TNP-T4 cannot be attributed to antigenic competition. Furthermore, the experiments also indicate that this failure is not due to masking of T4 determinants by TNP. In addition to attempting to detect anti-T4 antibody by phage neutralization, attempts were made to detect anti-T4 antibody by passive hemagglutination according to the method of Walker, Liu and Adler (1969). However, even by this means no evidence was found of anti-T4 antibody in supernatant fluids from cultures challenged with either TNP-T4 or T4 (data not shown).

As seen in Table IV, when T4 was injected into mice there was a resultant antibody response. Therefore, T4 is immunogenic *in vivo* and

does prime for secondary challenge *in vitro* with either T4 or TNP-T4 since culture supernates from the experiments summarized in Table III showed low levels of T4 neutralizing activity. Control, non-challenged cultures showed no such activity. Therefore, the lack of an anti-carrier response in spleen cell cultures from non-primed mice could be due to either too few phage-specific B cells or to lack or limiting numbers of auxiliary cells necessary for an anti-phage response. This *in vitro* deficiency, however, is associated only with the primary response.

Katz, Bluestein, Rouques, and Pierce (1971) and Bluestein and Pierce (1973, 1973a) used a similar *in vitro* model to study the anti-TNP response to TNP-ϕX174. However, no indication was made whether or not an anti-carrier antibody response was detected, although, as will be seen below, their system appears to differ considerably from that employed here with respect to its requirement for T cell help.

Lack of a Carrier Effect

The results of the experiments performed to see if the carrier effect was operative in the *in vitro* response to TNP-T4 were also unexpected. Based on the work of others investigating the anti-TNP response to TNP-ϕX174 (Katz et al., 1971; Bluestein and Pierce, 1973, 1973a) it seemed likely that at some point after *in vivo* priming with T4, the subsequent *in vitro* anti-TNP response to TNP-T4 would be enhanced and/or the concentration of TNP-T4 required for a maximum anti-TNP response would shift to a lower dose requirement.

In fact, early after *in vivo* carrier priming, the *in vitro* anti-hapten response to TNP-T4 was suppressed at day 4 and no enhancement was seen even 21 days after carrier priming (Table III). This observation may be explained by at least three different possibilities. First, it is possible that development of anti-T4 antibody in the mouse and subsequent release in tissue culture neutralized the TNP-T4 used as the *in vitro* immunogen. However, the experiments determining the neutralizing activity of the mouse serum at the time the spleens were removed for culturing (Table IV) showed an inverse relationship between suppression of the *in vitro* anti-TNP response and levels of serum T4 neutralizing activity (compare Tables III and IV). Furthermore, suppression of an immune response by antibody is generally determinant specific (Uhr and Möller, 1968; Brody, Walker, and Siskind, 1967) and in this case the antibody was anti-T4, but the response suppressed was anti-TNP. Therefore, the suppression of the *in vitro* response by carrier priming could not be explained in terms of anti-carrier antibody. The second possibility was that carrier priming caused migration of cells out of the spleen, resulting in a temporary decrease in carrier specific and/or hapten specific cells which would result in a decreased *in vitro* anti-hapten response. However, this possibility was not consistent with the observed lack of a carrier effect as late as 21 days after carrier priming and since others who have studied carrier priming *in vivo* have shown migration into, rather than out of the spleen shoftly after priming (Sprent, Miller, and Mitchell, 1971; Rowley, Gowans, Atkins, Ford, and Smith, 1972).

The third and most interesting possibility was that the anti-hapten response to TNP-T4 was independent of helper T cells and that carrier priming induced a population of short-lived suppressor T cells which were detected on day 4 after carrier priming. Evidence for the existence of suppressor T cells does exist. Baker, Barth, Stashak, and Amsbaugh (1970) found that depleting mice of T cells by treatment with antilymphocyte serum (ALS) produced a significant increase in the antibody response to type III pneumococcal polysaccharide (SSS-III). Baum, Lieberman, and Frenkel (1969) found that treatment of rats with ALS resulted in an increased antibody response to KLH. Kerbel and Eidinger (1971) found that mice treated with ALS alone or in conjunction with adult thymectomy gave an increased response to polyvinylpyrrolidone (PVP). SSS-III was shown to be thymus independent by Humphrey, Parrott, and East (1964) and by Davies, Carter, Leuchars, Wallis, and Dietrich (1970), and PVP was shown to be thymus independent by Andersson and Blomgren (1971).

It was decided to pursue the possibility that the anti-hapten response to TNP-T4 was T independent since it seemed necessary to characterize the immunogen further and since the suppressor effect was short lived. However, the induction of suppressor T cells by carrier priming is a possibility that should be investigated further, since it appears that the carrier involved (T4) does not play an active role in the induction of the anti-hapten response.

Is the Anti-Hapten Response to TNP-T4 Thymus Independent?

