REGULATION BY ANTIGEN OF THE IMMUNE RESPONSE *IN VITRO*

by

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INTRODUCTION

How Does Antigen Control Immunological Activity?

Antigen or immunogen can be defined as that which induces an immune response, that is, induces the production of immunoglobulins or reproduction of cells which react specifically against portions of the immunogen. As stimulus, antigen is central to understanding immune phenomena.

Antigenic molecules may be divided into two functional groups: a) that portion which bestows immunogenicity, which has been termed carrier function, and b) small regions recognized by cell receptors or immunoglobulins, the antigenic determinants. Since determinant regions on natural antigens are integrated into the immunogen it is difficult to isolate and characterize them. To overcome this difficulty many immunologists have employed haptens: added artifical determinants of a chemically defined nature. The term hapten was introduced by Landsteiner (1919) 52 years ago to describe an alcohol extract of horse kidney which was not itself immunogenic but when mixed with pig serum induced antibody which would react with the alcohol-extracted material itself (Landsteiner and Simms, 1921). The molecule required with the hapten for sensitization was termed the schlepper or carrier. Haptens have proved valuable in studies on the specificity of immune responses (Landsteiner, 1962), and on the interaction between antigens and antibodies (Pauling, Campbell, Pressman, 1941).

The separate roles of carrier and antigenic determinants or haptens in inducing, controlling, or preventing immune responses are poorly understood (Plescia, 1969). Understanding will require a map of the

"antigenic odyssey" from initial *in vivo* introduction to activities occurring several months following immunization.

Persistence of Antigen and Duration of Immunogenicity.

Antigen can enter an organism by various routes; most often it is injected intraperitoneally, subcutaneously, or intravenously. Antigen can be retained, removed or altered by various serum or cellular constituents. Substances such as pneumococcal polysaccharide can persist throughout an animal's lifetime (Felton, Prescott, Kauffmann, Ottinger, 1955). Purified pneumococcal polysaccharide is poorly antigenic in rabbits and is maintained in the circulation for a long time (Avery, Goebel, 1933). However if rabbits are immunized with whole pneumococci a specific rapid clearance of polysaccharide from the circulation occurs (Downie, 1937). Landsteiner defined a hapten as a compound requiring a helper substance to induce antibody specific for that compound. Thus the pneumococcal polysaccharide is a macromolecular hapten. Secondly, the study indicates that antibody enhances clearance of antigen from the circulation, confirmed by later studies with diphtheria toxin (Glenny, Hopkins, 1924) and with radioactively labeled bovine gamma globulin (Talmage, Dixon, Bukantz, Dammin, 1951). Clearance from the circulation occurs in three stages: a) a rapid loss due to equilibrium with extravascular spaces, b) a slow loss due to normal catabolic processes, and c) a more rapid loss due to the appearance of antibody and subsequent clearance of antibody-antigen complexes (Dixon, Talmage, 1951).

Particulate antigens are removed rapidly from circulation via phagocytosis in reticuloendothelial tissue (Ingraham, 1955). Some soluble antigens such as keyhole limpet hemocyanin are removed from the circulation

of normal rabbits within six hours after injection (Garvey, Campbell, 1954). Other smaller soluble antigens such as bovine albumin may remain in circulation in normal rabbits for longer periods of time, 10% at nine days following injection (Knox, Endicott, 1950).

Soluble antigens given to immune animals form insoluble antigen-antibody complexes which localize mainly in the liver. As antibody titers rise the lung also becomes an important antigen depot (Garvey, Campbell, 1954). Ingraham (1951) observed small amounts of keyhole limpet hemocyanin (KLH) persisting in mouse liver and spleen for 200 days. Garvey and Campbell (1957) detected ^{35}S-sulfonilate labeled KLH in rabbits up to 310 days after last antigen injection. They extrapolated the data to estimate that at three years following antigen injection there were 2,000 molecules of ^{35}S-KLH per liver cell (Garvey, Campbell, 1958).

The presence of residual antigen does not insure continued immunogenic potential. However, several investigators found that retained antigen can be immunogenic. Mitchison (1969) reported spontaneous production of antibody in mice given incubated spleen cells from donor mice sensitized 200 days previously with haptenated chicken gamma globulin. He concluded that the *in vitro* incubation prior to cell transfer unmasked antigen bound to cell surfaces thereby renewing its immunogenic activity. Britton, Wepsic, and Möller (1968) investigated the persistence of antigenic action with bacterial lipopolysaccharide (LPS) and sheep red blood cells (SRBC) *in vivo*. Mice were immunized with LPS and/or SRBC. At various periods later they were given 900 R whole body radiation to destroy their own immunocompetent cells. These animals were then injected with 4×10^7 normal lymphocytes. The normal donor lymphocytes were stimulated by antigen remaining in the recipients even when the donor cells were

injected 45 days after the recipients had been primed with LPS. Donor cells were stimulated by SRBC only if they were injected within 10 days after priming. This not only indicated that immunogenicity can persist but that the time of persistence depends on the antigen. In a second set of experiments following the responses of mice given LPS, the number of the animals own cells producing anti-LPS fluctuated widely in a cyclic pattern peaking at 10-day intervals throughout a 46-day period (Britton, Möller, 1968). This suggested that as antibody was formed it masked antigenic determinants preventing further stimulation; however, with catabolism of the antibody, antigen was unmasked and again stimulated lymphocytes.

Passively injected antibody can prevent antigen for which it is specific from inducing an immune response (to be detailed later). Wigzell (1966) has shown blocking by passive antibody added 40 days after immunization. Thus antigen not only persists in animals for long periods of time, but maintains its immunogenicity, depending on the antigen and its subsequent *in vivo* modification. An important depot and source of antigen modification may be the macrophage.

Macrophages and Antigen.

Nossal, Abbot, and Mitchell (1968) followed radioactive iodine labeled flagella antigen in rats using electron microscope autoradiography. Antigen was found to be located largely in the medullary macrophage of the lymph nodes. This antigen was rapidly fused into "phagolysomes" and did not appear to be released subsequently. The lymph node cortices also contained antigen, particularly in nodes of previously immunized animals.

However here it remained extracellular, associated with fine membranous processes between lymphocytes, and the number of macrophages present was not related to the amount of retained antigen.

Askonas, Auzins, and Unanue (1968) reported macrophages digest 90% of the KLH antigen presented to them. However, small amounts persist bound to membranes; this antigen is not phagocytosed or extensively degraded. Macrophages with surface antigen, when introduced into normal animals, induce anti-KLH antibody. Removal of surface-membrane bound KLH with trypsin prevents immunization even though most of the KLH or its digested by-products remain within the cells (Unanue, Cerottini, 1970). It has been popular periodically to consider that macrophages may process antigen and add a ribonucleic acid component essential for immunogenicity (Garvey, Campbell, 1957) (Fishman, 1961). However, Roelants and Goodman (1969) showed that binding between antigen and RNA was nonspecific in all respects. In light of the above work and that of Cohn and Ehrenreich (1967) showing that once injected antigen is degraded into small non-immunogenic fragments, it seems unlikely that macrophage digestion of antigen is essential for immunogenicity. This does not rule out possible *in vivo* functions such as sequestering undigested antigen on their surfaces. Macrophages do appear to increase immunogenicity of some particulate antigens. Lysis of macrophages during inflammatory processes could release soluble fragments of increased immunogenicity or macrophages may, by removal of excess antigen, prevent tolerance induction (Burnet, 1959). Pierce and Benacerraf (1969) used particulate SRBC antigen and found macrophage-antigen interaction to be critical to primary *in vitro* responses. Another particulate antigen *Brucella abortus* requires macrophages to obtain large immune responses (Theis, Thorbecke, 1970). Palmer

(1970) and Shortman, Diener, Russell, and Armstrong (1970) found that by adding macrophages to macrophage-depleted cultures of lymphocytes the response to whole SRBC increased 100-fold, responses to SRBC membranes increased 4-5 fold, but responses to soluble SRBC antigen or soluble polymerized flagellin were not enhanced. Enhancement of SRBC response with macrophages is likely nonspecific since macrophages from tolerant animals can enhance the response of normal lymphocytes but normal macrophages are unable to evoke a response with tolerant lymphocytes (Mitchison, 1969). However, Boyden and Sorkin (1961) described cytophilic antibody that can bind specifically to macrophages (Lay, Nussenzweig, 1969). This surface antibody, not synthesized by the macrophage, may add specificity to macrophages during formation of antigen depots. Macrophages can also inhibit the immune response (Parkhouse, Dutton, 1966). Thus macrophages can manipulate the concentration and/or solubility of antigen. Both of these factors are important to the immune response (Dresser, 1962) (Mitchison, 1964).

Antigen Recognition.

Paul Ehrlich (1900) proposed that cells expose specific chemical receptors on their surfaces which are overproduced and enter the circulation as antibodies when toxins react with them on the cell surface. Knowledge of the existence, nature and location of such receptors is essential to understanding antigenic action. Modification of Ehrlich's model resulted in the "clonal selection theory" which current experimental work appears to be substantiating (Jerne, 1955)(Talmage, 1957) (Burnet, 1959).

Blastogenesis and Allelic Exclusion.

Lymphocytes undergo blastogenesis recognized by mitotic transformations

resulting in reorganized and enlarged cytoplasm. Sell and Gell (1965) induced blastogenesis in rabbit lymphocytes using antiserum directed against antigenic determinants present on immunoglobulins produced by those cells. Rabbit lymphocytes with passively adsorbed immunoglobulins of an allotype differing from that of the lymphocytes, did not undergo blastogenesis when exposed to anti-allotype serum against the adsorbed antibody. This demonstrated that specific antisera stimulate blastogenesis by reacting with surface immunoglobulins synthesized by the lymphocyte. Two-fold or greater increases in blastogenesis occur when separate antisera, each with specificity for a different determinant, are simultaneously incubated with rabbit lymphocytes heterozygous for the two alleles (Gell, Sell, 1965). This finding was interpreted to indicate that separate cells were stimulated by each antiserum suggesting each cell produces and exposes only one immunoglobulin allele. This finding has been termed "allelic exclusion" and has been substantiated by staining rabbit lymphoid tissue with fluorescent anti-allotype antibody (Pernis, Chiappino, Kelus, Gell, 1965).

Allelic Suppression.

Anti-Allotype serum may also suppress cells capable of producing antibodies of a given allotype. Dray (1962) demonstrated this effect in newborn rabbits delivered by mothers possessing anti-allotypic activity. Mage and Dray (1967) reported suppression of paternal allotype up to three years in offspring of mothers immunized with paternal immunoglobulins.

A smaller but similar effect was demonstrated in mice (Herzenberg, Herzenberg, Goodlin, Rivera, 1967). It was suggested that since animals escape from allotype suppression anti-allotype serum may block surface receptor molecules of paternal allotype thus preventing stimulation by

antigen when it is administered*. These experiments support the idea that cells have surface receptors prior to antigen contact and that these receptors are immunoglobulins with only one allotype per cell.

It is not clear yet which classes of immunoglobulins are present as surface receptors. Mäkelä and Kostiainen (1967) reported that some antigen binding characteristics distinguishing humoral IgG and IgM antibodies corresponded to similar characteristics of those cell receptors inducing IgG and IgM upon stimulation. Recently conflicting reports have appeared as to the presence of different immunoglobulin classes on lymphocyte surfaces (Rabellino, Colon, Grey, Unanue, 1971) (Pernis, Forni, Amante, 1970). Kincade and Cooper (1971) found that if, at a period prior to hatching, anti-chicken IgM serum was administered to chicken embryos the appearance of both IgM and IgG protein was inhibited in hatched chicks. Secondly, they found a large percentage of individual cells in the bursa, several days after hatching, which stained for the presence of both IgM and IgG immunoglobulins. Their results suggest that IgG cells arise from precursor IgM cells. A second line of evidence suggesting that IgG and IgM producing cells can arise from one precursor cell is the finding of a patient with serum possessing two paraproteins, an IgM and an IgG protein with apparently identical L chains and thus far identical variable regions on their H chains (Wang, Wilson, Hopper, Fudenberg, 1970). These findings may be due to: a) a step in the differentiation of immunocytes with a common cell giving rise to two separate cells, one producing IgG and one producing IgM, but both possessing common antibody reactive sites and L chains, or b) a switching over from IgM to IgG synthesis by single cells following antigenic stimulation.

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*L. Herzenberg, Personal communication. 1969.

Lymphocyte Surface Reactivity to Antigen.

Cells potentially responsive to a determinant can bind that determinant prior to antibody induction. Chemically reactive haptens may affinity label surface receptors specific for those haptens (Plotz, 1969) (Segal, Globerson, Feldman, Haimovich, Givol, 1969). This hapten-oriented reaction blocks immune responses to subsequent challenge with similar haptens on protein carriers, but did not prevent responses to unrelated antigens. Non-covalently bound haptens can also block immune responses but only at 10-1000X higher molar concentrations (Paul, Siskind, Benacerraf, 1968), (Brownstone, Mitchison, Pitt-Rivers, 1966). Lower concentrations of hapten on heterologous carriers are also effective and can produce immune tolerance to the hapten (Borel, 1971) (Havas, 1969) (Rittenberg and Bullock, 1970).

Normal spleen cell populations have small subpopulations which bind protein antigens at 4° centigrade (Naor, Sulitzeau, 1967) in the presence of sodium azide which prevents phagocytosis (Byrt, Ada, 1969). This binding of protein by lymphocytes was inhibited 75-94 percent with anti-immunoglobulin serum, suggesting that antigen specific immunoglobulins are responsible for the binding.

Wigzell and Andersson (1969) coated glass beads with haptenated albumin or albumin to prepare columns for separating cells. Wigzell and Mäkelä (1970) filtered lymphocytes through these columns testing for specific binding of cells to haptenic or protein determinants on the glass beads. Cells immunologically responsive to a hapten were selectively removed by columns possessing that hapten. Binding was prevented by excess free hapten. In addition anti-allotype antibody prevented cells capable of synthesizing anti-hapten antibody of that allotype from binding

to the column, again suggesting that cells bind via immunoglobulin receptors on their surface which are determinant specific. Even after the induction of antibody synthesis most studies indicate that individual cells continue to produce antibody of only one class and specificity (Nossal, Lederberg, 1958) (Nordin, Cosenza, Sell, 1970). Possible switching by a single cell from IgM to IgG synthesis may be an exception brought about by loss of regulatory processes such as appears to have occurred in some myelomatous individuals (see Wang, Wilson, Hopper, Fudenberg, 1970, above). However, this topic is beyond the scope of this review.

In summary, the data reviewed support the proposal that each immunologically reactive lymphocyte possesses surface receptors resembling a single allotype and class of antibody. The receptor has a distinct ligand specificity which when properly stimulated induces the cell to secrete an immunoglobulin with similar or identical structure and specificity.

Cell-Cell Interaction.

Thus far it can be seen that stimulatory antigen can persist *in vivo* presumably between the free and cell-bound states. The immune response is further complicated by antigen induced interaction between cells. Miller, (1962) demonstrated that neonatal thymectomy removes the capacity for graft rejection (cellular immunity) and lowers the response to *Salmonella*, A antigens (humoral immunity). Glick, Chang, and Jaap (1956) reported that bursectomy in chickens induced agammaglobulinemia. Bursectomy does not affect graft rejection but thymectomy prevents graft rejection (Aspinall, Meyer, Graetzer, Wolfe, 1963). This indicates that bursa derived cells in chickens and presumably cells from a mammalian equivalent of the bursa produce immunoglobulins. The human counterpart of this

dichotomy in tissue origin of humoral and cellular immunity has been emphasized by Cooper, Peterson, and Good (1965) who believe it is reflected in human immune deficiency diseases. They suggested the existence of bursa-like organs in the gut lymphatics of mammals.

Claman, Chaperson, and Triplett (1966) demonstrated that local areas of antibody formation were detectable within the spleen of irradiated mice given immune thymus cells and/or bone marrow cells. They reported that the number of areas of antibody formation in mice given both thymus and bone marrow cells was 6-40 times the number produced with equal amounts of either cell type alone. Klinman (1969) has shown that a single such area of local antibody activity within the spleen represents homologous antibody secreted by a clone of cells apparently arising from a single precursor cell.

Mitchell and Miller (1968) studied sublethally irradiated mice which were unresponsive to antigen unless injected with additional normal spleen cells. They demonstrated using anti-H2 (histocompatibility) antiserum and chromosomal markers that the cells which produced antibody were host cells. It was further shown that the required helper cells in the population of injected spleen cells were of thymic origin (Mitchell, Miller, 1968). This immune enhancement due to synergism between thymus derived (T) cells and bursa derived (or the bursa equivalent) B cells may or may not be essential to a normal immune response. A major argument against the essential nature of this interaction are immune deficiency diseases of man such as DiGeorge's syndrome or thymic aplasia (DiGeorge, 1965). The syndrome is characterized by the absence of detectable T cell development, i.e. cellular immunity, whereas B cells are present since normal or low humoral responses occur. Therefore T cells may not be essential for an

immune response but may act as helper cells by increasing the immunogenic effectiveness of low antigen concentrations or by increasing the mitotic capacity of B cells since a) higher concentrations of antigen can circumvent the T cell requirement during antibody formation (Sinclair, Elliot, 1968) and b) some polysaccharide antigens appear to lack a carrier function (Paul, Siskind, Benacerraf, Ovary, 1967). When T cells are required for an immune response it is generally believed that they are bridged to B cells through the carrier function (Rajewsky, Schirrmacher, Nase, Jerne, 1969). This polysaccharide induces antibody without concomitant cell division and further memory cell development (Paul, Siskind, Benacerraf, Ovary, 1967).

Lymphocyte surfaces possess antigenic determinants which distinguish T cells from B cells. Antiserum to these determinants, plus complement can remove either cell type from a population of lymphocytes without damaging the immune capacity of the other cells (Raff, 1970) (Schlesinger, 1970) (Schimpl, Wecker, 1970) (Raff, Nase, Mitchison, 1971) (Chan, Mishell, Mitchell, 1970).

Chiller, Habicht, and Weigle (1970) injected thymus and bone marrow cells from normal and human γ-globulin (HGG) tolerized animals into irradiated syngeneic hosts, followed by challenge with HGG. Using this model they showed that both cell types were required to give a large immune response and furthermore tolerance of either cell type prevented the response. This indicates either cell type can be tolerized specifically and that each cell type recognizes antigen.

Carrier Function.

Rajewsky, Schirrmacher, Nase and Jerne (1969) proposed that carrier function of antigen is partially the bridging of T cells to B cells via

separate determinants recognized by each cell. Their experiments consisted of priming rabbits with haptenated bovine albumin (BSA) and challenging them with hapten-BSA or hapten-HGG. No secondary response occurred upon challenge with hapten-heterologous (HGG) carrier, but good responses were obtained with hapten or the homologous carrier (BSA). Good response could be obtained, however, with hapten on the heterologous carrier if, prior to secondary hapten challenge, the animals were sensitized to the heterologous carrier. Presumably under conditions used the animals lacked sufficient T cells to mount a large response against the hapten on a new carrier. Prior priming to the heterologous carrier induced a larger clone of carrier (HGG) specific T cells, enabling a measurable response. Similar results were obtained by Paul, Katz, Goidl, and Benacerraf, (1971) using adoptive transfer techniques, that is transferring cells from carrier primed animals to lethally irradiated syngeneic recipients and challenging with hapten-carrier. Successful challenges with haptens on heterologous carriers have been reported also without preimmunization by carriers (Rittenberg, Campbell, 1968) (Steiner, Eisen, 1967) (Paul, Siskind, Benacerraf, Ovary, 1967) (Brownstone, Mitchison, Pitt-Rivers, 1966). Such results again suggest either that anti-carrier cells are not an absolute requirement or that a population of T cells develop which recognizes hapten. Ability to recall the anti-hapten responses with heterologous carriers seems to parallel the development of cells with higher "affinity" for the hapten (Paul, Siskind, Benacerraf, Ovary, 1967). This suggests a possible second carrier function, that is, increasing the binding strength of antigen to lymphocytes through the multivalency of haptenated carriers or through the nonspecific weak secondary binding forces inherent in large molecules.

Preimmunization with carrier can either enhance (Rajewsky, Schirrmacher, Nase, Jerne, 1969) (Paul, Goidl, Benacerraf, 1970) or inhibit anti-hapten responses subsequent to challenge with hapten on that carrier (Levine, 1967) (Rittenberg, Amkraut, 1966) (Dubert, 1956)(Ashley, Ovary, 1965). These differences may be due to levels of hapten or carrier antibody or the ratio of cellular to humoral response at the time of secondary challenge.

The above reports suggest that additional determinants on a molecule may increase its antigenicity, if the animal recognizes them as foreign. Green, Paul and Benacerraf (1966) worked with strains of guinea pigs which respond poorly to the synthetic macromolecule dinitrophenyl-poly-L-lysine (DNP-PLL). However, the animals produce anti-DNP antibody if DNP-PLL is presented ionicly bound to methylated bovine albumin (new determinants) but not when bound to methylated guinea pig albumin (no new antigenic determinants since guinea pigs are tolerant to this protein). The foreign bovine albumin determinants may permit additional helper cell interactions. Here again the requirement for auxillary carrier activity may be overcome with higher concentrations of DNP-PLL (Stone, Goode, 1970).

An additional carrier is also required in the immune response to lactic acid dehydrogenase. This enzyme is composed of four peptides of type A and/or type B. Some rabbits respond immunologically to A_4 enzyme but not to B_4 enzyme. However, A_2B_2 hybrid enzyme induces anti-B peptide antibody. The A_4 enzyme does not induce anti-B antibody (Rajewsky, Rottländer, Peltre, Müller, 1967) (Rajewsky, Rottländer, 1967).

Added determinants do not always increase the immune response to

preexisting determinants and can compete with previously immunogenic determinants by directing antigen to new cells, producing a response lower than that obtained without the additional determinant (Amkraut, Garvey, Campbell, 1966).