It is generally accepted that the immune response is considered to be T-cell independent if the response of congenitally hypothymic nude mice is not significantly different from control animals or if anti-θ plus complement treatment of cells does not interfere with their ability to respond (Greaves, Owen, and Raff, 1973). Using these criteria the anti-hapten response to TNP-T4 is at least partially T-cell independent, in that spleen cell cultures from hypothymic nude mice were able to respond to TNP-T4 (Table VII), and treatment of spleen cells with Br-anti-θ plus complement did not alter the response to TNP-T4 as significantly as the response to TNP-KLH (Table VI).

Additional evidence which is consistent with the response to TNP-T4 being T-cell independent is that the addition of excess free carrier to spleen cell cultures challenged with an optimum dose of TNP-T4 does not inhibit the anti-hapten response. This is in sharp contrast to the anti-TNP response to TNP-KLH, which is severely suppressed by excess free carrier (Table V). Bluestein and Pierce (1973) found that the anti-TNP response to TNP-ϕX174 was suppressed about 70% by the addition of 10-fold excess free carrier. It should also be recalled that in the TNP-ϕX174 system the carrier effect is operative so that carrier priming not only increases the anti-hapten response but also permits maximal *in vitro* responses to doses of immunogen which were suboptimal for spleen cell cultures from non-primed mice (Katz, Bluestein, Rouques, and Pierce, 1971; Bluestein and Pierce, 1973a). Thus, TNP-T4 and TNP-ϕX174 differ in at least three ways associated with helper or T cell function.

At present the concept of thymus independent antigens is controversial in two ways. First, there is the evidence that for some antigens usually considered T-cell independent, there exists a population of T cells which suppress the response to that antigen. Examples of this are discussed above on the antibody response to PVP and SSS-III. The possible role for amplifier T cells in B cell expression will be discussed below.

The second approach in which the idea of "T independent antigens" is being questioned is in regard to the $B_1$, $B_2$ concept. Gershon (1973) differs from most other investigators in that he suggests that instead of T independent "antigens" one should think in terms of T independent "antibodies". He suggests that there are classes of antigens which preferentially stimulate either $B_1$ cells or $B_2$ cells and would substitute this classification of thymus dependency or independency.

Krüger and Gershon (1972) distinguished, on the basis of stimulation of DNA synthesis in T cells, two classes of antigens which are considered thymus independent. One class, which includes POL, is a good stimulator of DNA synthesis in T cells. The second class, which includes SSS-III, PVP and LPS, is a poor stimulator. On this basis they suggested that the antibody response to the good T cell stimulators was not thymus independent, but that these antigens produced good thymus independent antibody responses ($B_1$ type response) which in the absence of T cell control obscured the lack of a thymus dependent component ($B_2$ type response).

Effect of *In Vitro* Addition of TNP-T4 and TNP-KLH

The observation that simultaneous challenge of spleen cell cultures with TNP-T4 and TNP-KLH resulted in an additive response to TNP (Table VIII) provides the greatest potential for future work based on this thesis because it offers the possibility of establishing the B cell characteristics which distinguish thymus dependency from thymus independency.

The first and simplest explanation for the additive response was that each carrier formed a part of the antigenic determinant which included the TNP group and that the hapten on different carriers selected different subpopulations of B cells. This concept has been termed the local environment hypothesis and was discarded by Mitchison (1971) on the basis of experiments in which spacer groups were placed between hapten and carrier. Nevertheless, the specificity of the antibody produced in cultures was investigated by hapten inhibition. The rationale was that if different hapten-carrier local environments stimulated different B cells the products might be distinguished by their ability to be inhibited by different concentrations of various haptenated amino acids. This was investigated by both hapten inhibition of plaque formation by the method of Claflin and Merchant (1972) and hapten inhibition of neutralization of trinitrophenylated coliphage R17 (TNP-R17) by culture supernates using the method of Barber and Rittenberg (1969). In both cases several different TNP-amino acid haptens were used (TNP-ε-aminocaproic acid, TNP-ε-amino lysine, TNP-glycine, and TNP-phenylalanine) at molar concentrations ranging from $10^{-4}$ to $10^{-10}$. However, the results of these experiments were

uninformative (data not shown); there was no difference in hapten inhibition of antibodies stimulated by either immunogen alone or by the mixture of immunogens. Therefore, even if distinct subpopulations recognizing different local environments were the explanation for the additive phenomenon, each immunogen was able to stimulate anti-TNP antibody with such a range of affinities that hapten inhibition could not reveal differences between them.

Although these negative experiments had to be viewed with caution, they were interpreted to indicate that more distinct differences than the local environment of the hapten existed between B cells stimulated by TNP-KLH and those stimulated by TNP-T4. It was decided to pursue the possibility that the results were an expression of the $B_1$-$B_2$ concept, that is, anti-TNP cells which responded to thymus independent antigens and cells which responded to thymus dependent antigens respectively (Gershon, 1973).

The findings that partial immune paralysis to TNP-KLH had no effect on the anti-hapten response to TNP-T4 (Table IX) was in accord with this concept since it was shown that the anti-TNP response to TNP-KLH is thymus dependent and that to TNP-T4 is thymus independent (Table VI).