In summary cell-cell interaction between helper T cells and potential antibody producer B cells can enhance the immune response by an as yet unknown mechanism. This cell interaction is likely due to both cells reacting specifically with antigenic determinants via surface receptors which resemble immunoglobulins. Antigens with multivalent and multiple types of determinants can react with and possibly bridge an increased number and variety of cells, evoking a detectable immune response. The helper cell's effect can be duplicated by transferred allogeneic cells participating in a graft vs. host reaction suggesting that the helper cell's function is unidirectional, i.e. from helper to producer cell and not vice versa (Katz, Paul, Goidl, Benacerraf, 1971).

Antigen-Induced Increases in Cellular Avidity.

Lymphocytes have a variable halflife depending on the differentiation they undergo. This may range from several days to several years (Gowens, McGregor, 1965) (Caffrey, Rieke, Everett, 1962) (Ottesen, 1954). New lymphocytes arise with differing frequency. Chiller, Weigle, and Habicht (1971) obtained evidence that new B cells arise much more frequently than T cells. Therefore antigens retained *in vivo* for long periods of time can continue to stimulate both cells resulting from initial sensitization and new cells arising during the period of antigenic effectiveness. If antigen is gradually lost due to catabolism or blocked by developing antibody; less antigen will be available to stimulate cells. The resulting

"competition" between cells for antigen should allow stimulation of only those cells with high avidity receptors for antigen. Due to proposed similarities between cell receptors and antibody product, this should result in a gradual shift in the specific immunocompetent population until late in immunization; it would consist mainly of cells bearing high avidity receptors and synthesizing high affinity antibody. This hypothesis was presented by Eisen (1966) and it is in accord with the following experimental observations. Antibodies with increased "avidity" for antigen appear following immunization (Jerne, 1951). Ligand affinity of anti-hapten antibody can rise several orders of magnitude in serum samples taken a few weeks apart (Eisen, Siskind, 1964). High affinity antibody appears earlier when smaller antigen doses are used for immunization and the increase results from synthesis of new molecules (Steiner, Eisen, 1967). Similarly thymidine incorporation *in vitro* indicates that animals sensitized by low antigen doses yield cells more responsive to low dose challenge than do animals sensitized by high doses, although in these studies the cells were not shown to be making antibody (Paul, Siskind, Benacerraf, 1968). Thus not only is the amount of retained antigen changing with time but presumably the avidity of the cells and the affinity of the antibody as well.

Antigen-Antibody Interaction.

There is strong evidence that the complexing of antigenic determinants with antibody can drastically alter the immunogenic potential of immunogen. Antibody passively added during antigenic challenge can greatly suppress immunization (Glenny, Südmerson, 1921) (Finkelstein, Uhr, 1964) (Möller, Wigzell, 1965) (Henry, Jerne, 1968). This suppression is specific for the

determinants against which the passive antibody reacts, but the same antibody can enhance the response to other determinants on the same molecule with which the antibody does not react (Brody, Walker, Siskind, 1967) (Pearlman, 1967) (McBride, Schierman, 1970) (Pincus, Nussenzweig, 1969).

There is a large population of lymphocytes with receptors for complement-activated antigen-antibody complexes (Bianco, Patrick, Nussenzweig, 1970). Since antigen-antibody complexes can induce division in normal and immune cell populations (Möller, 1969), these complexes may be activating receptors on lymphocytes. This may produce enhancement of antigenicity by antibody as previously discussed or these complexes may augment the inflammatory response. Very low or very high levels of hapten per antigen molecule can result in low anti-hapten responses (Mäkelä, 1970) (Rittenberg, Amkraut, 1966). Since the extent of hapten substitution can affect immunogenicity, altering the number of exposed determinant groups with antibody could increase or decrease the response to a particular antigen. It has been proposed that for an immunogen to induce immunity some determinants must be complexed with small amounts of antibody (Jerne, 1955) present due to leakage of receptors into the plasma (McBride, Schierman, 1970). Other workers have shown an enhancing effect by the passive transfer of anti-carrier cells to recipients immunized with hapten on that carrier. This enhancement did not occur when anti-carrier antibody was substituted for cells (Katz, Paul, Goidl, Benacerraf, 1970). It can be concluded that antigen-antibody interaction is a complicating variable during immunization and the high levels of antibody possible in the *in vivo* milieu present a formidable block to the study of antigenic action.

Immune Tolerance.

When an animal makes antibody against his own tissue severe disease or death can result (Doniach, Roitt, 1957) (Billingham, Brent, 1957). Tissue can be removed prior to development of tissue specific antigens and "stored" *in vivo* in another animal where it undergoes development of its normal antigen. The original tissue will be treated as foreign when later placed back into the original animal even though it could be shown that it did not acquire abnormal antigens while residing in the "storage" animal (Triplett, 1962). This demonstrates the ability of animals to respond immunologically to their own tissues. Thus the normal inability of an animal to respond to its own antigens is not genetically determined but due to somatic process which is abrogated in the absence of antigen. Immunological tolerance, paralysis, or immune unresponsiveness can be defined as a specific immunological non-reactivity to certain "antigenic" determinants. A primary source of insight into the phenomenon occurred when Owen (1945) demonstrated that vascular anastomosis *in utero* between non-identical bovine twins resulted in reciprocal tolerance after birth. The calves developed into adult erythrocyte chimeras although by breeding it was possible to show that genetically the twins were distinct. This finding led Burnet and Fenner (1949) to propose that early exposure to antigens resulted in tolerance but later exposure produced immunity. Billingham, Brent and Medawar (1953) demonstrated that the injection of antigen into embryos could result in tolerance. However, it has since been shown that even unborn animals can respond to small doses of antigen (Silverstein, Uhr, Kraner, Lukes, 1963). Thus age *per se* does not explain the induction of tolerance. Injection of additional lymphocytes

into newborn animals makes the induction of tolerance difficult (Cohen, Thorbecke, 1963). This suggests that the low number of immune cells in early life may explain the ease of tolerance induction in newborns. This is supported by data showing that immunosuppressive drugs make adult animals more susceptible to tolerance induction, presumably by lowering the number of immunocompetent cells to newborn levels (Schwartz, Dameshek, 1963). High doses of antigen can induce tolerance in adult animals both *in vivo* and *in vitro* (Dixon, Maurer, 1955) (Weigle, 1966) (Sercarz, Coons, 1963) (Wells, Osborne, 1911) (Diener, Armstrong, 1969) (Mäkelä, Mitchison, 1965). Diener, Shortman and Russell (1970) conclude that "the only parameter which decides whether immunity or tolerance to POL (polymerized flagellar antigen) is induced *in vitro* is a quantitative one, the antigen dose".

Dresser (1962) has demonstrated that bovine γ-globulin contains two fractions, a particulate immunogenic fraction and a soluble tolerogenic fraction. Mitchison (1954) found that very small subimmunogenic amounts of bovine serum albumin (BSA) induced tolerance. It was reasoned by Thorbecke and Benacerraf (1965) that this may be due to diluting out of the immunogenic particulate form, leaving the tolerogenic material analogous to Dresser's finding with BGG, since the particulate form represents a small portion of BSA preparations. One reason given for the increased immunogenicity of particulate material was enhanced macrophage processing (Thorbecke, Benacerraf, 1965). Another reasonable explanation is some proteins in their soluble form have a low number of foreign-appearing determinants. This low valency prevents effective cell bridging and masks receptors to subsequently administered multivalent particulate protein complexes.

Two types of tolerance have been reported, receptor blocking and cell exhaustion. As has been discussed, receptors can be covered with anti-γ-globulin antibody, blocked by haptens on non-foreign proteins (Borel, 1971) (Havas, 1969) or blocked by very high concentrations of simple haptens (Paul, Siskind, Benacerraf, 1968) (Brownstone, Mitchison, Pitt-Rivers, 1966). The combined effects of determinant valency and molar concentration may decide whether the tolerance is a reversible type reported by Byers and Sercarz (1970) or a permanent form of tolerance due to an exhaustion of immune cells (Byers, Sercarz, 1968). Cells can be induced to form antibody without concomitant cell division, indicating that induction of antibody and multiplication of cells may be separable events (Sterzl, 1968) (Paul, Siskind, Benacerraf, Ovary, 1967). Other reports have concluded that mitosis is required for antibody induction (Dutton, Mishell, 1967a). The process leading to tolerance is frequently preceded by low levels of antibody and small numbers of detectable antibody-synthesizing cells (Kerman, Segre, 1970) (Glenny, Hopkins, 1924). Recently Segal, Globerson and Feldman (1969) reported that only one cycle of cell division is required to induce antibody synthesis, possibly explaining the above conflicts. T cell-B cell interaction may be required for extensive immune cell mitosis. Conditions capable of blocking interaction but simultaneously stimulating receptors such as may occur with excess antigen might turn on antibody synthesis and cell maturation to end cell stages without concomitant cell division. This would exhaust the immune population while producing a small immune response. This is however speculation and it is still unknown how excess antigen affects tolerance. It is important to know the separate roles, if any, of carrier and individual antigenic determinants in this process and how the

concentration of immunogen and the concentration of determinants determine whether paralysis or immunity develops.

The *In Vitro* Model.

In vitro studies of immune cells have shown increased DNA synthesis, antibody synthesis and cell proliferation resulting from *in vitro* antigen exposure (Dutton, 1967). Quantitative studies of the *in vitro* cellular immune response have been limited to the use of complex antigens (Mishell, Dutton, 1967b) (Marbrook, 1967) (Armstrong, Diener, 1969). *In vitro* anti-hapten experiments utilized either assays of antibody in culture fluids (Steiner, Eisen, 1967) which did not permit enumeration of individual responding cells, or tests of single cells obtained by micro-manipulation (Mäkelä, 1967). Only recently have kinetics of an anti-hapten response *in vitro* utilizing the plaque technique been reported (Bullock, Rittenberg, 1970a,b,c) (Rittenberg, Bullock, 1971) (Kettman, Dutton, 1970) (Katz, Bluestein, Rouques, Pierce, 1971). All of these studies have utilized the same hapten and detection system (Rittenberg, Pratt, 1969). The use of haptens to study immune cell kinetics *in vitro* offers two main advantages for detecting the induction of immunity and the induction of tolerance, a) stimulating the anamnestic response *in vitro* insures exposure of the entire immunocompetent population to a known concentration of antigen permitting a comparison of dose effects; b) detecting only cells synthesizing anti-TNP avoids variability resulting from additive responses to multiple, uncharacterized determinants on complex immunogens.

The thesis problem was to develop an anti-hapten *in vitro* culture model and to use this model to probe the roles of carrier and hapten in regulating immune cells.

MATERIALS AND METHODS

Animals.

Adult Balb/c female mice were used. They were caged in groups of eight with free access to food and water. Mice were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. and first immunized when 2-9 months old.

Glassware.

Glassware was washed in 7X (Linbro Chemical Co., Inc., New Haven, Conn.) rinsed in tap water and then rinsed five times with double distilled water. Pasteur pipets were presoaked in 0.85% saline for one hour at 37°C, rinsed in distilled water, plugged and autoclaved. Polystyrene 35 X 10 mm culture dishes, 60 X 15 mm culture dishes, and 100 X 15 mm quadrant petri plates were purchased from Falcon Plastics, Los Angeles, Calif.

Red Blood Cells.

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

Cell Preparation and Assay Medium. (Mishell, Dutton, 1967)

Eagle's powdered minimal essential medium (MEM-P) for suspension cultures, without glutamine, Cat. no. F-14, from Grand Island Biological Co., Grand Island, N. Y. was dissolved in double distilled water.

Solutions, 2X or IX, were prepared with 2.14 or 1.07 gm/100 ml respectively. The pH was adjusted to 7.2 with 1 N sodium hydroxide.

Culture Medium. (Mishell, Dutton, 1967)

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

1. Minimal Essential Medium (Eagle) for suspension cultures, without L-glutamine, cat. no. 12-126, stored at 4°C (43.0 ml).
2. L-glutamine 200mM solution, cat. no. 17-605, stored frozen (0.5 ml).
3. Sodium pyruvate 100mM solution, cat. no. 13-115, stored at 4°C (0.5 ml).
4. Non-essential amino acids mixture-supplement for minimal essential medium (Eagle) 100X concentrate, cat. no. 13-114, stored at 4°C (0.5 ml).
5. Penicillin-streptomycin mixture (5,000 units/ml), cat. no. 17-603F, stored frozen (0.5 ml).
6. Fetal calf serum, stored frozen, obtained from either Colorado Serum Co., Denver, Colo., cat. no. CS-1190, or Reheis Chemical Co., Chicago, Ill., cat. no. 268 (5.0 ml).

Culture medium was used on the day prepared.

Nutritional Cocktail. (Mishell, Dutton, 1967)

Ingredients for 227 ml of nutritional cocktail:

1. Autoclaved MEM-P, IX solution (140.0 ml).
2. Essential amino acids -- amino acid mixture for minimal essential medium (Eagle) 50X concentrate, cat. no. 13-606, Microbiological Associates, Albany, Calif. (20.0 ml).

3. Non-essential amino acids, as used in culture medium (10.0 ml),same conc.
4. Dextrose, reagent grade, cat. no. 72623, Merck and Co., Inc., Rahway, N.J., 200 mg/ml solution was prepared in double distilled water, sterilized by filtration through an 0.45μ Millipore membrane, and stored frozen (10.0 ml).
5. L-glutamine, as used in culture medium (10.0 ml), same conc.
6. Penicillin-streptomycin mixture, as used in culture medium (3.6 ml).

The above cocktail was adjusted to pH 7.2 with sterile 1 N sodium hydroxide.

7. Sodium bicarbonate solution, 7.5%, cat. no. 17-603, Microbiological Associates, Albany, Calif., was added to the adjusted pH 7.2 cocktail (30.0 ml).

The completed cocktail was aliquanted and frozen to be thawed on the day used.

Hapten Reagent. (Rittenberg, Amkraut, 1966)

Picryl-sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid (TNBS) from Nutritional Biochemicals Corp., Cleveland, Ohio, was recrystallized once from 1 N hydrochloric acid after dissolving 10 gm. TNBS in 20 ml of hot hydrochloric acid.

Radiochemicals.

L-Valine ^{14}C (U), specific activity 219 mCi/mM, 0.0134 mg/2.5 ml sterile saline, NEC-291 and L-isolucine ^{14}C (U.I.), specific activity 247 mCi/mM, 0.0133 mg/2.5 ml sterile saline, NEC-279, were purchased from New England Nuclear Corp., Boston, Mass.

Plaque Assay Reagents.

Difco-Bacto Agar, cat. no. 0140-01 was purchased from Difco Laboratories, Detroit, Mich. DEAE-Dextran, cat. no. D-2000 was purchased from Sigma Chemical Co., St. Louis, Mo., dissolved in 0.85% saline to 30 mg/ml and adjusted to pH 7.2 with 1 N sodium hydroxide. Sodium chloride, cat. no. 74073, biological grade, was purchased from Merck and Co., Inc. Rahway, N.Y.

Buffers.

1. Modified barbital buffer (MBB), pH 7.3-7.4, was stored as a 5X concentrate at 4°C (Campbell, Garvey, Cremer, Sussdorf, 1963).

2. Cacodylate buffer, 0.28 M, pH 6.9, was stored at room temperature (Rittenberg, Amkraut, 1966). Cacodylate saline, the same buffer diluted 1:10 in 0.85% saline.

3. Phosphate buffer, 0.1 M, pH 7.1 (Gomori, 1955). Phosphate saline, the same buffer diluted 1:10 in 0.85% saline.

4. Borate buffer (Campbell, Garvey, Cremer, Sussdorf, 1963) 0.08 M, pH 8.8. Borate saline, the same buffer diluted 1:20 in 0.85% saline.

Hemocyanin.

Hemocyanin (KLH) was obtained from the keyhole limpet *Megathura crenulata* purchased from Pacific Bio-Marine Supply Co., Venice, Calif. The animals were bled and the hemocyanin prepared by the method of Campbell, Garvey, Cremer, Sussdorf (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 one hour at 8,000 rpm and sterilized by filtration through an 0.45μ Millipore membrane and stored at 4°C.

Bovine Albumin.

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

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 was discarded by centrifuging at 1500 rpm for 10 min. The clear amber supernatant 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, 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 in three changes of saline (12 hr each). This removes the arsenic in cacodylate buffer and any residual unbound hapten. This procedure usually produces 850-1000 M TNP/1 M KLH, assuming a molecular weight of 8×10^6 for KLH. Other TNP/KLH ratios were obtained by altering the concentration of TNBS, the temperature, and the time of reaction (Rittenberg, Amkraut, 1966). The mole ratios used are noted in the results.

Trinitrophenyl Bovine Albumin (TNP-BSA).

Five hundred mg of BSA were dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.1, containing 120 mg of TNBS and stirred for 1 hr. The coupling reaction was stopped by the addition of 10 moles of glycylglycine per mole of hapten (Macris, Chase, 1965). The conjugated protein was precipitated by the addition of cold 1 M acetic acid and centrifuged. Borate buffer pH 8.6 dissolved the precipitated conjugate. After centrifugation the clarified protein was passed through a Sephadex G-50 column equilibrated in borate-saline to remove free TNBS and TNP-glycylglycine. The cycle of precipitation and gel filtration was repeated once or twice more until the 350/280 nm O.D. ratio of the conjugate in borate-saline was the same after two successive column passages. The mole ratios used were TNP_{13}BSA and TNP_{15}BSA as noted in the results (Rittenberg, Campbell, 1968) (Rittenberg, Amkraut, 1966).

Hapten:Carrier Ratios.

The degree of hapten substitution was estimated by spectrophotometric measurement at a wave length of 350 nm. The molar extinction coefficient of TNP-ε-aminocaproic acid (15,400 at 350 nm (Benacerraf, Levine, 1962)) was used to determine the average number of hapten groups bound per molecule of protein as determined by Nesslerization. Because of its absorbance at 350 nm a correction had to be applied for the contribution of KLH to the optical densities obtained with TNP-KLH conjugates. For TNP_{830}KLH this correction was approximately 30%. Addition of hapten does not appear to alter significantly the underlying absorption of the protein. A molecular weight of 8×10^6 was used for all hemocyanin preparations and 7×10^4 for BSA, thus all mole ratios are relative to these assumed values (Rittenberg, Amkraut, 1966).

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

The KLH and TNP-KLH were adsorbed onto bentonite (Fisher Scientific Co., St. Louis, Mo.) as Gallily and Garvey (1968) did with KLH. Their method was modified in that the bentonite sediment 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 X in cacodylate saline. The amount of protein coated on the particles was determined by Nesslerization (Campbell, Garvey, Cremer, Sussdorf, 1963).

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

60 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 X with 0.85% saline, were added dropwise with stirring. The mixture was stirred slowly at room temperature for 10 min. Reacted cells were brought to 50 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 glycylglycine (Nutritional Biochemicals Corp., Cleveland, Ohio). Glycylglycine 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 MBB and stored at 4°C until used in MBB. Haptenation and subsequent handling were carried out with foil wrapped containers to prevent photodecomposition (Okuyama, Satake, 1960).

Benzidine Stain for Assay Plates.

Benzidine base 0.2 gm, reagent grade (Hartman Leddon Co., Phila., Pa.) was mixed with 10 ml of 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 1-2 ml of 30% hydrogen peroxide. The final mixture was mixed well and used within a few hours after preparation (Jerne, Nordin, Henry, 1963).

Immunization.

For primary *in vitro* experiments mice were given one injection intraperitoneally with 100 µg protein of hemocyanin-bentonite in 0.5 ml 0.85% saline. For secondary *in vitro* experiments mice were given intraperitoneal injections of T-K-B every two weeks for a total of three injections. Each injection contained 100 µg protein in 0.5 ml 0.85% saline (Bullock, Rittenberg, 1970).

Cell Culture.

Mice were killed by cervical dislocation. Their spleens were removed aseptically and placed into a 60 mm petri dish with 5-8 ml of 1X room temperature MEM-P. The spleens were gently disrupted with sterile forceps and the cells dispersed with a capillary pipet. The cells were transferred to a tube and placed into an icebath for 3-5 min to allow particles to settle. Cells remaining in suspension were transferred to a conical tube and centrifuged at 1500 rpm for 15 min. After centrifugation the supernatant fluid was discarded and the pellet resuspended in culture medium to 0.9-1.4 X 10^7 nucleated spleen cells/ml. Cells were counted on a Coulter Counter, model F. They were cultured in 1 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 7-8 back and forth cycles per minute (Mishell, Dutton, 1967).

Feeding.

Every day each culture was given three drops from a capillary pipet of a 2:1 mixture of nutritional cocktail and fetal calf serum.

TNP-Plaque Assay.

A localized hemolysis in gel plaque assay (Jerne, Nordin, 1963) was modified to detect cells synthesizing anti-TNP antibody (Rittenberg, Pratt, 1969). Guinea pig serum was diluted 1:10 or 1:20 with MEM-P as a source of complement. Cultured cells were scraped from the culture dishes and packed by centrifugation at 1500 rpm for 15 min; culture supernatants were stored frozen for separate analysis. Cells were washed by resuspending in 5 ml of MEM-P/culture and centrifuged. The second supernatant was discarded and the cells were resuspended in appropriate volumes (1-4 ml) of cold MEM-P, counted, and plated in quadruplicate with TNP-SRBC and with non-haptenated SRBC. The assay plates consisted of base layers of agar, 1.4% in 0.85% saline. A 0.47% suspension of red blood cells and $<10^6$ spleen cells were added as thin 1 ml 0.62% agar layers on top of the base layers. This upper 1 ml layer contained 0.022 ml of 35 mg DEAE Dextran/ml saline to prevent the anti-complementary action of agar. The plates were incubated 3 hr at 37°C with complement to detect plaques due to cells producing IgM antibody. Indirect plaques were determined two ways: a) by incubating plates 3 hr with complement and anti-mouse IgG at a dilution revealing the maximum number of plaques (Sterzl, Riha, 1965) (Dresser, Wortis, 1965); this varied with the batch of anti-mouse IgG used; b) by incubating the plates 1.5 hr with complement alone and then replacing the complement with anti-mouse IgG diluted with complement and reincubating for 1.5 hr. Guinea pig serum and anti-globulin serum were absorbed with SRBC prior to use (Kabat, 1963). The anti-globulin serum was heated at 56°C for 30 min

prior to use (Bullock, Rittenberg, 1970). After incubation the plates were stored overnight at 4°C and stained the following day with benzidine stain and counted on a New Brunswick Scientific colony counter with electronic probe.

Supernatant Titration.

The lytic spot test of Hübner and Gengozian (1969) was modified to titer small amounts of anti-TNP antibody in culture supernatants. Soft agar layers containing 0.47% TNP-SRBC were used. Drops of diluted supernatant were placed on these layers, allowed to soak in, and incubated 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 supernatant. Supernatant samples were treated with 0.1 M 2-mercaptoethanol for 30 min at 37°C to inactivage IgM and dialyzed against 0.01 M iodoacetamide and then against 0.85% saline (Pearlman, 1967). Supernatants so treated were then compared with comparable untreated supernatants.

^{14}C Amino Acid Incorporation.

Cultures were tested for protein synthesis by adding 1μCi each of ^{14}C isoleucine and ^{14}C valine to cultures on day 4. Supernatants were concentrated by incubation with polyacrylamide gel (Lyphogel, LKB Instruments, Inc., Rockville, Md.). ^{14}C amino acid incorporation in total supernatant protein was estimated by the method of Timourian and Denny (1968) (Williams, Chase, 1968). The supernatants were dialyzed against three changes of buffered saline prior to adsorption on chromatography paper strips. The protein was precipitated on the strips with 10% trichloracetic acid. Duplicate samples were prepared and the counts

averaged after subtraction of background. Background was approximately 1% of the total counts. Incorporation of ^{14}C amino acids into specific anti-TNP was determined by adding TNP-BSA to six-fold concentrated culture supernatants and incubated at 37°C for 1 hr. Rabbit anti-BSA was then added at approximate equivalence to coprecipitate anti-TNP - TNP-BSA complexes. Antigen-antibody precipitates were allowed to form at 37°C for 1 hr followed by 4°C for 3 days. The precipitates were then washed twice in cold 0.85% saline, digested in NCS (Nuclear-Chicago Corp., Des Plaines, Ill.), and counted in a Beckman Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, Calif.).

Radioimmunoelectrophoresis.

The method of Thorbecke and Hochwald (1968) was used. Culture supernatants were concentrated three-fold and electrophoresed with unlabeled mouse serum containing anti-TNP antibody. Following electrophoresis TNP-BSA, TNP-KLH, BSA, and KLH were used to develop precipitates. Radioautographs were prepared on Kodak RPS negative film and exposed for two months.

Subclass Analysis of Plaques.

Various anti-class and anti-subclass specific antisera were kindly supplied by L. Herzenberg. These sera were used in place of the goat anti-mouse IgG sera to permit me to distinguish specifically the immunoglobulin class and subclass of individual cells producing anti-TNP antibody.

RESULTS

Anti-TNP Response *In Vitro.*

Figure 1 illustrates the general procedure used. The appearance of 7S and 19S plaque-forming cells (PFC) is seen in Figure 2. Notice that the 19S plaques are larger than the 7S plaques; the reason for this is unknown.

In vitro anti-TNP responses obtained varied quantitatively in terms of the maximum PFC attained, presumably due to subtle differences in culture conditions (different lots of fetal calf serum for example) and to differences in batches of mice which were usually purchased in groups of 100. Figure 3 demonstrates this quantitative variability. However, the important feature of the model is that regardless of the magnitude of the response in a given set of cultures the qualitative and quantitative relationships were consistent throughout. The peak responses occurred on day 5 or 6 (Figures 4 and 11). An optimum antigen dose was always detectable with both higher and lower doses stimulating smaller responses (Figures 3, 4, 9, 12, 13, 16, 17), and both 19S and 7S anti-TNP secondary responses occurred (Figures 3, 4, 8, 9, 11). Thus although the conclusion drawn could be reproduced from experiment to experiment it was essential in this system to make comparisons between responses of cultures prepared simultaneously with the same pool of cells and to include all essential controls with each set of cultures.

Day of Peak Response.

Spleen cell suspensions were placed in culture with 0.2 or 0.02 µg protein TNP-KLH (particulate or soluble) 6-7 weeks following the last

Figure 1.

Outline of culture method used to determine antigen sensitivity of memory cell population. TNP-KLH was injected or added to cultures in micrograms of protein (P) as described in the text. Each cell (PFC) forming anti-TNP antibody is found at the center of a lytic area termed a plaque. Formation of these plaques requires the presence of complement. The plaques are inhibited by excess free hapten.

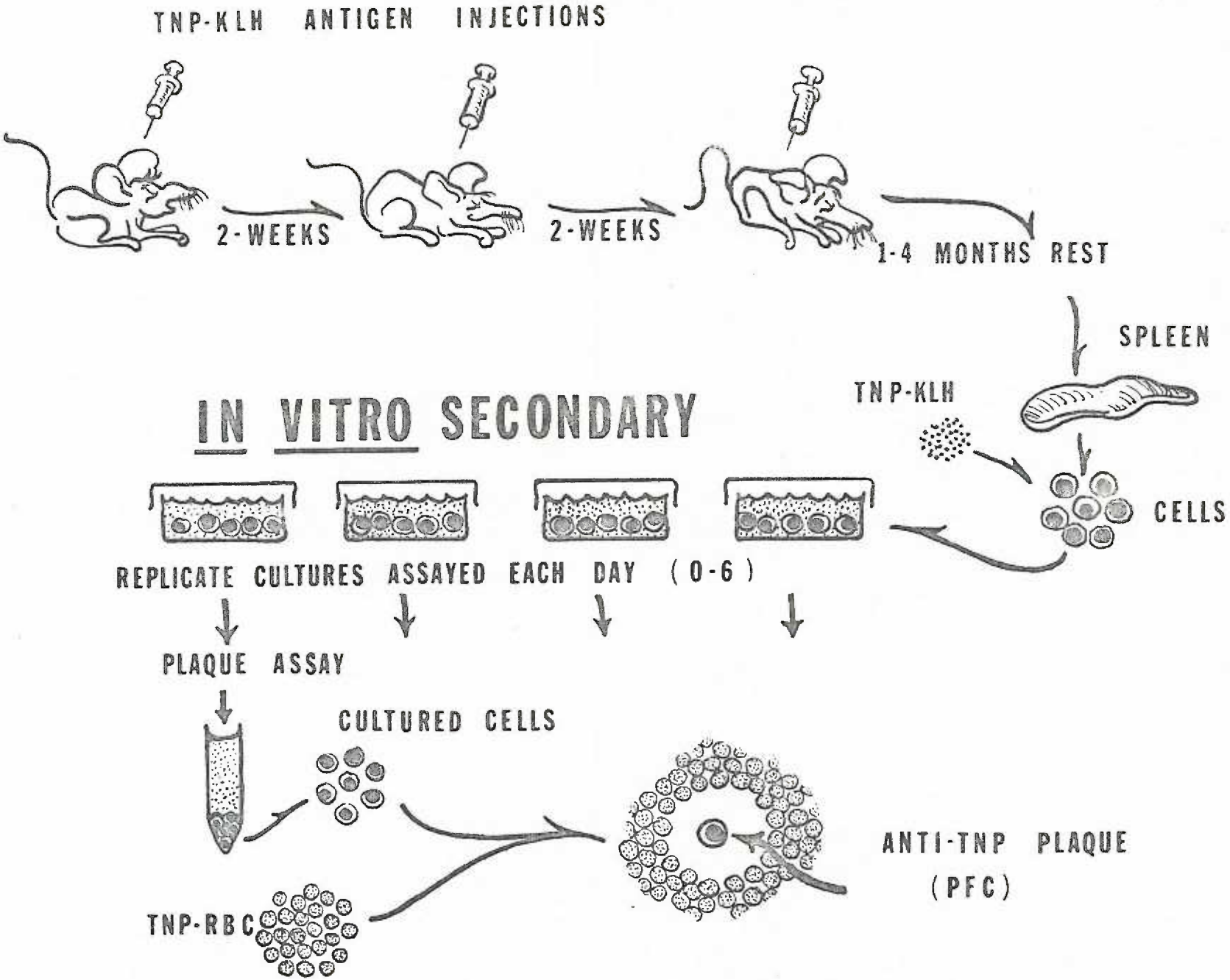

TNP-KLH ANTIGEN INJECTIONS
2-WEEKS
2-WEEKS
1-4 MONTHS REST
SPLEEN
IN VITRO SECONDARY
TNP-KLH
CELLS
REPLICATE CULTURES ASSAYED EACH DAY (0-6)
PLAQUE ASSAY
CULTURED CELLS
TNP-RBC
ANTI-TNP PLAQUE
(PFC)

Figure 2.

These photographs demonstrate the appearance and size of plaques as they are counted on benzidine stained assay plates. All three plates were initially treated to develop 19S (IgM) plaques by incubating at 37°C for 1.5 hr with a 1:20 dilution of guinea pig complement. The 7S (IgG) plaques were then developed by incubating plate C an additional 1.5 hr with goat anti-mouse IgG serum in fresh complement.

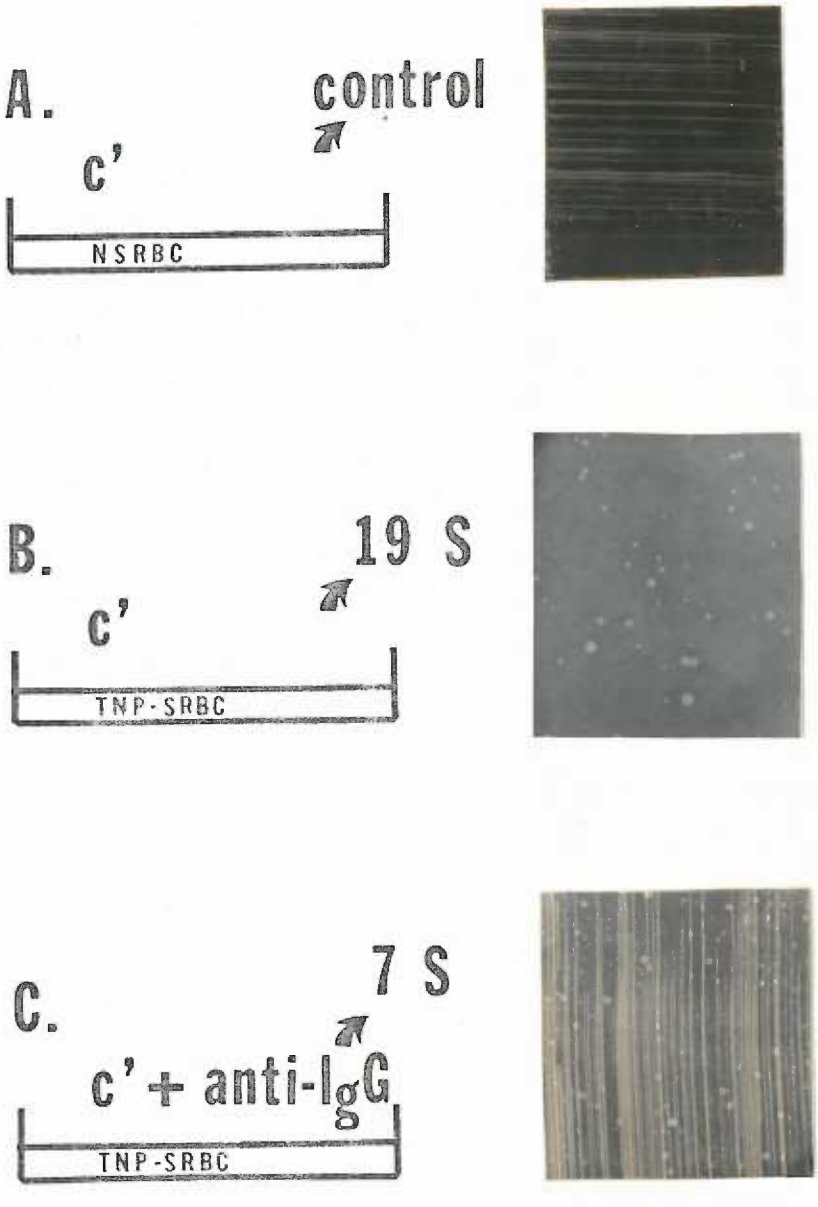

A.
control
c'
NSRBC
B.
19 S
c'
TNP-SRBC
C.
7 S
c' + anti-IgG
TNP-SRBC

Figure 3.

Two separate experiments are shown plotted on a log-log scale, which exhibited widely different quantitative (PFC/10^6 cells assayed) response. In experiment A (●----●) cells were cultured 6 weeks after *in vivo* priming. A TNP_{1000}/KLH antigen preparation was used *in vitro*. In experiment B (●____●) cells were cultured 12 weeks following *in vivo* priming. A TNP_{1430}/KLH antigen preparation was used *in vitro*. Each value is the mean of three cultures plated in quadruplicate. Response on day 5 of culture is shown for both experiments.

Note that in both experiments both 19S and 7S responses were obtained, high antigen doses were suppressive, low antigen doses produced sub-optimal stimulation and that the quantitative relationships are similar within each experiment.

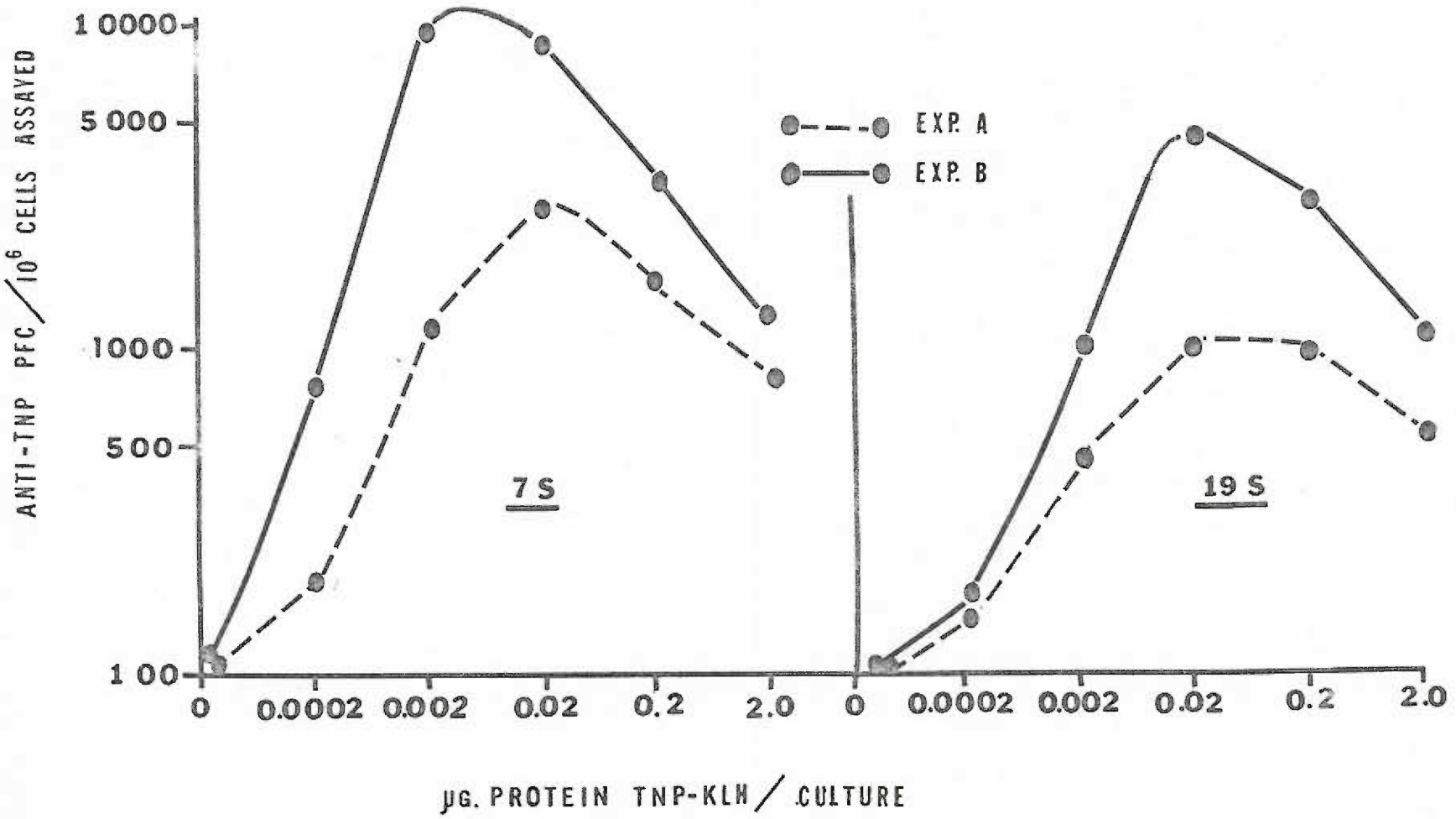
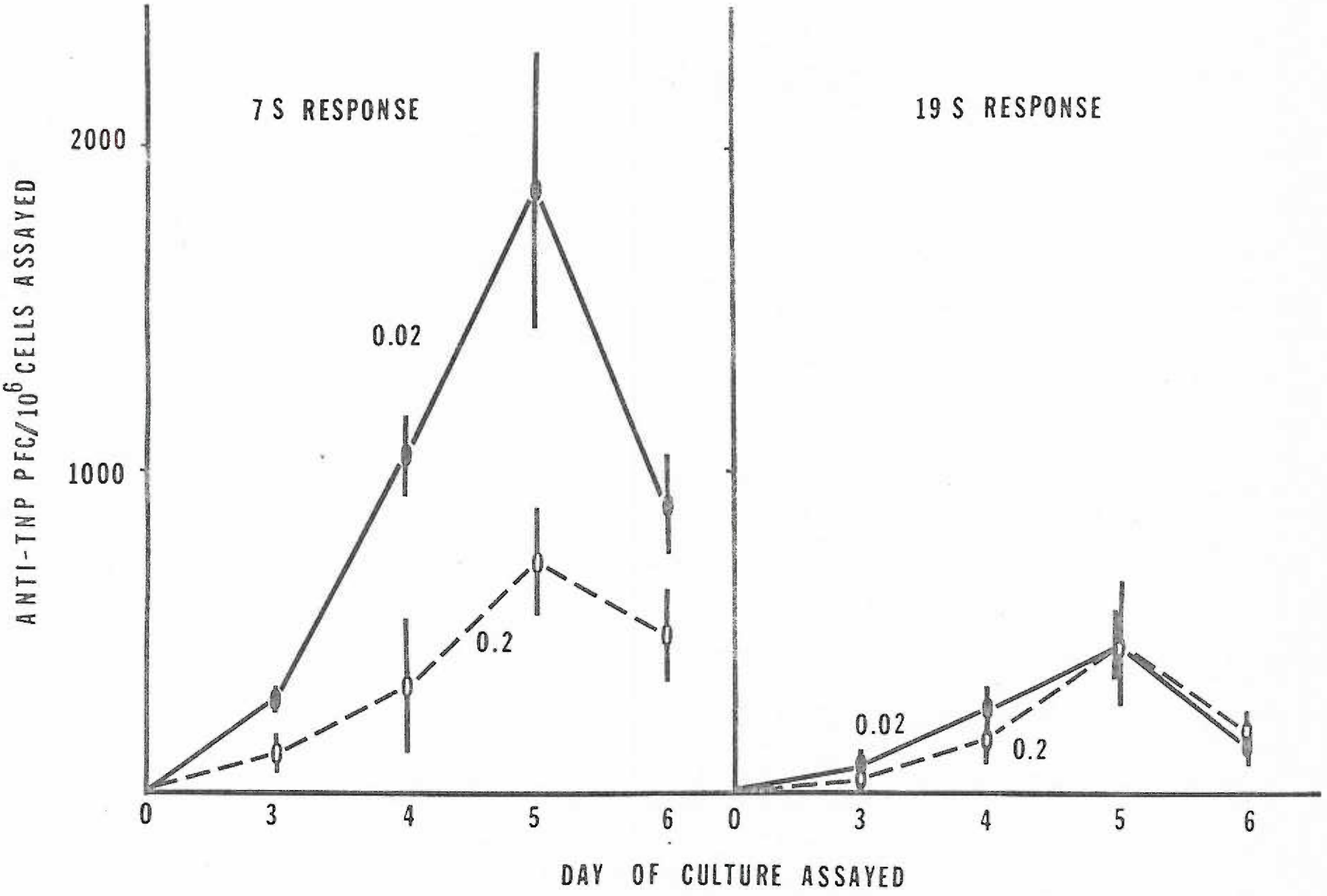

ANTI-TNP PFC / 10^6 CELLS ASSAYED
1 0000
5 000
1000
500
1 00
EXP. A
EXP. B
7 S
19 S
0
0.0002
0.002
0.02
0.2
2.0
0
0.0002
0.002
0.02
0.2
2.0
µG. PROTEIN TNP-KLH / CULTURE

Figure 4.

Semi-log plot of the increase in cells producing anti-TNP antibody after *in vitro* initiation of secondary immune response. Cells were placed in culture 6-7 wk after primary immunization. Antigen was added to cultures at day 0. Either soluble or particulate TNP-KLH was used and the results were treated together since at the same concentration both forms were shown previously to induce similar responses (Bullock, Rittenberg, 1970a). Antigen added: 0.2µg protein/culture O----O; 0.02 µg protein/culture ●——●. The response was evaluated by anti-TNP plaque assay. Backgrounds resulting from primary stimulation have been subtracted (7S <201 PFC/10^6, 19S <190 PFC/10^6). Each point represents the mean of duplicate cultures (plated in quadruplicate) from four separate experiments; therefore, n = 8 except for day 4 and 5 (0.02 µg 7S) where n = 7. Vertical bars represent 95% confidence intervals. 19S response was detected by direct plating. 7S response was detected by facilitation with antiglobulin serum.