Use of Additional Hapten-Carrier Complexes

It is obvious from the addition experiments in which TNP-T4 and TNP-KLH were used as *in vitro* immunogens (Tables VIII and IX) that either immunogen used alone was unable to allow for the expression

of the maximum number of anti-hapten plaque forming cells, hence the additive effect. The critical question was whether the additive effect would be observed when other hapten-carrier complexes were used and most importantly, would the additive effect correlate with the thymus dependence or independence of the immunogen used. As seen in Tables X and XI the additive effect was apparent only when the combinations of immunogens used correlated with the $B_1$, $B_2$ concept. No addition of the anti-hapten response was seen when either two T dependent immunogens (TNP-KLH and DNP-CGG) or two T independent immunogens (TNP-T4 and DNP-POL) were used together. However, any combination of a T dependent and a T independent immunogen did result in an additive effect. These findings provide the strongest evidence that addition results from stimulation of separate B cell populations, one responding to TNP on a thymus dependent carrier and one on a thymus independent carrier.

In some experiments (Tables X and XI) the resulting anti-hapten response was considerably greater than simple addition. The reason for this "super-additive" effect is not known, but may be speculated upon based on other data available from Dr. Rittenberg's laboratory.

Regulation By the Carrier Molecule

Recent experiments by M. Baltz (unpublished observations) in which KLH primed mice were given an additional booster injection of KLH two to four days prior to the spleen cells being placed in culture resulted in the anti-TNP response to TNP-T4 being significantly suppressed. Additionally, Rittenberg and Pratt (unpublished observations) found that TNP-KLH primed spleen cells can be induced to respond *in vitro* to

suboptimal doses of TNP-KLH if they are additionally challenged with low doses of free carrier. These results, along with the suppressive effect of carrier priming on the *in vitro* response to TNP-T4 (Table III) reflect the delicate balance between helper and inhibitory regulation of antibody responses by T cells. Further experiments will be required to determine if perhaps the same T cell factor can both suppress or enhance an immune response depending on whether the factor reacts with $B_1$ or $B_2$ cell types. An alternative possibility is that both cell types are susceptible to immune modulation by the same factor, but differ in their response to this factor on the basis of dose. That is to say, if T cells release large amounts of a factor, one cell type could be suppressed and the other cell type could be enhanced and vice versa. Such a bifunctional T cell factor(s) has recently been postulated by Katz, Paul, and Benacerraf (1973).

Recently, Zolla, Naor, and Tanapatchaiyapong (1974) have suggested that B cells may release factors which modulate the response of other B cells. The presence of either T cell factors and/or B cell factors in spleen cell cultures is a possible explanation for the observed super-additive effect seen in Tables X and XI.

# SUMMARY AND CONCLUSIONS

An experimental model was used to study the primary immune response to a chemically defined haptenic determinant, trinitrophenyl (TNP), coupled to bacteriophage T4 (TNP-T4). The response was initiated under controlled *in vitro* conditions with mouse spleen cells and was followed by determining with a plaque assay the number of anti-TNP-synthesizing cells arising on days 0-6 of culture.

The anti-hapten response to TNP-T4 was determined to be thymus (T cell) independent since a) the response of spleen cell cultures from congenitally hypothymic nude mice was not significantly different from the response of control animals; b) treatment of spleen cells with Br-anti-θ plus complement did not significantly suppress their ability to respond to TNP-T4, but abolished the response to a thymus dependent antigen by 99%; c) free carrier (T4) did not suppress the response to TNP-T4; and, d) priming of spleen donors *in vivo* with T4 before the spleen cells were challenged with TNP-T4 did not increase the response to TNP *in vitro*. Points c) and d) indicate that there is no carrier or helper cell requirement for an *in vitro* primary response to TNP-T4. The helper cell effect is a T cell function.

It was found that the *in vitro* responses to TNP in spleen cells primed *in vivo* to hemocyanin (KLH) and challenged *in vitro* simultaneously with both TNP-T4 and TNP-KLH, a T dependent immunogen, yielded responses that were greater than that obtained to either immunogen alone. In some cases the response was double that to either immunogen alone ("addition") and sometimes greater than double ("super addition").

These results were interpreted to suggest that each immunogen triggered separate subpopulations of hapten-specific B cells. Consistent with this was the finding that if the anti-hapten response to either TNP-immunogen alone was paralyzed by a high dose, the same cell suspension was still able to respond to an optimal dose of the other TNP-immunogen.

The additive phenomena were investigated further by simultaneous challenge of spleen cell cultures with various combinations of two T dependent immunogens, TNP-KLH and DNP-CGG, and two T independent immunogens, TNP-T4 and DNP-POL. Addition was not observed when cultures were challenged simultaneously with either two T dependent or two T independent immunogens. However, addition was observed when the double challenge consisted of one T dependent and one T independent immunogen. These results are consistent with the hypothesis that there may be two distinct subpopulations of B cells ($B_1$ and $B_2$) producing antibody to the same determinant, but which respond to thymus independent or to thymus dependent antigens respectively.

The additive phenomenon is interesting in itself in that it raises a cautionary note in estimating population sizes among antibody-forming cells since the response to a given determinant may be limited or determined by the choice of carrier.

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