7 S RESPONSE
19 S RESPONSE
2000
1000
0.02
0.2
0.02
0.2
0
3
4
5
6
0
3
4
5
6
ANTI-TNP PFC/10^6 CELLS ASSAYED
DAY OF CULTURE ASSAYED

in vivo injection of T-K-B. The number of cells synthesizing anti-TNP at the start of the cultures and during the period of maximum response (days 3-6) is shown in Figure 4. The number of cells producing 7S antibody is compared to the number producing 19S antibody. The higher antigen dose suppressed the 7S response 60 percent whereas both doses stimulated the same 19S response. Primary 19S responses of this magnitude, with normal mice, have not occurred under similar conditions in our laboratory, indicating that the 19S response is a secondary one. A small increase in cells producing anti-TNP occurred in control cultures usually less than 100 PFC/10^6 cells cultured, possibly due to the release or unmasking of sequestered antigen persisting from the *in vivo* priming (Byers, Sercarz, 1968) (Mitchison, 1969).

Unhaptenated carrier protein added to cultures did not induce an anti-TNP response greater than that in cultures not given carrier. The anti-TNP plaques were inhibited with excess antigen or hapten in the assay mixture. In addition, cells did not produce anti-TNP if heat-killed at 56°C for 30 min (Schrek, 1966), if treated with 10^{-5} M Methotrexate (Rueckert, Mueller, 1960), or by the addition of 100 µg chloramphenicol/culture at day 0 (Hartman, Pettengill, Sorenson, 1969)* both shown to inhibit cell division. These experiments indicated specific induction and a requirement for viable and actively metabolizing cells.

The high *in vitro* secondary "7S" anti-TNP responses shown in Figures 3 and 4 are comparable to those obtained with TNP-KLH *in vivo* (Rittenberg, Pratt, 1969) and demonstrate the capacity of this *in vitro* model to

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*Methotrexate assay by S. E. Edney

follow the response to a chemically defined determinant on a complex carrier.

Cell Loss During Culture.

Since the culture medium was supplemented each day rather than replaced, it can be assumed that toxic products build up in the medium and result in cell degradation. As shown in Figure 5 the total number of cells remaining by days 5 and 6 is only 40-50 percent of the starting population. This loss is much too small to explain the increase in anti-TNP cells after exposure to antigen *in vitro*. There appears to be two rates of cell loss, an initial rapid loss during the first 24 hours followed by a slower steady decline. The number of viable cells was not determined because cell counts by Coulter Counter are more reliable and consistent than hand counts and vital staining. If anything, utilization of total cell counts would tend to decrease responses as reported in plaques/10^6 cells assayed. As can be seen control, high and low antigen dose treated cultures all lost cells at approximately the same rate.

Antibody Synthesis *In Vitro*.

Anti-TNP antibody was detected in unconcentrated culture supernatants on day 6 at endpoint dilutions of 1:512 in antigen-stimulated cultures and 1:32 in controls using the lytic spot test (Hübner, Gengozian, 1969). ^{14}C labeled amino acids were used to determine *in vitro* synthesis of antibody. Table I compares the ability of antigen-stimulated and control cultures to form plaques and to incorporate ^{14}C isoleucine and ^{14}C valine into protein and specific anti-TNP antibody. Antigen stimulation *in vitro* resulted in a 30-fold increase in anti-TNP PFC. It also caused a 16-fold increase in the incorporation of ^{14}C label into anti-TNP, as compared to non-stimulated

Figure 5.

This figure shows the percent cell survival occurring with time under standard culture conditions. Each point is the average cell count of 3 similar cultures. Spleen cells from 10 mice were cultured 14 wk following *in vivo* priming with T-K-B. During this experiment a peak response of several thousand PFC/10^6 cells assayed was obtained with TNP-KLH stimulated cultures assayed on days 5 and 6. Cells were scraped from the plastic culture dishes with rubber policeman, centrifuged and washed as outlined in the Methods section. Total nucleated cell counts were determined for each day of culture with a Coulter Counter. The controls (●----●), 0.2 µg protein TNP-KLH stimulated (○——○), and the 0.2 µg protein TNP-KLH stimulated (●——●) cultures all lost cells at approximately the same rate. Similar results have been consistently obtained with the other experiments reported. Each experiment is checked to insure that gross cell loss did not occur. The survival values shown are similar to the 35-45% cell survival on day 5 reported by Mishell and Dutton (1967) with a similar *in vitro* system.

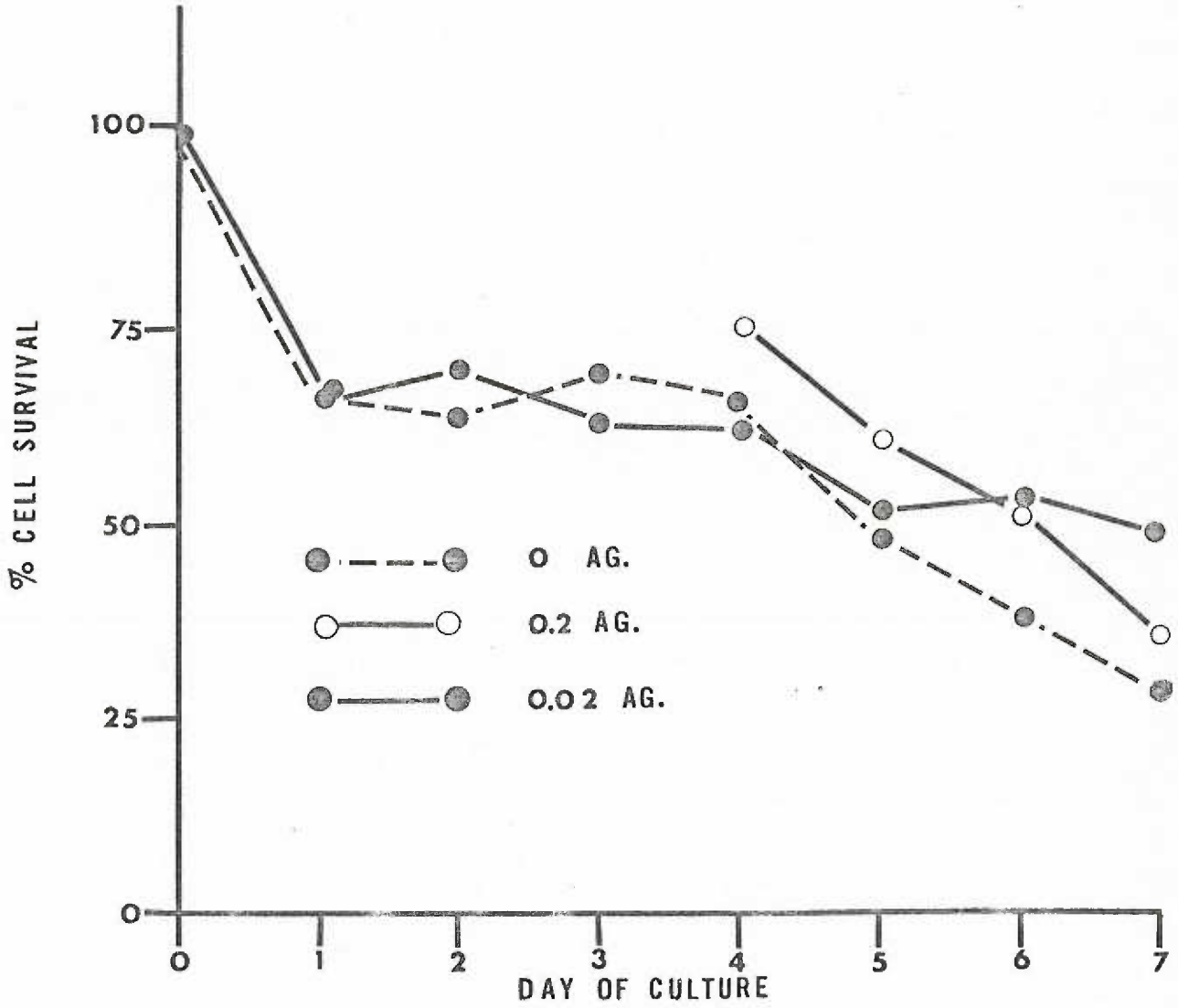

% CELL SURVIVAL
100
75
50
25
0
0 AG.
0.2 AG.
0.02 AG.
0
1
2
3
4
5
6
7
DAY OF CULTURE

Table I.

This table gives data showing that antigen was required for PFC formation but antigen presence did not affect the overall level of protein synthesis. However, antigen was required for *in vitro* synthesis of anti-TNP antibody from labeled amino acids and synthesis of this antibody correlated with PFC formation as also seen in Figure 10.

Table 1

Correlation of Secondary Anti-TNP Immune Response *In vitro*.* Comparison of Plaque Assay and Incorporation of ^{14}C Isoleucine and ^{14}C Valine into Antibody

Culture Tested	+Antigen	-Antigen
7S PFC/10^6 cells	1860	62
Net CPM in supernatant protein	3250	3428
Net CPM precipitated from supernatant† by TNP-BSA and Anti-BSA	491	30

*Spleen cell response to 0.002 µg P TNP-KLH; assay on Day 5.

†Supernatant protein after concentration 5.5 mg N/ml.

cultures. Antigen-stimulated and control cultures incorporated approximately equal amounts of ^{14}C label into total protein.

Figure 6 shows radioimmunoelectrophoretic patterns of three-fold concentrated, dialyzed supernatant from antigen-stimulated cultures. The patterns indicate ^{14}C-labeled anti-TNP antibody in the supernatant. Labeled antibody was not apparent against either unhaptenated carrier. A more intense line developed with the haptenated homologous carrier protein (TNP-KLH) than with the haptenated heterologous protein (TNP-BSA), suggesting antibody against TNP-modified KLH.

7S vs. 19S Antibody Induction.

The ratio of 7S PFC to 19S PFC was found to vary widely between experiments. The cause of this variation is still largely unknown although it appears to involve the antigen concentration and the ratio of moles of hapten per mole of carrier. Figure 7 illustrates the affect of antigen concentration on the 7S PFC/19S PFC ratio in three separate experiments. Similar results were found consistently in other experiments. As can be seen, lower antigen concentrations evoke approximately four times as much 7S antibody cells whereas high concentrations of antigen induce more 19S than 7S cells. To determine if this could be explained by a lower concentration of TNP determinants per molecule of antigen, TNP-KLH was prepared with different hapten to carrier ratios. Figure 8 compares the responses to two antigen preparations, TNP_{500}/KLH and TNP_{1430}/KLH. The relative effectiveness in inducing a 19S or 7S response is shown by plotting that percentage of the total response due to 7S PFC. Seven experiments are shown with cultures which were initiated at different times after the last *in vivo* injection. The major difference between high

Figure 6.

Radioimmunoelectrophoretic demonstration of *in vitro* synthesized anti-TNP in supernatants from antigen-stimulated cultures. Supernatants were obtained and pooled on day 6 of culture and concentrated three-fold prior to electrophoresis with unlabeled mouse anti-TNP-KLH antiserum as carrier. One μCi each of ^{14}C isoleucine and ^{14}C lysine was added to cultures 48 hr before assay. Cultures were stimulated by 0.002 μg TNP-KLH on day 0.

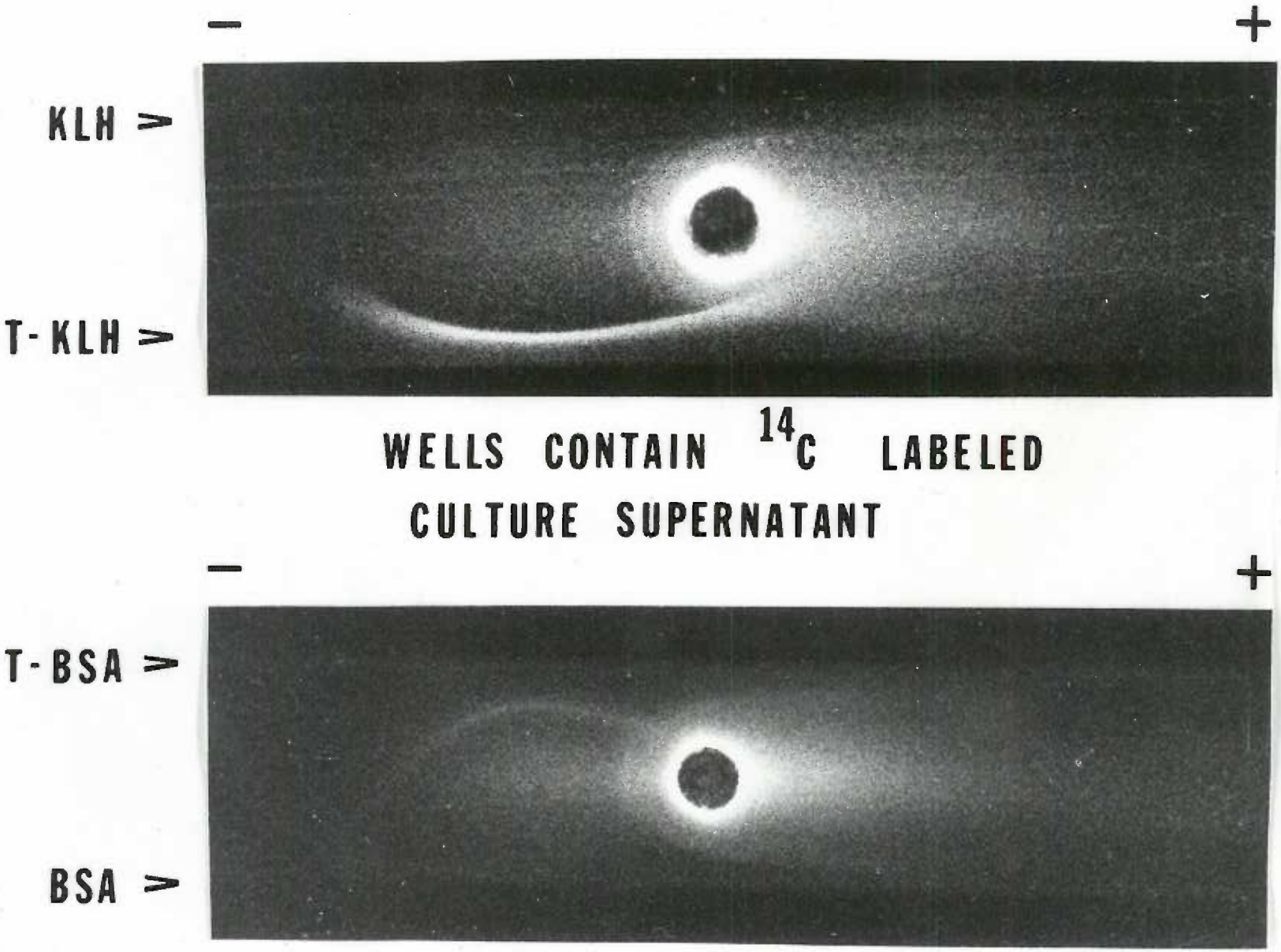

–
+
KLH >
T-KLH >
WELLS CONTAIN ^{14}C LABELED
CULTURE SUPERNATANT
–
+
T-BSA >
BSA >

Figure 7.

Three separate experimenta (A, B, and C) are plotted (log-log) as the 7S PFC/19S PFC ratio obtained from cultures stimulated with different antigen doses. 19S PFC and 7S PFC values used were each the means of three similar cultures plated in quadruplicate. The rise in the proportion of 7S PFC with lower antigen doses has been consistently observed with other experiments. This finding is consistent with the suggestion that low antigen concentrations favor the induction of 7S antibody because of its higher binding capacity.

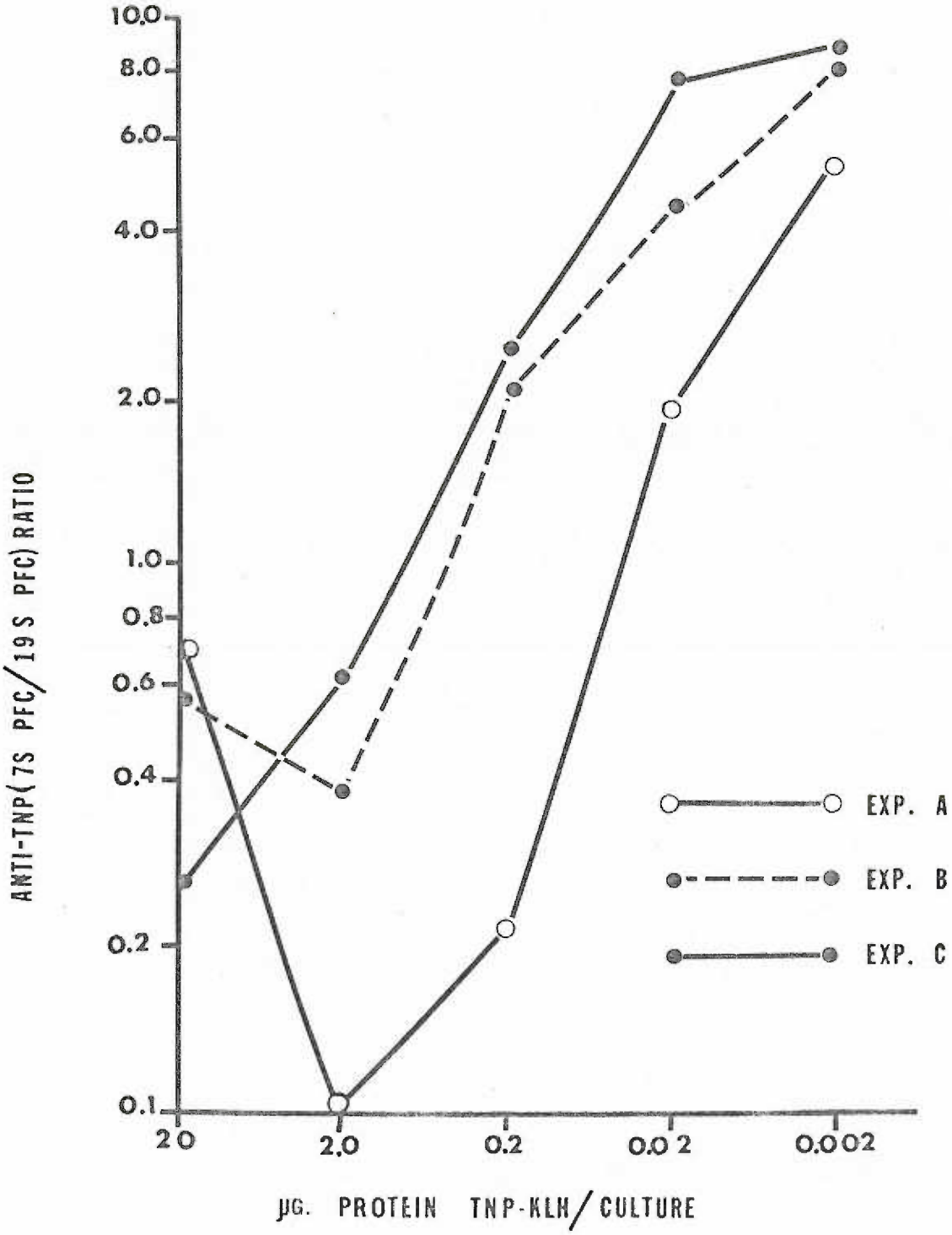
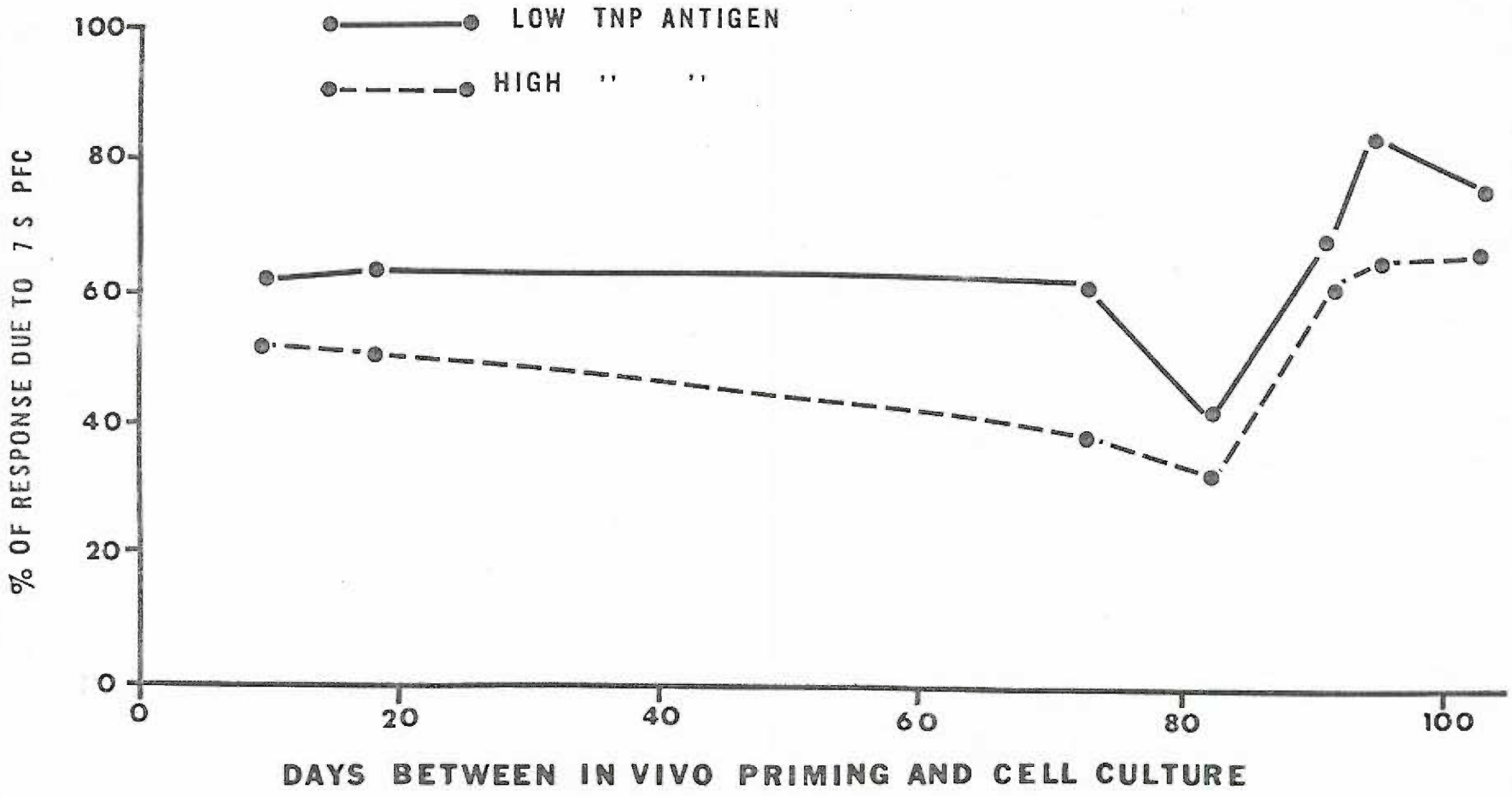

10.0
8.0
6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
ANTI-TNP(7S PFC/19S PFC) RATIO
2.0
0.2
0.02
0.002
μG. PROTEIN TNP-KLH/CULTURE
EXP. A
EXP. B
EXP. C

Figure 8.

This graph plots seven separate experiments which each compared the anti-TNP responses induced with TNP_{500}/KLH antigen (●——●) to those obtained with TNP_{1430}/KLH antigens (●----●). The maximum values obtained with each experiment were used to calculate the percent 7S response shown. With time after *in vivo* priming the antigen concentration inducing maximum values decreased from 2.0 µg protein TNP-KLH at 9 days to 0.002 µg protein TNP-KLH in the last two experiments shown. This phenomenon is covered in the text and Figures 12 and 13. Note that in each experiment the low hapten substituted preparation (TNP_{500}/KLH induced a greater percentage of 7S PFC. Each value plotted was obtained with triplicate cultures plated in quadruplicate.


LOW TNP ANTIGEN
HIGH " "
% OF RESPONSE DUE TO 7 S PFC
100
80
60
40
20
0
0
20
40
60
80
100
DAYS BETWEEN IN VIVO PRIMING AND CELL CULTURE

and low substituted antigens usually seen in an increased 7S response with low substituted antigens. To overcome the variability seen in Figure 8 two TNP-KLH antigens were prepared having a 10-fold difference in hapten substitution, TNP_{228}/KLH and TNP_{2260}/KLH. Animals were primed *in vivo* as usual with either high or low substituted antigen and later challenged *in vitro* with high or low substituted antigen. The results are shown in Figure 9. The low TNP/KLH preparation shown in Figure 9b induced a much greater 7S response and a slightly larger 19S response substantiating the tendency of low hapten/carrier antigens to produce increased 7S PFC/19S PFC ratios. However, this effect occurred only in animals primed with high substituted antigen. Animals primed with low substituted antigen yielded cells which were equally responsive to both high and low substituted antigens and did not produce as large a 7S response (Figure 9a).

Figure 10 indicates the value of this model during the above experiments in enabling an accurate quantitation of the number of cells synthesizing 19S or 7S antibody. It can be seen that titration of antibody would result in an inaccurate estimate particularly with regard to the number of cells producing each antibody class. In addition, Figure 10 illustrates that the absence of PFC seen with high and low doses of TNP-KLH antigen correlates with the absence of or lower levels of anti-TNP antibody.

Particulate vs. Soluble Antigen.

Figure 11 gives the means and 95 percent confidence intervals for results of three experiments using the particulate antigen T-K-B and three experiments using TNP-KLH *in vitro*. Duplicate cultures plated in

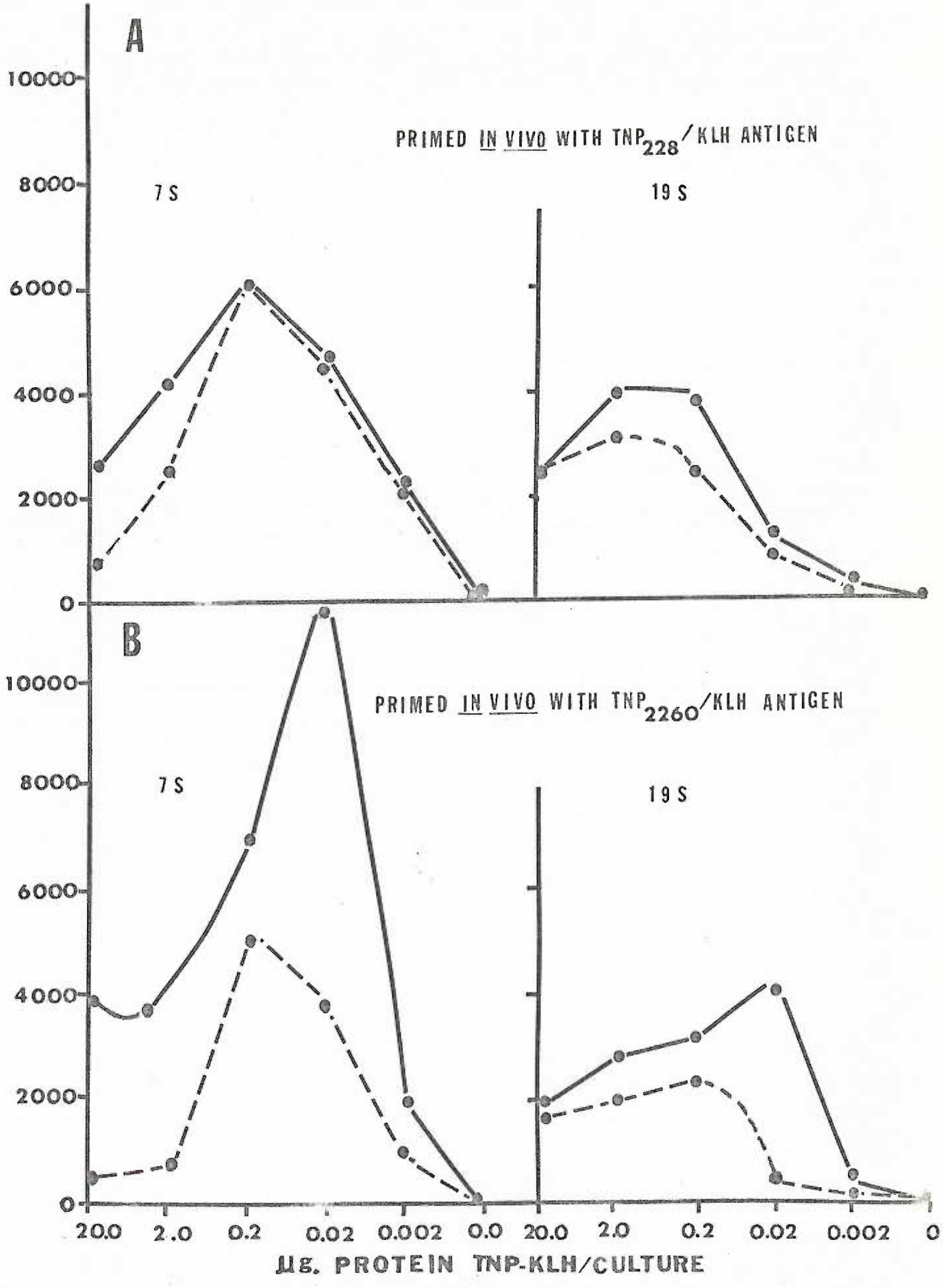
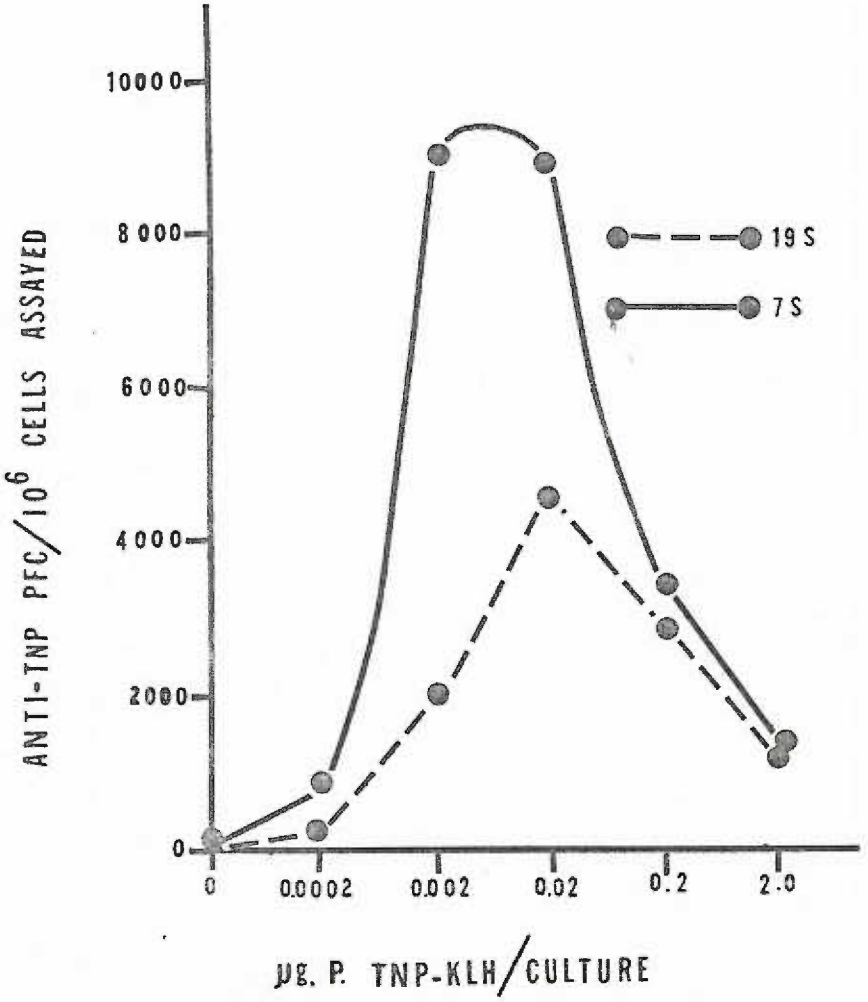
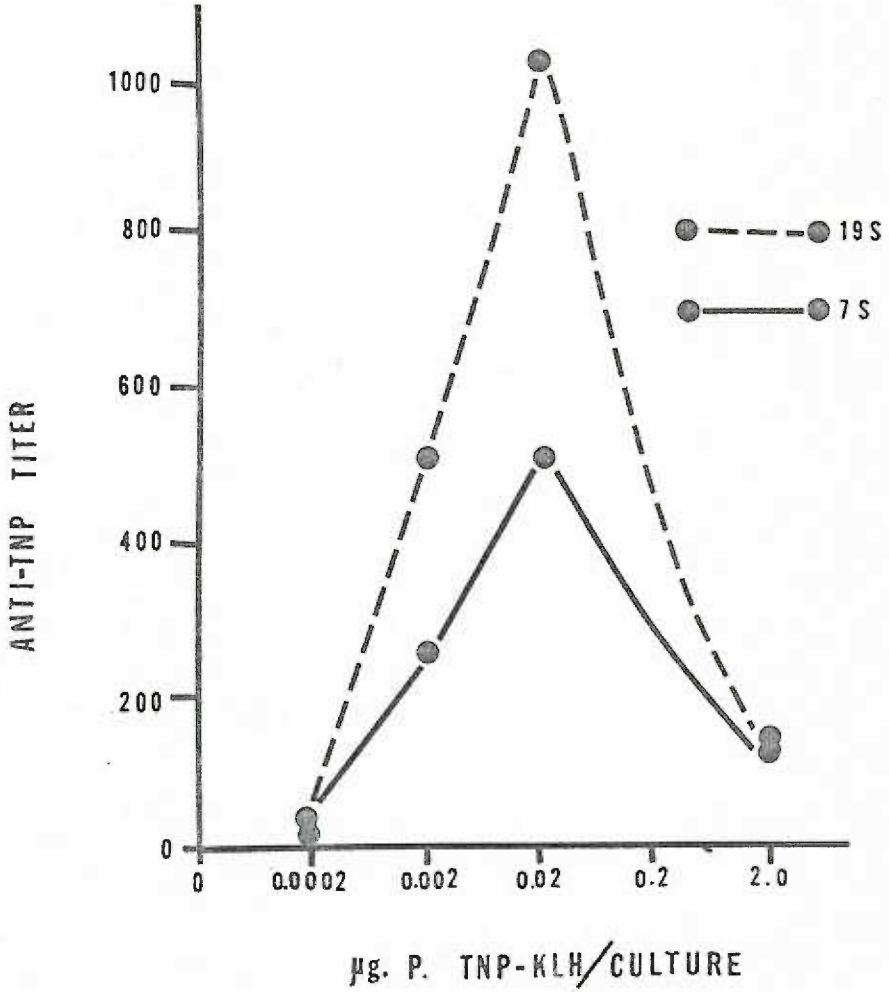

A
PRIMED IN VIVO WITH TNP228/KLH ANTIGEN
7 S
19 S
10000
8000
6000
4000
2000
0
B
PRIMED IN VIVO WITH TNP2260/KLH ANTIGEN
7 S
19 S
10000
8000
6000
4000
2000
0
20.0
2.0
0.2
0.02
0.002
0.0
20.0
2.0
0.2
0.02
0.002
0
µg. PROTEIN TNP-KLH/CULTURE

Figure 10.

This figure compares the anti-TNP PFC responses with the anti-TNP antibody titer (detected in undiluted culture supernatants) obtained on day 5 of culture over a wide scale of antigen concentration. The anti-TNP PFC values shown are the means of triplicate cultures plated in quadruplicate. The anti-TNP titer values were each obtained from pooled supernatants of 3 cultures titered by the method of Hübner and Gengozian (1969). It can be seen that while the majority of cells were 7S producers, most of the titerable antibody was 19S. This presumably reflects both quantity of antibody and the hemolytic efficiency of the two antibody classes.


ANTI-TNP PFC/10^6 CELLS ASSAYED
10000
8000
6000
4000
2000
0
19S
7S
0
0.0002
0.002
0.02
0.2
2.0
µg. P. TNP-KLH/CULTURE


ANTI-TNP TITER
1000
800
600
400
200
0
19S
7S
0
0.0002
0.002
0.02
0.2
2.0
µg. P. TNP-KLH/CULTURE

quadruplicate were treated separately for computing means and confidence limits. Particulate T-K-B is required for vigorous primary anti-TNP formation in Balb/c mice *in vivo* (Rittenberg, Pratt, 1969). However, Figure II indicates the secondary response is initiated equally by either form *in vitro*. Thus, the results indicate that the particulate form is not required for *in vitro* processing in secondary stimulation. In five of the six experiments shown in Figure II, 10-fold higher and lower antigen concentrations were tested but were less stimulatory than the 0.02 µg dose reported.

Does Altered Antigen Concentration, or the Particulate or Soluble Nature of Antigen Affect the Ratio of Immunoglobulin Classes or Subclasses Induced?

This question was investigated by culturing cells with either of two concentrations of antigen: 0.002 µg protein or 0.02 µg protein in a particulate (T-K-B) or soluble (TNP-KLH) form. The cells were assayed on day 5. IgG plaques were developed with anti-total IgG antisera or with subclass specific anti-IgG_1 or anti-IgG_{2_a} anti-sera donated by Dr. L. Herzenberg. It can be seen that the majority of 7S PFC produce IgG_1. In addition, the anti-IgG_1 antiserum is more effective in developing IgG_1 PFC than the anti-$IgG_{(total)}$ antiserum usually used to develop plaques. The different types of antigen (soluble or particulate) or different antigen doses used, however, did not affect the ratios of immunoglobulin class or subclass of anti-TNP induced (see Table II).

7S Response: Time Dependent Development of Increased Cell Sensitivity to Antigen.

Two preliminary experiments revealed the development of increased sensitivity to antigen during the postpriming period. Six weeks and 18 weeks after the last *in vivo* injection of priming antigen, sets of mice

Figure 11.

A log-log plot of increase in cells producing anti-TNP antibody as a result of *in vitro* initiation of secondary immune responses with soluble or particulate antigen. Cells were placed in culture 3-7 weeks after primary sensitization. Antigen was added on day 0. Antigen added (0.02 µg protein/culture) ●——●. No antigen added O----O. Response is evaluated by anti-TNP hemolytic plaque assay. Each point represents mean of duplicate cultures from 3 separate experiments. Therefore, n = 6 except for day 4 where particulate n = 5 and soluble n = 4. Vertical bars represent 95 percent confidence intervals. 19S response was detected by direct plating while 7S response was detected by the action of antiglobulin serum.

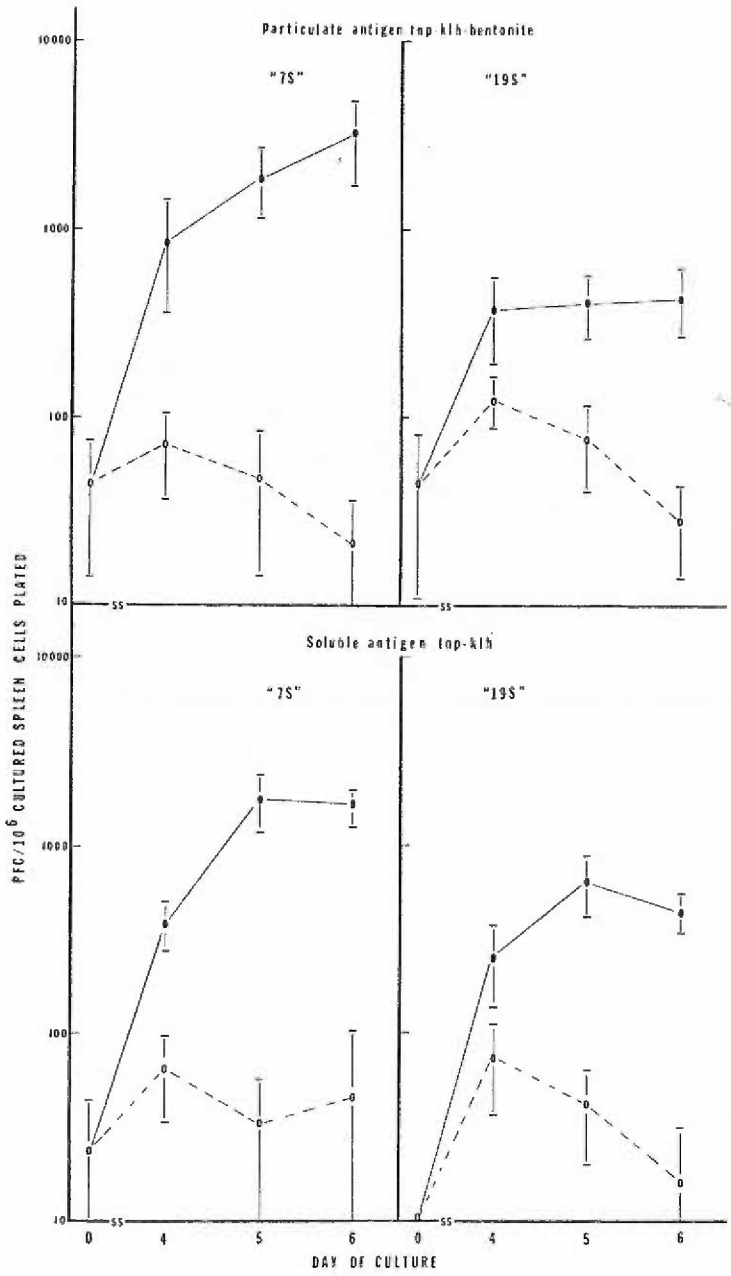

Particulate antigen tnp-klh-bentonite
"7S"
"19S"
Soluble antigen tnp-klh
"7S"
"19S"
PFC/10^6 CULTURED SPLEEN CELLS PLATED
10000
1000
100
10
SS
0
4
5
6
DAY OF CULTURE

Table II

This experiment indicates that the original priming antigen is more effective in determining the antibody class response than is the subsequent challenge antigen.

Table II

Percent Anti-TNP Class Response to High and Low Antigen or T-K-B and T-KLH*

	Percent of Total Response	
	0.2 µg Protein TNP-KLH	0.02 µg Protein TNP-KLH
IgM	23	13
$IgG_{(Total)}$	78	87
IgG_1	101	122
IgG_{2b}	0	1
	T-KLH	T-K-B
IgM	18	18
$IgG_{(Total)}$	82	87
IgG_1	124	98
IgG_{2b}	1	0

* $\frac{\text{Class PFC}/10^6}{\text{IgG PFC}/10^6 + \text{IgM PFC}/10^6} \times 100 = \%$ of response.

The IgM values have been subtracted from the IgG_1 and IgG_{2b} values given since these specific antisera do not suppress the IgM response.

were killed and their spleen cells placed in culture with log dilutions of antigen. The 7S anti-TNP PFC response obtained to each dose on day 5 is shown in Figure 12. The experiments indicated: a) The number of antibody-forming cells induced at 18 weeks was one-half that at six weeks; b) concentrations of antigen greater than those yielding maximum stimulation suppressed the response. The 18-week cultures were suppressed by lower antigen concentrations than the six-week cultures. c) Cells cultured soon after *in vivo* priming responded to a narrower range of antigen dose than cells cultured 12 weeks later. d) Cell sensitivity to antigen increased with time *in vivo* with maximum stimulation of 18-week cells possible with 1000-fold less antigen than that which maximally stimulated the six-week group.

To further investigate this latter point a series of experiments were conducted during the 6-18 week period after priming. These experiments are summarized in Figure 13 which reveals the continual development of increased memory-cell sensitivity during the 6-18 week period after priming. The ratio of response (low TNP-KLH dose/high TNP-KLH dose) is given for each of eight sequential experiments. The change in ratio with time is due to increasing effectiveness of the lower antigen dose. Cell cultures from mice six weeks postpriming were twice as responsive to the high antigen dose; however, by 10 weeks both concentrations were equally effective and by 12 weeks the response was completely reversed with the low dose twice as effective as the high dose. This transition in antigen sensitivity at these doses ceased at 12 weeks. This cessation appeared to result from further increase in cell sensitivity concomitant with suppression by higher, previously stimulatory doses. A similar transition is shown for 10-fold higher antigen concentrations in Table III.

Figure 12.

Shift in anti-TNP memory cell sensitivity to antigen dose with time (semi-log plot). Values are means of 7S anti-TNP PFC from duplicate cultures assayed in quadruplicate on day 5 of culture. Cells placed in culture 6 weeks after priming (●——●); 18 weeks after priming (O----O). Note the increased sensitivity of the 18-week culture.

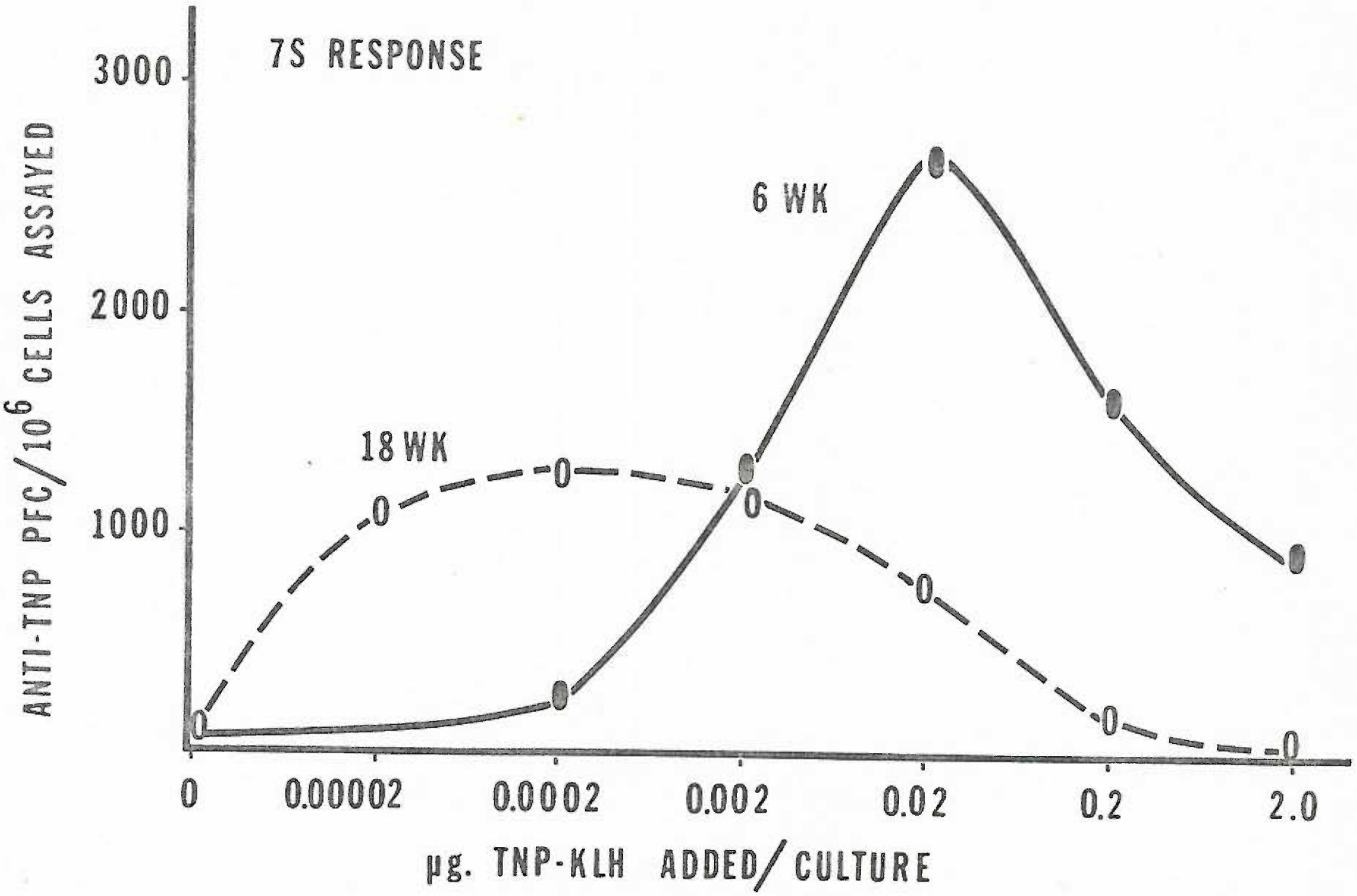

7S RESPONSE
3000
2000
1000
ANTI-TNP PFC/10^6 CELLS ASSAYED
6 WK
18 WK
0
0.00002
0.0002
0.002
0.02
0.2
2.0
µg. TNP-KLH ADDED/ CULTURE

Figure 13.

Temporal transition in memory-cell sensitivity to high and low antigen doses. Relative shift in maximum anti-TNP response. Ratio of 7S response to 0.002 μg TNP-KLH/0.02 μg TNP-KLH. Each point is the mean of two cultures assayed on day 5 and plated in quadruplicate.

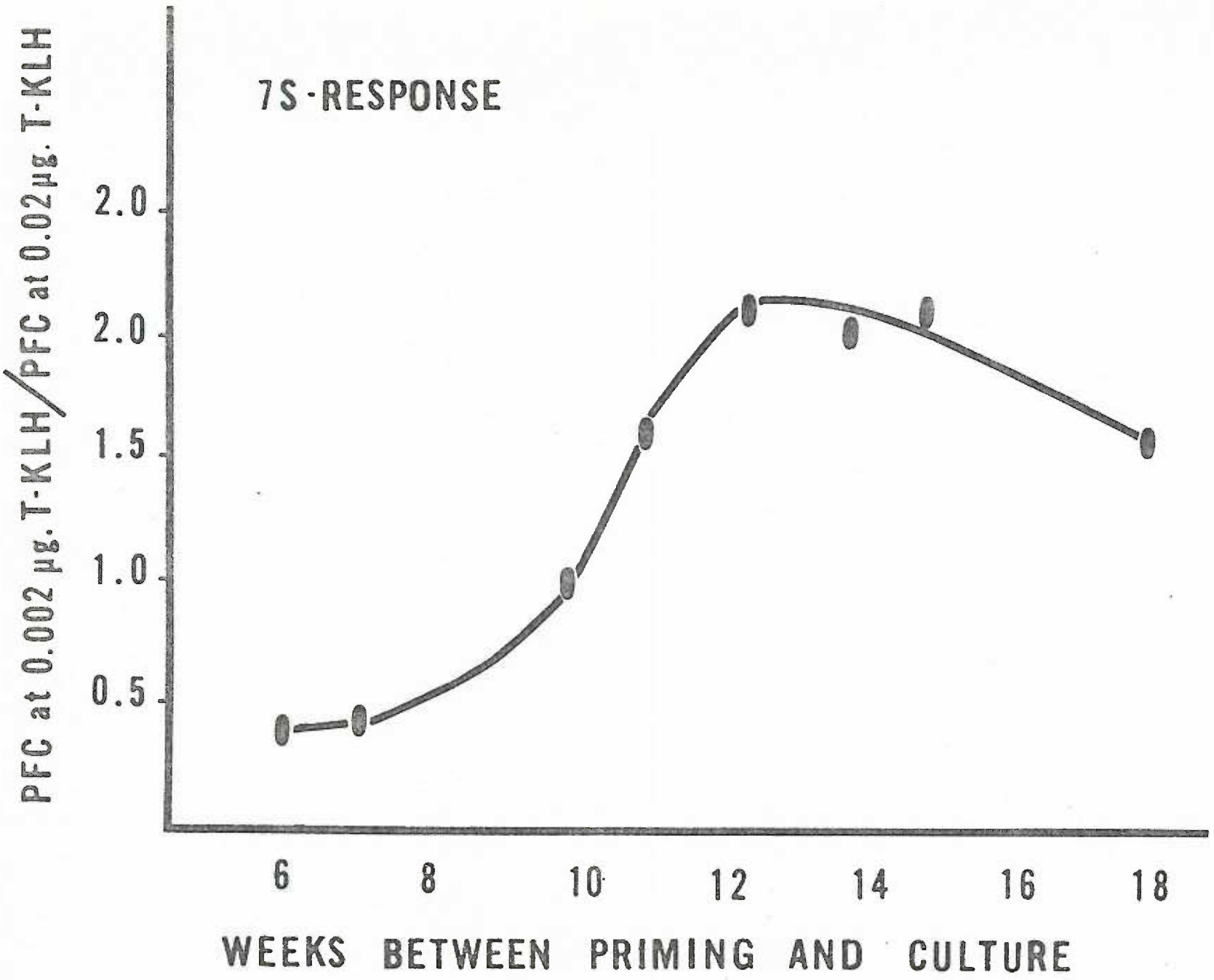

7S-RESPONSE
PFC at 0.002 µg. T-KLH/PFC at 0.02µg. T-KLH
2.0
2.0
1.5
1.0
0.5
6
8
10
12
14
16
18
WEEKS BETWEEN PRIMING AND CULTURE

Table III.

It can be seen that with time the lower antigen concentration becomes more stimulatory for 7S cells and maintains its stimulatory capacity for 19S cells; whereas the high antigen dose becomes suppressive for both 19S cells and 7S cells.

Table III

In Vitro Anti-TNP Response to 0.2 µg P TNP-KLH and 0.02 µg P TNP-KLH*
During two Intervals after Priming

Period after Priming		21-52 Days		70-93 Days	
Antigen concentration		0.2 µg P	0.02 µg P	0.2 µg P	0.02 µg P
Mean† PFC/10^6	7S	1106 ± 346	2029 ± 396	433 ± 209	972 ± 209
spleen cells	19S	695 ± 190	548 ± 150	354 ± 134	518 ± 139
Ratio: $\frac{\text{PFC 0.02 µg P}}{\text{PFC 0.2 µg P}}$	7S	1.83		2.24	
	19S	0.79		1.5	

*Culture conditions and assay as in Figure 4. Cells assayed on day 5 of culture.

†Mean of n cultures plated in quadruplicate ± 95% confidence interval, where n = 16 for 0.2 µg P, n = 15 for 0.02 µg P (days 21-52); n = 11 for 0.2 µg P, n = 10 for 0.02 µg P (days 70-93). Maximum 19S background from primary stimulation was 102 PFC/10^6 in the 21-52 day period and 160 PFC/10^6 in the 70-93 day period. There was no detectable 7S background.

19S Response: Increasing Antigen Sensitivity.

A more limited change in antigen sensitivity was observed with cells synthesizing 19S anti-TNP. 19S responses at two periods after priming are shown in Table III. Cells tested early after priming were twice as responsive to the higher antigen dose. It is of interest that the 19S response of cells to low antigen concentration remained constant between the observed periods whereas the 7S response to the low antigen dose during the same interval decreased indicating the suppression of 7S cells through increased sensitivity to antigen. In the early period IgM memory cells responded better to ten times the antigen concentration which stimulated maximum IgG responses (Table III).

Tolerance Induction *In Vitro*.

The suppression by high doses of antigen seen in Figures 3, 4, 9, 11 and 12 could be a non-specific toxic effect or a specific immunological effect (neutralization of antibody during the plaque assay or switching off of cells, i.e. immune tolerance). The *in vitro* stimulation of recently primed cells, by the same concentration of antigen which suppressed immune cells from animals long after priming (Figure 12), argues against nonspecific toxicity since nonspecific toxicity should apply equally to both sets of cells. Also, as will be shown later, TNP-KLH prevents only anti-TNP PFC and not other immune responses. Therefore either tolerance or neutralization of antibody during the plaque assay by high antigen concentration, or both, were likely causes of the observed suppression.

Immune Cell Rescue Experiments.

Attempts were made to rescue cells suppressed by high antigen doses by washing away the suppressing antigen. Cultures were initiated with high suppressive doses or low stimulating doses of TNP-KLH antigen.

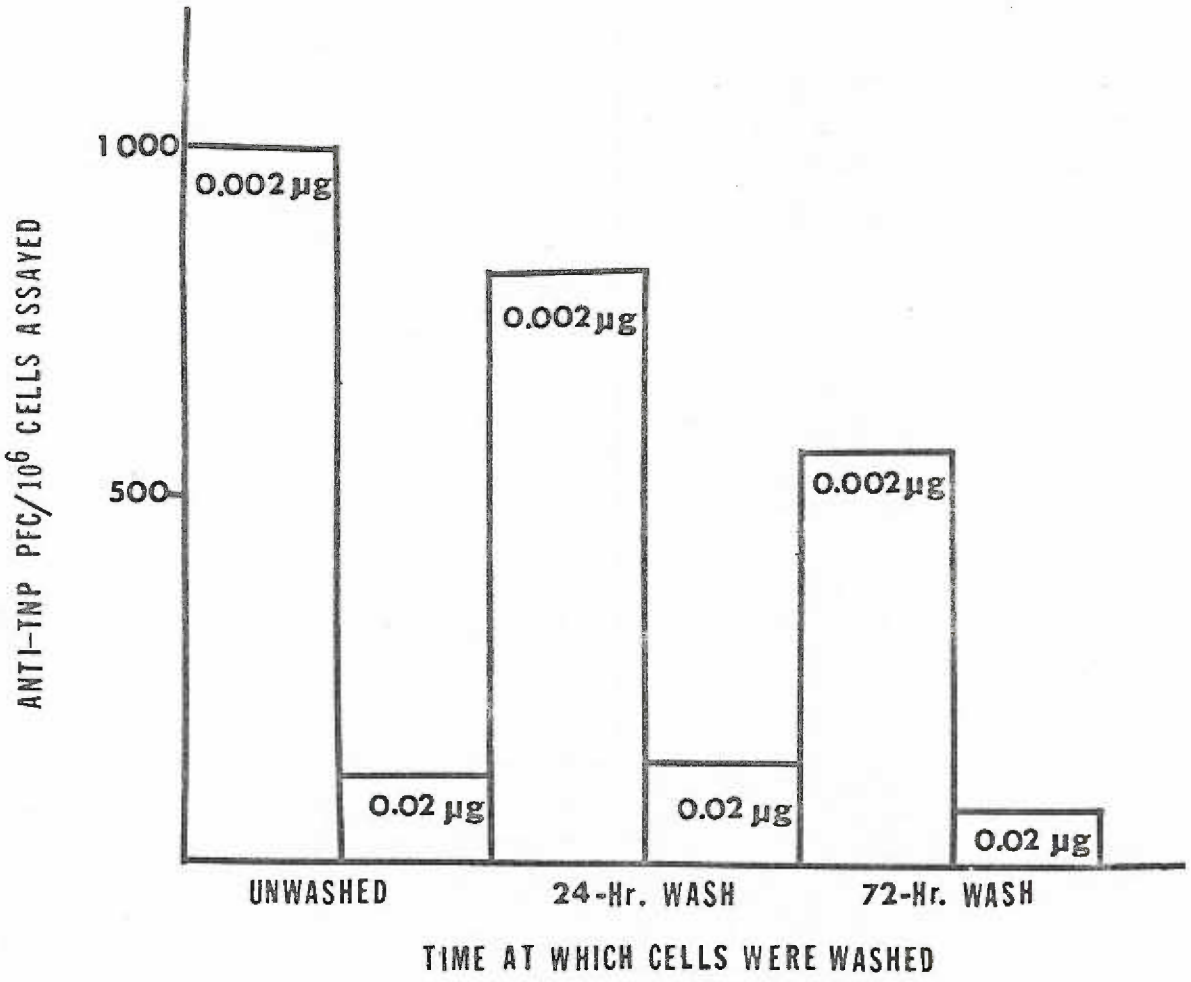
Separate cultures were then left unwashed, washed at 24 hr or at 72 hr (Figure 14). As can be seen, washing did not remove the suppression by high dose or the stimulation by low dose. Presumably as seen in Figure 5 the cells become more fragile with time which may explain why the washing procedure caused a small loss in PFC during the 24 and 72 hr washes. Furthermore several subsequent washing experiments showed that suppression was irreversible within three hours after the addition of high suppressive doses of antigen. This indicated that suppressive antigen was tightly bound to the cells, or had already suppressed the cells within a three-hour period, but in either case the antigen was not free to neutralize antibody during the PFC assay. Although improbable, some antigen may elute off the cells and cause some anti-TNP neutralization.

When is High Antigen Dose Most Suppressive?

To further distinguish between early tolerizing of cells during antibody induction and late neutralization of anti-TNP by the high antigen dose I compared suppression by high antigen doses added at initiation of culture (and washed out 3 hours later) with cultures to which the high dose was added 3 hours prior to plaque assay on day 5. Cultures were stimulated with optimum inducing doses of TNP-KLH added to washed cultures 3 hours after initiation. Cultures were washed prior to assay to make them comparable at assay. Figure 15 shows the level of suppression of PFC with high antigen doses added during culture initiation and just prior to assay as indicated. It was reasoned that if neutralization were occurring antigen would be least degraded and therefore most

Figure 14.

This experiment compares the effects of washing cells from cultures stimulated with optimum or suppressive doses of TNP-KLH antigen. Mouse spleen cells were cultured 13 weeks after *in vivo* priming. Cultures were given an optimal stimulatory antigen dose (0.002 µg protein TNP-KLH), or a suppressive antigen dose (0.2 µg protein (TNP-KLH). Cultures were then incubated and left or the cells were removed at 24 or 72 hours, washed 2 times and placed into fresh medium and new culture dishes and reincubated. Cultures were assayed on day 5 for anti-TNP PFC. As can be seen both the suppression and the stimulation were maintained in spite of the washing procedure. Some loss in PFC was observed in the stimulated cultures presumably due to excess cell handling during the wash procedure.


1000
500
ANTI-TNP PFC/10^6 CELLS ASSAYED
0.002 µg
0.02 µg
0.002 µg
0.02 µg
0.002 µg
0.02 µg
UNWASHED
24-Hr. WASH
72-Hr. WASH
TIME AT WHICH CELLS WERE WASHED

suppressive when added on the day of assay. However if the high antigen dose was interfering with initiation of antibody formation it would be most suppressive when added on day 0. Figure 15 indicates that a high antigen concentration added at day 0 is much more suppressive than when a high dose of antigen is added on day 5.

Burro Red Blood Cells (BRB) vs. TNP-KLH.

TNP-KLH primed mice were injected intraperitoneally with 0.5 ml of 20 percent wet packed BRBC in saline. The mice were rested six weeks and their spleen cells cultured. Cultures were each treated with one of a series of TNP-KLH doses or left untreated as controls, incubated three hours at 37°C, washed three times and cultured with optimal immunogenic doses of BRBC and TNP-KLH. All cultures were washed prior to assay. The harvested cells were assayed separately with TNP-SRBC and BRBC plaque assays. BRBC and SRBC do not crossreact. The results are shown in Figure 16. This experiment demonstrates that the suppression seen with high doses of TNP-KLH is antigen specific and not due to nonspecific toxicity since the anti-BRBC response was not suppressed by high doses of TNP-KLH whereas the anti-TNP response was suppressed 76 percent. The anti-BRBC response was slightly depressed by the immunogenic doses of TNP-KLH. A similar experiment is shown in Figure 17 in which the various doses added were left in the cultures throughout the culture period. Optimum immunogenic doses of BRBC were added on day 0. Again it can be seen that high TNP-KLH concentrations inhibit anti-TNP and have little effect on the anti-BRBC responses. In this experiment, as in that shown in Figure 16, the BRBC response was slightly suppressed by optimal doses of TNP-KLH, indicating possible antigenic competition.

Figure 15.

This experiment demonstrates that high suppressive doses of antigen are most inhibitory when added during the early stages of antibody initiation rather than when added just prior to assay. Cells from mice were primed to TNP-KLH and BRBC were used. Cell cultures were prepared and incubated at the start of culture with the antigen doses shown on the abscissa for 3 hours and then washed 2 times (●——●). A second set of cultures from the same cell pool were incubated without antigen and washed. Both sets of cells were then given an optimal dose of TNP-KLH antigen (0.02 µg protein TNP-KLH) and incubated in fresh medium and new culture dishes, for 5 days. Three hours prior to assay the second set of cultures were treated with the various doses of TNP-KLH shown on the abscissa for three hours (●----●). All cultures were washed and assayed on day 5. Each value shown is the mean of three cultures plated in quadruplicate. Values are plotted on a log-log scale.

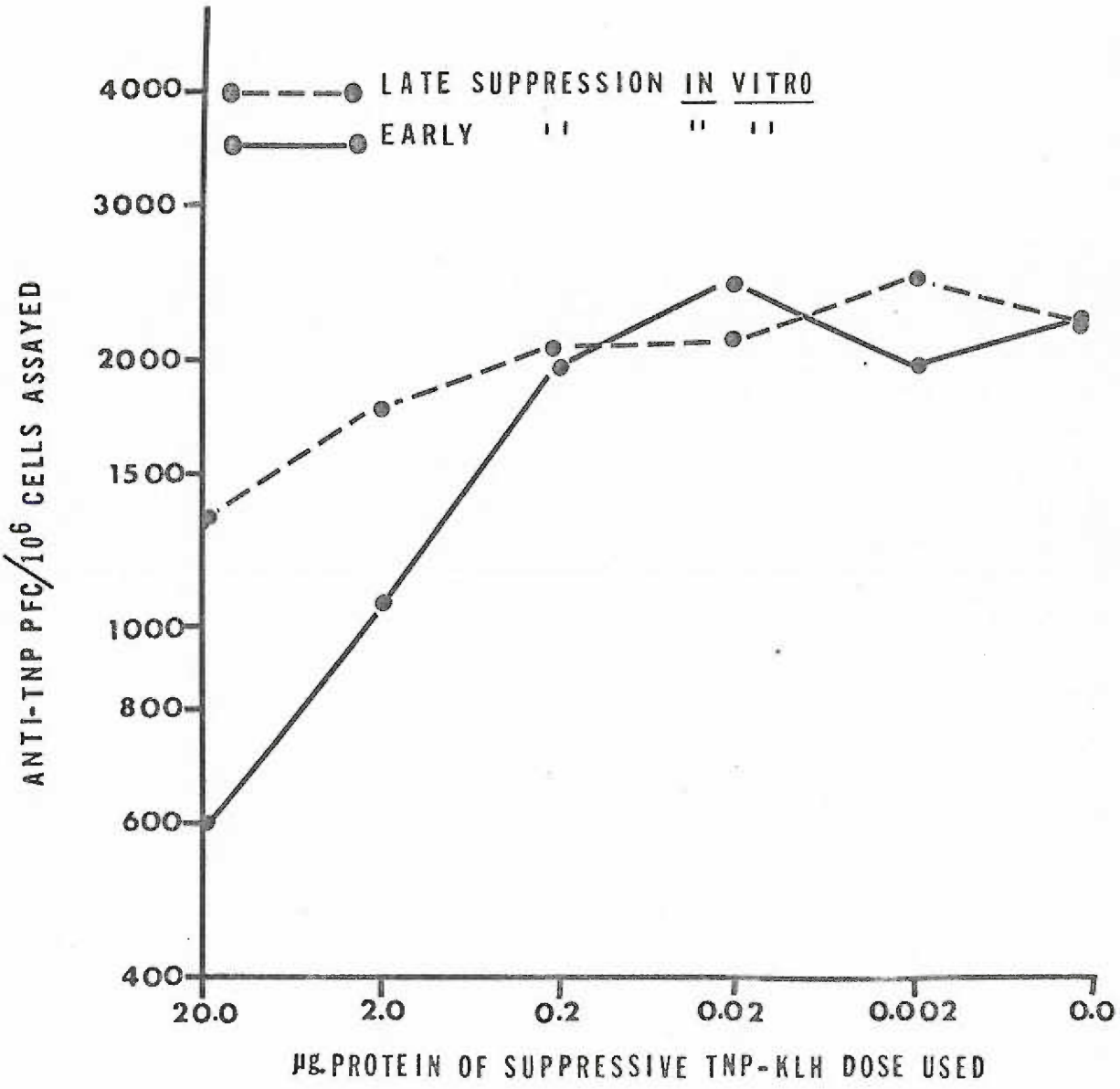

LATE SUPPRESSION IN VITRO
EARLY " " "
4000
3000
2000
1500
1000
800
600
400
ANTI-TNP PFC/10^6 CELLS ASSAYED
20.0
2.0
0.2
0.02
0.002
0.0
µg. PROTEIN OF SUPPRESSIVE TNP-KLH DOSE USED

Figure 16.

This is a log-log plot of an experiment comparing the suppressive activity of TNP-KLH on induction of anti-TNP and anti-BRBC responses occurring simultaneously in the same cultures. Cell cultures were prepared and incubated for 3 hours at the start of culture with the antigen doses shown on the abscissa and then washed 2 times. Cell cultures were then placed in fresh medium and new culture dishes with the doses of TNP-KLH (0.02 µg protein) and BRBC, (one drop of a one percent suppression) which would produce maximum antibody responses. All cultures were washed just prior to assay on day 5. The total PFC response is shown. Note that high doses of TNP-KLH suppressed only the anti-TNP response and not the BRBC response.

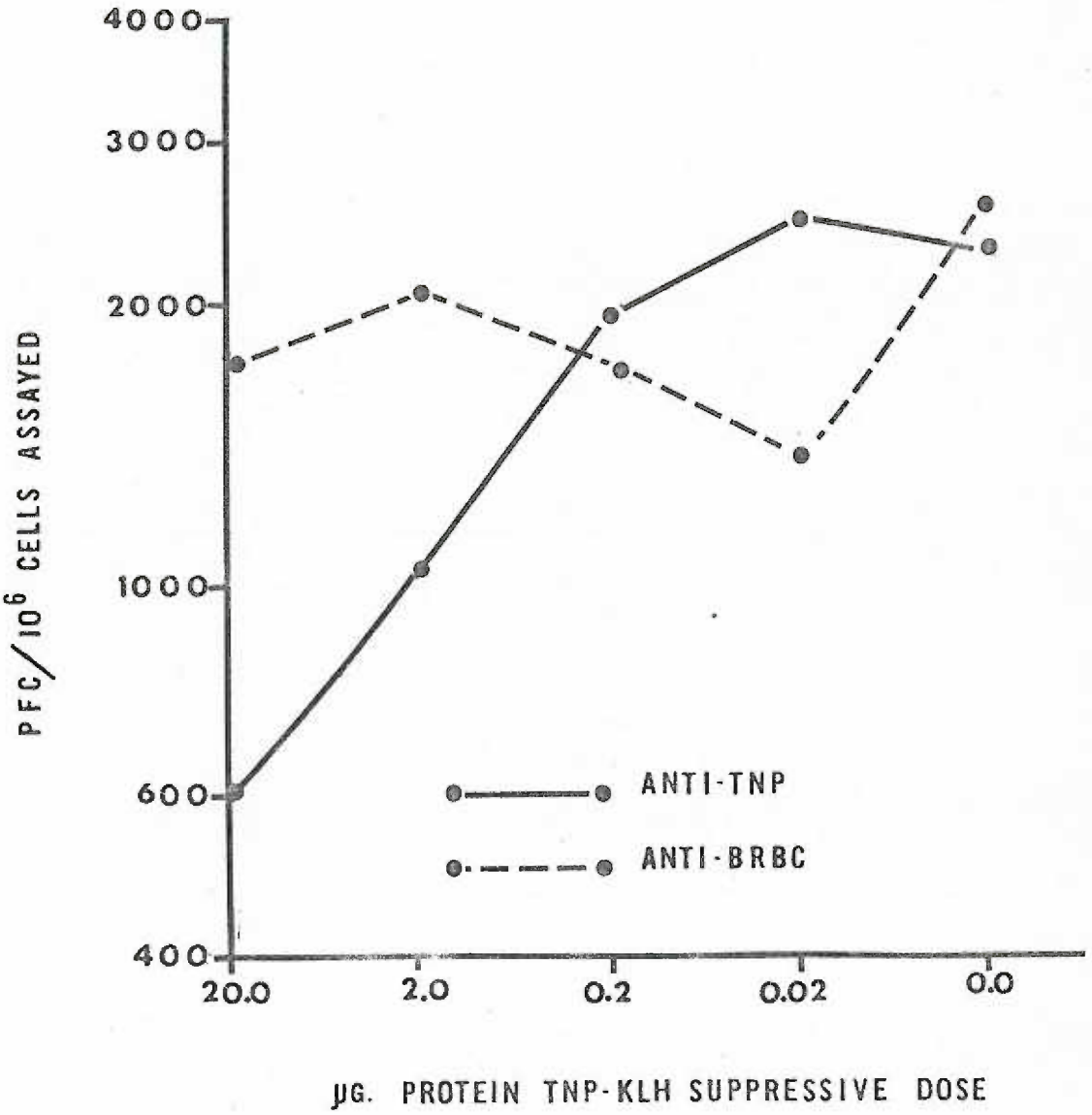
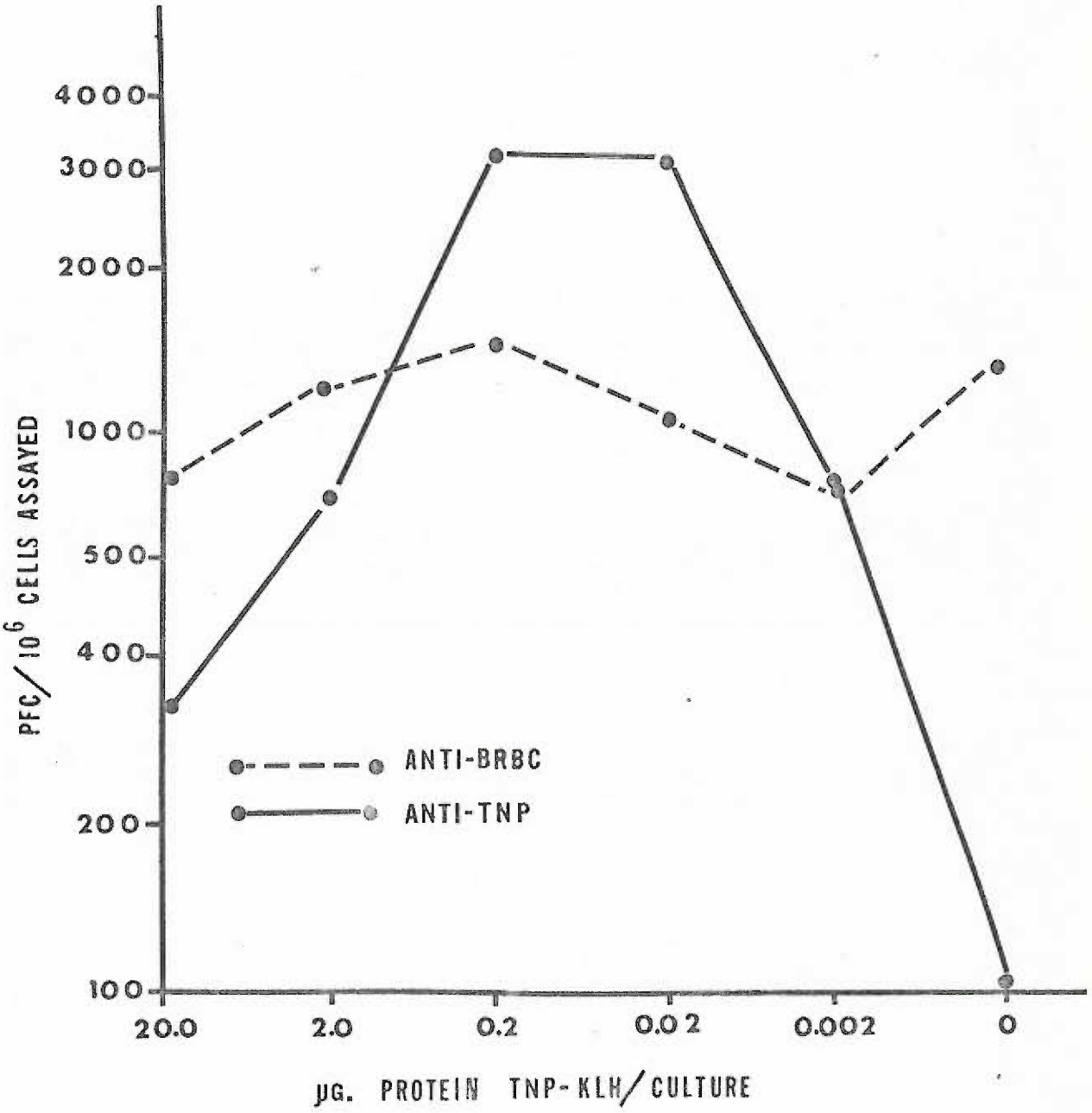

4000
3000
2000
1000
600
400
PFC/10^6 CELLS ASSAYED
ANTI-TNP
ANTI-BRBC
20.0
2.0
0.2
0.02
0.0
µG. PROTEIN TNP-KLH SUPPRESSIVE DOSE

Figure 17.

This is a log-log plot of an experiment in which several doses of TNP-KLH were separately added with an optimal dose of BRBC to cultures of sensitized mouse spleen cells. This experiment is identical to that shown in Figure 16 except that both the suppressive doses of TNP-KLH tested and the optimal BRBC dose were added at time 0 and left in culture until the cultures were assayed on day 5. In this experiment it can be seen that the entire dose range from 0 - 20.0 µg protein TNP-KLH had little affect on the response to BRBC.


4000
3000
2000
1000
500
400
200
100
PFC/ 10^6 CELLS ASSAYED
ANTI-BRBC
ANTI-TNP
20.0
2.0
0.2
0.02
0.002
0
µG. PROTEIN TNP-KLH/CULTURE

Which Components of the TNP-KLH Antigen Are Suppressive?

High doses (2.0 µg protein) of either KLH and/or TNP-BSA were added to cultures together with an optimal immunogenic dose of antige, 0.002 µg protein TNP-KLH. Other cultures were incubated first with the high doses for 4-5 hours and then washed after which they were challenged with optimal immunogenic doses of TNP-KLH antigen. Table IV indicates that 2.0 µg KLH or 2.0 µg TNP-BSA were suppressive but that neither reactant alone was as suppressive as 2.0 µg of the intact antigen TNP-KLH. Preliminary experiments showed that doubling the concentration of either component alone did not increase its paralytic effectiveness.

When administered together suppression by KLH and TNP-BSA was additive as shown in the last column of Table IV. In two of three cases suppression with both components exceeded that caused by intact antigen. These results indicate that hapten and carrier determinants are separable and that each attacks a different site in establishing immunological unresponsiveness.

Is Suppression Due to the Concentration of Hapten or the Concentration of Protein Molecules?

Two TNP-KLH preparations with a 10-fold difference in hapten substitution were used to determine whether equal moles of protein or equal moles of TNP produced similar degrees of suppression. In Figure 9 two sets of cultures are compared. One set contained cultures each of which was challenged with one of a range of high hapten-substituted antigen concentrations and the other set of cultures was similarly challenged with low hapten-substituted antigen. It can be seen that suppression is dependent on this concentration of protein rather than the number of

Table IV.

*Mean of 8 cultures. Total anti-TNP plaque count after facilitation with goat anti-mouse IgG ± 95% confidence interval. The mean background anti-TNP plaque count in cultures not challenged with antigen was 111 ± 32/10^6. †p derived from student t test comparing mean of separate KLH or TNP-BSA paralysis with mean of cultures paralyzed with KLH + TNP-BSA. All cultures were treated with paralyzing antigen were suppressed significantly compared to unparalyzed control (all $p < 0.001$).

Table IV. *Additive effects of separate hapten and carrier components in inducing secondary anti-hapten immune unresponsiveness in vitro*

Experiment Number		Paralyzing antigen (2.0 µg protein)				
		None	TNP-KLH	KLH	TNP-BSA	KLH + TNP-BSA
1. Cells incubated with paralyzing antigen and challenged by 0.002 µg TNP-KLH. No washing.	Mean* PFC/10^6	9723 ± 1956	625 ± 267	3421 ± 613	1731 ± 486	1046 ± 200
	p†			<0.01	<0.01	
2. Cells incubated with paralyzing antigen 4-5 hr/37°C, washed and challenged by 0.002 µg TNP-KLH	Mean* PFC/10^6	6804 ± 1486	1662 ± 427	3118 ± 806	2568 ± 796	370 ± 170
	p†			<0.001	<0.001	
3. Cells incubated with paralyzing antigen 4-5 hr/37°C, washed and challenged by 0.0002 µg TNP-KLH	Mean* PFC/10^6	11,299 ± 1720	2251 ± 580	3995 ± 865	4987 ± 1401	1262 ± 551
	p†			<0.001	<0.001	

TNP hapten groups added to culture. In fact, if anything, the low hapten-substituted antigen was more effective at lower concentrations. However two potential complicating factors must be considered when interpreting these data. Coupling high amounts of TNP to KLH induces increased denaturation and secondly high TNP substitution may increase dissociation of the tertiary structure of KLH into more numerous smaller subunits. Since preparation of KLH and TNP-KLH involved pelleting the materials in a like manner with a preparative ultracentrifuge, it is unlikely that the KLH underwent a significant loss in sedimentation value (Rittenberg, Amkraut, 1966). High TNP substituted KLH was able to induce a large response in cells from animals primed to antigen with a low hapten ratio, thus indicating that the immunogenic configuration was not grossly altered.

Requirement for Anti-KLH Carrier Cells During the *In Vitro* Response to TNP-KLH.

Several experiments in this laboratory by J. Jennings, M. B. Rittenberg and K. Pratt showed that primary responses to TNP-KLH could be obtained *in vitro* with cells from normal mice. However, these responses were generally low and infrequent. To determine whether the inconsistency of these primary responses might result from a deficiency of anti-KLH (carrier) in the spleens of immunologically virgin mice, mice were primed with KLH prior to culture. Figure 18 indicates that anti-KLH cells induced by preimmunization with KLH alone were able to help in inducing a large primary response to TNP. The suppressive capability of excess KLH in removing this helper effect seen in Table V indicates the specificity of the helper cell effect. A 7S response was not observed indicating the primary nature of the anti-TNP response.

Figure 18.

This is a semi-log plot of an *in vitro* primary response to TNP obtained with cells from mice primed to the carrier used, KLH. The results of two experiments were pooled. Each value is the mean of 4 cultures (except: 0.002 µg - day 5, 6 = 3 and 0.02 µg - ay 6 = 6). All antigen stimulated cultures were significantly higher than the control on both day 5 (●——●) and day 6 (●----●). Mice were injected intraperitoneally 2-3 weeks prior to culture with 100 µg protein KLH or bentonite. Normal unprimed mice did not form significant anti-TNP responses when challenged *in vitro* with TNP-KLH.

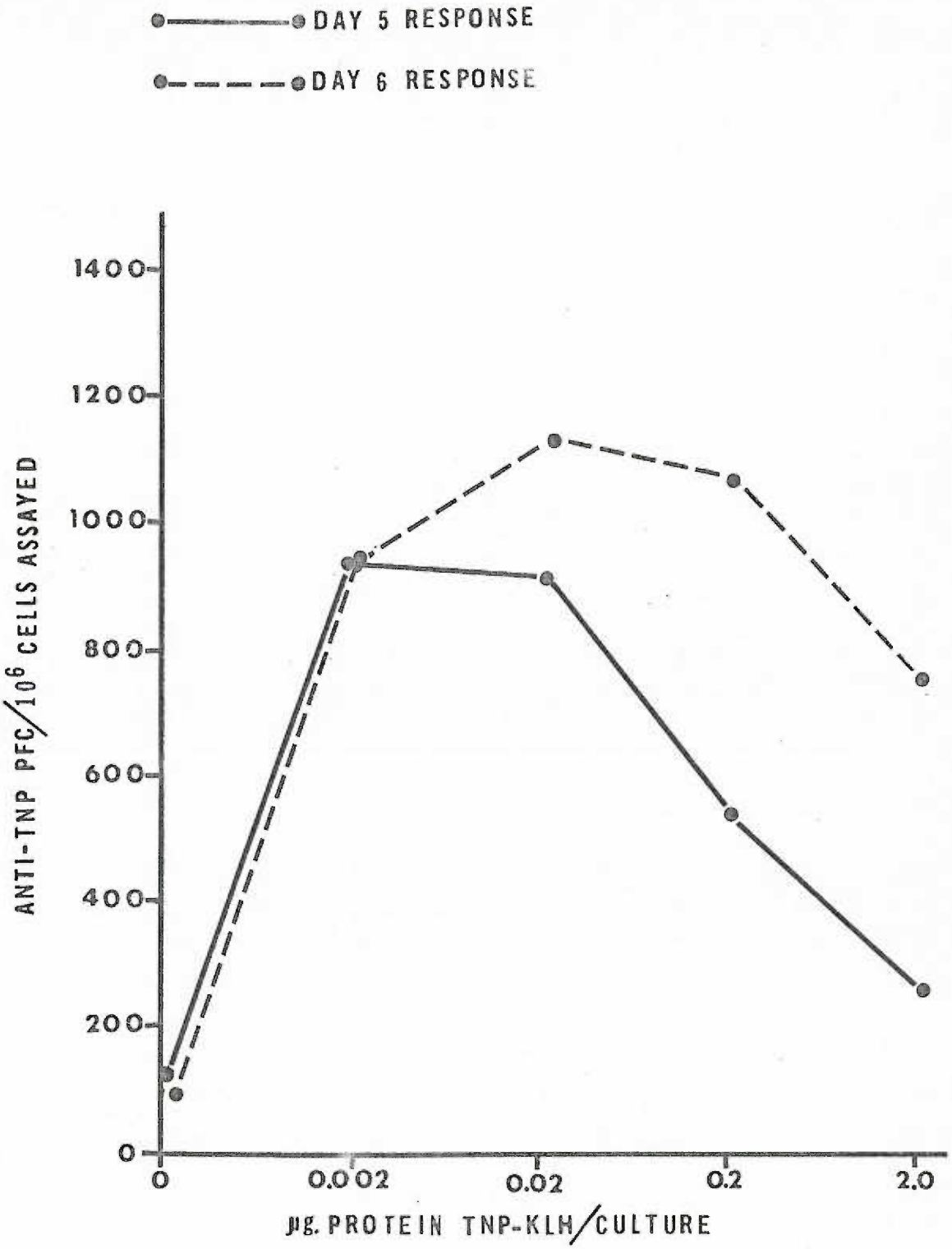

DAY 5 RESPONSE
DAY 6 RESPONSE
ANTI-TNP PFC/10^6 CELLS ASSAYED
1400
1200
1000
800
600
400
200
0
0
0.002
0.02
0.2
2.0
μg. PROTEIN TNP-KLH/CULTURE

Table V.

Spleen cells from animals primed intraperitoneally with 100 µg protein KLH-bentonite were challenged 3 weeks later *in vitro* with 0.002 µg protein TNP-KLH and assayed on days 5 and 6 for anti-TNP PFC. To determine if KLH recognizing cells were in fact responsible during augmentation of the anti-TNP response, some of the responding cultures were each treated with one of the three doses of soluble KLH shown. It can be seen that all doses of KLH suppressed the anti-TNP response significantly.

Table V

Suppression of the Primary *In Vitro* Anti-TNP Response to TNP-KLH with Excess Concentrations of the Carrier KLH*

	µg Protein/Culture of KLH added day "0"	Anti-TNP PFC/10^6 cells assayed†	
		Day 5	Day 6
Cultures of KLH sensitive cells were all challenged *in vitro* with 0.002 µg protein TNP-KLH at day "0"	None	932 (1083-781)	926 (1216-636)
	2.0	106 (149-63)	32 (44-20)
	0.2	63 (82-44	59 (74-44)
	0.02	163 (192-134	107 (156-58

*Background PFC values of control cultures without TNP-KLH or KLH were 110 (146-74) on day 5 and 34 (53-15) on day 6.

†Values are the mean of 3 cultures, plated in quadruplicate with the 95% confidence intervals shown in parenthesis.

DISCUSSION

Anti-TNP *In Vitro* Model.

This thesis presents data obtained with an experimental model which permits a study of the *in vitro* induction of an anti-hapten secondary immune response. This response is detected as an increase in the number of cells synthesizing anti-TNP antibody (plaque-forming cells or PFC) on days 0-6 of culture. Passive release rather than active synthesis of antibody can cause false plaques (Roseman, Lesserman, Fitch, Rowley, 1969). However, the data indicate that PFC formation requires actively metabolizing viable cells since a) plaques were prevented by heat-killing although the temperature used (56° for 30 min) does not affect antibody, b) 10^{-5} M Methotrexate and 100 µg chloramphenicol/culture, both known to inhibit mammalian cell division, prevented plaques, and c) isotope-labeled anti-TNP recovered with TNP-BSA-anti-BSA precipitates and also detected by radioimmunoelectrophoresis demonstrated that antibody was actually synthesized *in vitro*. I interpret the chloramphenicol and Methotrexate data to most likely indicate a cell division requirement in light of the supportive more extensive data obtained with a similar system by Dutton and Mishell (1967). The data probably reflect *in vivo* normal immune processes since the 7S and 19S results obtained *in vitro* are similar in size and kinetics to *in vivo* anti-TNP responses. The large IgG response obtained is typical of secondary responses. Earlier studies questioned the development of IgM memory (Uhr, Finkelstein, 1963) (Svehag, Mandel, 1964; however, subsequent reports have confirmed its existence (Borel, Fauconnet, Miescher, 1964) (Nossal, Austin, Ada, 1965) (Pierce, 1969). The data confirm that IgM memory exists as reflected by increased numbers of

cells synthesizing IgM antibody. This increase is many fold larger than that observed in primary anti-TNP responses *in vivo* (Rittenberg, Pratt, 1969) or *in vitro* (Jennings, Rittenberg, 1971).

19S vs. 7S Antibody.

Mäkelä and Kostiainen (1967) proposed a model to explain the increased propensity of both low hapten substituted antigen and low antigen concentrations to induce 7S antibody over 19S antibody (Figure 7, 8, and 9). They argued that 19S immunoglobulins have a greater valency than 7S immunoglobulins. This increased valency would give an advantage to cells with 19S surface-receptors for antigen during the binding of that antigen to the cell surface. This advantage would arise however only if there were enough hapten groups in a localized area on the antigen, permitting saturation of the valency provided by any one lymphocyte surface receptor. Thus high hapten substituted antigens would favor 19S stimulation. The only 7S cells to arise would be those with exceptionally high affinity antigen-receptors (therefore not requiring a high valency). Two events may then alter this selective process. 1) The resultant antibody may bind to many of the hapten groups on the immunogen retained *in vivo* thereby lowering its effective hapten concentration and reducing its tendency to favor 19S cells. 2) Simultaneously, decreasing antigen concentration resulting from catabolism and immune clearance would select for cells best able to compete for antigen (those with highest avidity, now the 7S population) causing an overall switch from 19S to 7S antibody induction. Mäkelä and Kostiainen's (1967) data were less direct since they only followed antibody titers

rather than numbers of cells. This can give an inaccurate measure of the relative numbers of 19S and 7S cells as seen in Figure 10. Presumably this difference is due to the rate of synthesis and catabolism of 19S and 7S immunoglobulins. The data seen in Figures 7, 8, and 9 confirm Mäkelä and Kostiainen's hypothesis although it is not clear why the animals primed to low substituted antigen (Figure 9A) did not produce cells as responsive to low substituted antigen as did animals primed to high substituted antigen.

Antigen Selection Model.

It is clear from the results seen in Figures 8, 12, 13, and Table III that the antigen dose giving maximum recall depends on the interval between *in vivo* priming and *in vitro* challenge. Although Iványi and Černý indicated recently (Iványi, Černý, 1969) that antigen dose is a key factor in the immune response, few systematic studies relate challenge-dose effectiveness with time after priming. It has been established that there is a critical time after priming in which a given dose will produce a maximum anamnestic response (Fecsik, Butler, Coons, 1964) and that less antigen is required for secondary than for primary stimulation (Tao, 1968) (Daniels, Weigle, 1968). These *in vivo* and *in vitro* observations suggested changes in the immune memory cell population but did not reveal whether these changes were quantitative or qualitative. The data presented in this thesis indicates that those memory cells which continue to develop have an increased avidity for antigen as evidenced by increasing sensitivity to that antigen. Paul, Siskind, and Benacerraf (1968) were unable to detect a consistent change in avidity for antigen (sensitivity to lower antigen doses) leading to thymidine incorporation in cells harvested for culture either 7-19 days or 5-7 months after immunization. In contrast Valantová, Černý,

and Ivanyi (1967) reported that the dose of human serum albumin required to give a maximum IgG serum titer at 16 weeks after priming was one tenth that required at 6 weeks. However, similar increased sensitivity was not observed with SRBC antigens after longer rest periods (Richardson, Moorhead, Reedy, 1969). Our results concur with and expand the findings of Valantová, *et al.* as can be seen in Figures 8, 12, 13 and Table III. Those animals which were rested longer developed memory cells requiring less antigen in culture both for detectable threshold stimulation and for maximal stimulation. The inability of Paul, *et al.* (1968) to find this change in sensitivity may have been due to their protocol which favors the detection of delayed sensitivity or T cells by thymidine incorporation and not antibody production of B cells. It is not known if T cells undergo antigen mediated selection for high avidity cells similar to that observed for B cells.

The ability to detect these cellular changes in sensitivity here may result from following the response to a restricted determinant, TNP and quantitating the number of individual cells synthesizing antibody *in vitro*. This *in vitro* model eliminates such *in vivo* complications as cell migration, antigen dilution, and catabolism or localization of antigen outside of the immune tissue being studied; however, it exposes the same tissue to antigen-antibody complexes which could be eliminated *in vivo* (Campbell, Garvey, 1963). However, washing cultures at 24 or 72 hours after initiation (Figure 14) to remove these complexes did not alter the results.

In this system with time after priming there are fewer anti-TNP memory cells (Bullock, Rittenberg, 1970b). This leads us to conclude that with time after priming only that portion of the memory cell population

with high sensitivity for antigen continued to develop under the continual *in vivo* selective pressure of antigen. Although we did not assay for *in vivo* KLH it has been detected in rabbits one year after injection (Campbell, Garvey, 1963). Furthermore, persistent stimulation by KLH was required for mouse anti-KLH memory cell development to continue for 30 days after priming (Cerottini, Trnka, 1970). Consequently the results in Figures 8, 12, 13, and Table III were interpreted to reflect the continued presence of and selective pressure by persisting immunogen. This conclusion is of theoretical interest because of the role of receptor affinity in the cell selection models proposed in recent years (Eisen, 1966) (Siskind, Benacerraf, 1969) (Talmage, 1957). This model was based on observations that with time after hapten immunization the serum contained antibody of high affinity. The model consists of the following proposals: a) that cells are stimulated by contact with antigen above a threshold concentration which is related directly to the affinity of the resultant antibody (Eisen, 1966); b) that each cell and/or its progeny make antibody of only one affinity (Eisen, 1966) (Klinman, 1969), and c) that competition for diminishing concentrations of antigen results in the selection of cells synthesizing high affinity antibody (Eisen, 1966) and/or nonsynthesizing helper cells implicated by cell interaction studies covered later in this discussion. As will be made clear it is possible that such helper cells possess similar receptor sites and also undergo antigen-selected proliferation.

Most experiments supporting the antigen selection model have utilized changes detected in the affinity of the antibody produced rather than on a direct demonstration of the development of cells with increased

sensitivity to antigen. The temporal increase in *in vitro* inductive capacity of lower TNP-KLH doses reported here provides direct cellular evidence for development of cells with increased avidity for antigen most likely reflecting the selective pressure of persisting antigen *in vivo*.

Tolerance.

Dose-response curves such as shown in Figures 3, 10, 12, 17, and 18 are similar to those obtained by Mäkelä and Mitchison (1965) *in vivo* when studying the suppression of anti-albumin memory cells in irradiated hosts. These figures all demonstrate that high TNP-KLH doses suppress the anti-TNP response. Failure of some investigators to detect suppressive effects with high antigen doses may have been due to overlapping optimum concentrations of the determinants on complex immunogens used to follow the immune response (Dutton, Eady, 1964). The suppression of *in vitro* anti-TNP responses by high antigen doses, as we have noted previously (Bullock, Rittenberg, 1970b), resembles the high zone immunological tolerance described by others for a variety of antigens *in vivo* and for flagellin and endotoxin *in vitro* (Dresser, Mitchison, 1968) (Diener, Armstrong, 1969) (Britton, 1969).

The failure of washing to remove the suppression (Figures 14 and 15) and relative failure of high doses of antigen added on day 5 of the culture (Figure 15) argue strongly for suppression of some inductive stage of antibody synthesis. This suppression is specific since high doses of TNP-KLH do not suppress a concomitant anti-BRBC immune response while simultaneously suppressing the anti-TNP response in the same culture (Figures 16 and 17). BRBC and SRBC antigens do not cross react (Dutton, Mishell, 1967). In addition an optimum BRBC dose did not

induce anti-TNP plaques (Figures 16 and 17). This strongly argues against suppression due to generalized nonspecific toxicity with high TNP-KLH doses. Also Figure 12 indicates that suppressive capacity also depends on time after priming and not just antigen dose, again arguing against nonspecific suppression. It appears as can be seen in Figure 9 that over the substitution and dose range tested the ability to suppress is dependent upon the number of separate molecules rather than the numbers of hapten units per molecule.

This suppression may or may not be reversible. As can be seen in Figure 5, the total number of cells is slowly decreasing and cultures beyond six days have not been attempted. However up to day 6 of culture paralysis could not be reversed even when cells were washed within 3 hours after adding the high antigen dose at time 0. A longer period of culture might show this paralysis to be reversible as Byers and Sercarz (1970) noted with cultured spleen fragments paralyzed with excess BSA although the pattern of distribution within a tissue fragment adds a degree of complexity which makes direct comparison with this model difficult. The above data show: a) that high TNP-KLH antigen doses suppress the anti-TNP response, b) that this suppression is antigen specific and not a general toxic effect, c) that the shift in dose required for suppression correlates with increased antigen avidity of anti-TNP memory cells and d) that the suppression is due to an initial step in the induction of immunity and not the suppression of antibody release or neutralization of released antibody. The above provide substantial evidence that the observed suppression is a form of high zone immunological tolerance.

Functional dissection of the immunogen TNP-KLH was carried out by investigating the high-dose tolerance phenomenon. As can be seen in Table IV high doses of both sensitizing hapten TNP (bound to a protein to which the mice were not sensitized, BSA) and carrier protein KLH were each suppressive when added to separate TNP-KLH low-dose-stimulated cultures. Neither component alone was as suppressive as the equivalent amount of complete antigen TNP-KLH even though the moles of TNP provided by TNP-BSA or protein provided by KLH equaled that provided by TNP-KLH. When both components, hapten and carrier (TNP-BSA + KLH) were added to the same culture, suppression was greater than with either component alone and in two of three cases was greater than that produced by the same concentration of complete antigen (Table IV). It can be concluded that carrier and hapten are separately paralytic when added *in vitro* to TNP-KLH sensitized cells and the increased suppression by both indicates each reacts at different receptor sites critical to the immune response to TNP.

Cell-cell interaction reportedly occurs between bone marrow derived B cells which can secrete antibody and thymus derived helper T cells (Claman, Chaperon, Triplett, 1966) (Mitchell, Miller, 1968) (Raff, 1970) (Chan, Mishell, Mitchell, 1970). Recently Chiller, Habicht and Weigle (1970) reported that paralysis of either cell type prevents antibody formation. Since the data demonstrate that paralysis can result from separate action by hapten or carrier it is reasonable to assume each acts on different cells. Tests of this assumption showed that cultures incubated separately with TNP-BSA or KLH, washed and then pooled, elicited an immune response 2-5 times that seen in cultures incubated simultaneously with KLH and TNP-BSA (Rittenberg, Bullock, 1970, in

preparation) supporting the supposition that hapten and carrier each paralyze a site on a separate cell.

Paralysis by KLH likely is due to inactivation of carrier cells since only the anti-TNP cell population was measured. Tolerance to proteins reduces their carrier capacity in anti-hapten responses (Cinader, Dubert, 1956) (Green, Paul, Benacerraf, 1968). Anti-KLH memory cells appear to play an important role in the induction of anti-TNP antibody even in the primary response as seen in Figure 18 and Table V. TNP-BSA could bind to antibody-producing cells and prevent bridging to carrier cells when the intact stimulatory antigen TNP-KLH is added. Even after completion of cell interaction high levels of TNP-BSA on the cell surface might affect anti-TNP production. However as previously stated suppression is greatest when high antigen is added at the initiation of cultures (Figure 15) and paralytic doses added to stimulated cultures on day 3 or 4 produced only 25-30% of the suppression obtained when paralytic doses were added on day 0 (Bullock, Rittenberg, 1970b). This indicates that a direct affect on inductive processes is the more important means of suppression. This conclusion is strengthened by the finding that KLH, which does not react with TNP receptors, also paralyzes.

A general model is presented in Figure 19 which would explain some of the observed phenomenon. Closely related models were proposed by Bretcher and Cohen (1970) and Plescia (1969). This model assumes that anti-carrier T cells are required to induce a large increase in the clone size by stimulating cell division of each specific immune cell population. If, however, a large responsive population of B cells existed (via prior immunization or broad specificity receptors), T cells would not be an absolute requirement for the induction of antibody.

Since this model assumes that binding of antigen by B cells induces that cell to produce and secrete antibody, increases in antigen concentration (allowing lower affinity receptors to bind antigen) would cause additional B cells with lower affinity to release antibody. This is testable since equal antibody titers induced in the absence of T cells would have a lower average affinity. Secondary responses to haptens or heterolgous carrier proteins have been repeatedly reported (Rittenberg, Campbell, 1968) (Steiner, Eisen, 1967) (Paul, Siskind, Benacerraf, Ovary, 1967) (Brownstone, Mitchison, Pitt-Rivers, 1966). This recall could occur via two different mechanisms: a) the hapten could simply turn on antibody production in a well-developed population of B cells without T cell intervention and therefore without cell division, or b) T cells could develop which recognize the hapten itself eliminating the need for carrier determinant recognition. Paul, Siskind, Benacerraf and Ovary, (1967) reported that challenge with hapten on a heterologous carrier produced antibody with increased affinity. This argues for T cell involvement with multiplication of a select subpopulation of cells of high avidity antigen selected cells. In addition, evidence that T cells may recognize the TNP hapten was recently presented by Jennings and Rittenberg (1971). They have shown that TNP on the bacteriophage T4 induced a primary response without prior carrier immunization and in addition anti-TNP responses were not increased by prior immunization with T4. This is not always the case as seen in Figure 18 and Table V. Without KLH *in vivo* priming, anti-TNP responses did not occur *in vitro*, when cells were challenged with TNP-KLH. This difference between TNP-T4 and TNP-KLH may be due to either the steric arrangement of the bound

hapten, concentrations of the hapten per antigen molecule, or both and is currently being studied.

In accord with the above model the data in this thesis show that regulation of the immune response can be modulated by antigen dose and by separable components of a macromolecular antigen. They show clearly that antigen components act separately in inducing immune paralysis; the separated components act at different sites and these sites appear to be on different cells. Ability to paralyze carrier cells (cellular immunity?) or determinant cells (humoral immunity) with separated components of a complex immunogen may prove useful in immune therapy when it is desirable to suppress selectively one limb of the immune apparatus such as the humoral response associated with tumor enhancement (Hellström, Hellström, Bill, Pierce, Yang, 1970) or the cellular response associated with graft rejection.

Figure 19.

This figure presents a model proposed to explain some of the tolerance data reported in this thesis. The normal response depicts a delayed immune cell or T cell being bridged to an antibody-producing or B cell via TNP-KLH antigen. The T cell is bound to a KLH determinant and the B cell is bound to TNP. The binding processes induce the T cell to release a localized effective concentration of mitogen which prevents further differentiation of the B cell and induces a clone of identical B cells through mitosis. The continuous binding of TNP to the B cell induces the synthesis and secretion of anti-TNP antibody.

Tolerance is shown as any process which caused stimulation but prevents a close localization of the reactive B cells and T cells and therefore prevents a large mitotic response. If this is brought about via high KLH concentrations the B cells may remain unaffected and can be stimulated with new T cells. Blocking of T cell interaction with hapten or an ineffective carrier removes B cell activity. Excess antigen can bring about both processes.

Symbols:

T = Thymus-dependent cell

B = Antibody-forming cell from bone marrow.

—● = TNP determinant

≷ = KLH

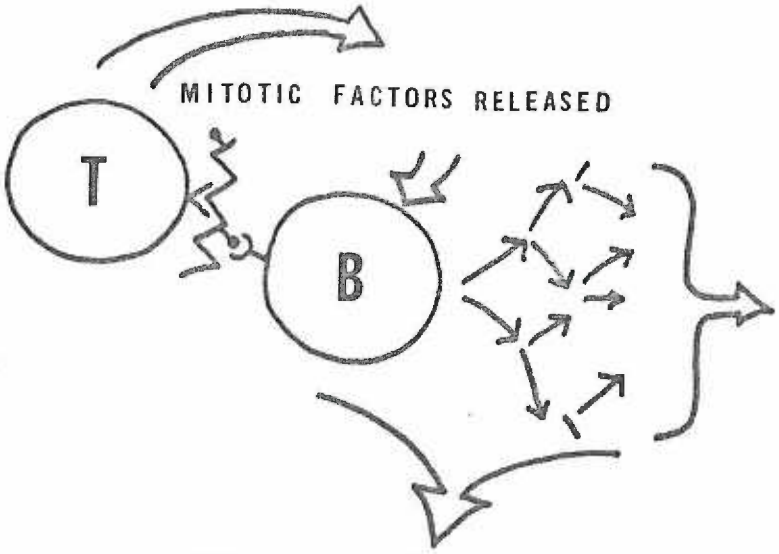
—(= TNP receptor on cell surface

< = KLH receptor on cell surface

▯ = BSA

P = End-stage plasma cell

IMMUNIZATION

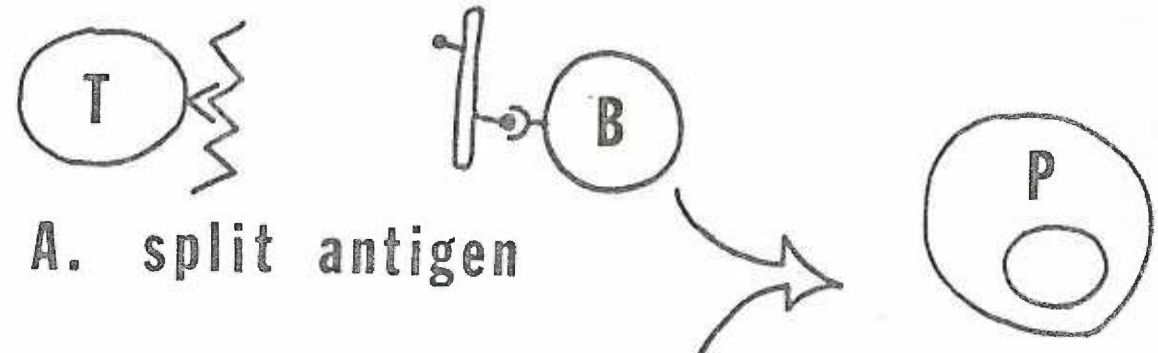


A. RAPID CELL DIVISION

B. SPECIFIC CLONE BUILDUP

C. MEMORY CELLS REMAIN

TOLERANCE



A. split antigen

B. excess antigen

A. DIFFERENTIATION TO END CELL

B. LIMITED CELL DIVISION

C. LOW ANTIBODY TITER

SUMMARY AND CONCLUSIONS

An experimental model was developed to study the secondary immune response to a chemically defined haptenic determinant (TNP). The response was initiated under controllable *in vitro* conditions with sensitized 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 induction of this response was shown to be hapten specific requiring viable cells capable of cell division and protein synthesis. Under stimulatory conditions the number of 7S and 19S anti-TNP synthesizing cells rose from 50-150 to 1,000-10,000. Using this model several variables were studied to better understand the action of antigen on immune memory cells.

Decreasing antigen *in vivo* may preferentially stimulate cells with the potential for synthesis of high affinity antibody through the activation of surface receptors with similarly high affinity. This selection should result in cells with increased sensitivity to lower antigen concentrations, cells with greater avidity for antigen. *In vivo* changes in anti-TNP memory-cell sensitivity were followed by initiating the secondary anti-hapten response *in vitro* at various times after priming.

The results indicate that cell populations with increased sensitivity for trinitrophenyl-hemocyanin (TNP-KLH) antigen continue to emerge with time after priming and that this sensitivity may increase 1000-fold in a 4-month period.

Greater than optimum doses of TNP-KLH antigen induce immunological tolerance since, a) high doses of TNP-KLH suppress the anti-TNP responses,

b) the suppression is antigen specific, and c) the suppression occurs maximally during the first few hours of culture. Unhaptenated carrier protein KLH or TNP on a different carrier also induce tolerance when added to cultures of TNP-KLH-sensitive cells prior to *in vitro* challenge with TNP-KLH. When the carrier (KLH) and hapten (TNP) components are both present, but separate, paralysis is additive and may equal or exceed that caused by a high dose of intact antigen. These findings indicate that hapten and carrier tolerize separate receptor sites in the induction of immune unresponsiveness.

These data have proved useful in developing models to understand antigenic action in both immunity and tolerance. Furthermore, studies on separation of the two antigenic functional components, carrier and determinant, may prove useful in developing ways to control the immune response in man.

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