EARLY SIGNALS IN LYMPHOCYTE ACTIVATION

by

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INTRODUCTION

ACTIVATION OF LYMPHOCYTES--GENERAL COMMENTS

An important goal of immunology is immunoregulation, the turning off or turning on of specific lymphoid populations. To achieve such regulation it is necessary first to understand the immune system, the types of cells involved and their interactions, and how these cells are activated to either immunity or tolerance. There are probably inter-dependent sequential signals which lead to lymphocyte activation and the subsequent expression of humoral or cellular immunity or tolerance. The initiating event in lymphocyte stimulation is thought to be the interaction of antigen or mitogen with a specific receptor on the lymphocyte surface. Signals delivered at the cell surface appear sufficient to begin the activation process as several experiments suggest it is unlikely that interiorization of antigen or mitogen is essential to the process; in these experiments, antigen or mitogen covalently bonded to insoluble matrices was capable of stimulating lymphocytes (Feldmann et al., 1974; Greaves and Bauminger, 1972; Andersson et al., 1972). Thus, events occurring at the lymphocyte plasma membrane are critical to the expression of lymphocyte function.

The Fluid Mosaic Model of the Plasma Membrane

The plasma membrane is a lipid bilayer composed of phospholipids and cholesterol with protein molecules embedded within the bilayer (for review see Ladoulis et al., 1975). Since the fluid-solid transition temperature of the lipids is approximately 16° to 18°, the lipid phase

is in a fluid state at mammalian physiological temperatures (Resch and Ferber, 1975). Singer and Nicolson (1972) described a fluid mosaic model of the cell membrane which allows protein molecules to diffuse freely within the plane of the lipid membrane. This model is supported by the observation that cell surface antigens can undergo redistribution within the plane of the membrane.

Redistribution and capping of surface components on murine lymphoid cells was first described by Möller (1961). Taylor et al. (1971) observed that anti-immunoglobulin (Ig) antibody induces surface Ig to redistribute, which suggested to many investigators that movement of membrane components might be involved in lymphocyte activation. Taylor and coworkers studied murine bone marrow-derived (B) lymphocytes which bear a dense coat of Ig molecules; these molecules presumably function as antigen receptors. Cells incubated with fluorescein-conjugated anti-Ig showed different staining patterns depending upon conditions used. If divalent anti-Ig was used to label Ig, the labelling was "patchy" at 0°, but if the temperature was raised to at least 20°, the label "capped" and congregated over one pole of the cell. If monovalent anti-Ig was used, the labelling was diffuse at all temperatures, suggesting that lattice formation or aggregation was required for cap formation. Capping was temperature dependent and required a metabolically active cell, since metabolic inhibitors such as dinitrophenol or sodium azide reversibly inhibited capping in a dose-dependent fashion.

Surface components on a variety of cells can be redistributed into caps indicating that cap formation involves a general cell process and is not restricted to lymphoid cells. Sundqvist (1972) examined blood

group, species and histocompatibility antigens of monkey kidney, human lymphoid and thyroid, HeLa and L cells and found capping to be a general phenomenon. Even though many surface antigens can be induced to cap by antibody, some components (H-2, θ, Tla, Concanavalin A [Con A] receptors) cap more readily when a second bivalent anti-globulin reagent is added (Stackpole et al., 1974).

Katz and Unanue (1972) have shown that capping of Ig receptors on murine B cells is not sufficient for B cell stimulation and generation of an immune response. However, Unanue (1974) suggested that movement of antigen-receptor (antibody?) complexes on the surface of B cells plays a role in lymphocyte activation. In these experiments anti-Ig antibody was used as a ligand interacting with surface Ig molecules to mimic antigen-receptor interaction. Under appropriate conditions the surface complexes redistributed into caps, were endocytosed and cell movement was stimulated. However, B cells so treated did not differentiate and produce antibody, indicating capping of antigen receptors is not a sufficient signal to trigger B cell responsiveness.

Capping of lymphocyte surface Ig molecules is energy dependent. However, capping of artificial membranes occurs in the absence of an energy source and results from lipid redistribution to an energetically more favorable environment or configuration. Yguerabide and Stryer (1971) demonstrated that artificial, fluorescent, spherical bilayer membranes initially showed uniform fluorescence, but within a minute at room temperature a fluorescent cap formed at the top of the sphere with a decrease in the fluorescence at the bottom. This capping resulted

from the spontaneous migration of octane, oxidized cholesterol and the fluorescent label to the cap region. The apparent energy dependence of lymphocyte capping may be a secondary effect; that is, migration of surface molecules may not be energy dependent, but maintenance of appropriate membrane fluidity to allow for such movement may require energy.

Since fluidity of the membrane depends upon lipid content, changes in membrane phospholipids during lymphocyte stimulation have been investigated. Resch and Ferber (1975) reported rapid changes (within 10 minutes) in phospholipid metabolism of plasma membranes during mitogen stimulation and hypothesized that binding of a stimulating ligand to receptors on the membrane causes enhanced turnover of phospholipid fatty acids, resulting in increased membrane fluidity. Increased fluidity allows for altered membrane permeability and enzymatic changes which serve in an unknown manner as signals for activation. Thus, binding of a stimulant to receptors causes increased membrane fluidity which is a catalyst for cell activation.

The Role of Cyclic Nucleotides

Lymphocytes contain membrane-bound adenylate cyclase and adenosine 3':5' cyclic monophosphate (cAMP) has been postulated to play a "second messenger" role in lymphocyte activation (reviewed by Watson, 1975). Thus, while activators may provide a variety of first signals depending upon the activator, all would function thereafter through a common intermediate second signal, cAMP. Upon activation of the adenylate cyclase system, cyclic nucleotides generated from ATP activate protein kinases leading to phosphorylation and enzyme activation. Such enzymes

would be required for the increased RNA, DNA and protein synthesis associated with activation.

Watson (1975) analyzed the effect of cyclic nucleotides on in vitro immune responses. Induction of immune responses was inhibited by cAMP, but inhibition could be reversed by guanosine 3':5' cyclic monophosphate (cGMP) which could also replace the requirement for T cells in responses to erythrocyte antigens (thymus-dependent antigens). Watson suggested that antigen binding activates the adenylate cyclase system, increasing cAMP levels; but, since both cAMP and cGMP are required for an inductive signal, antigen binding alone would lead only to tolerance. It is the action of cooperating T cells (in a thymus-dependent response) or a mitogenic signal (in a thymus-independent response) which increases cGMP levels and allows for an inductive signal (Watson, 1975). Since changes in the ratio of cyclic nucleotides occur during activation, it has been proposed that the guanylate cyclase system balances the adenylate cyclase system. However, there are discrepancies regarding early changes in cyclic nucleotide levels after mitogen activation and on their effects on lymphocyte activation. Some investigators found increases in cGMP (Hadden et al., 1972) and others found increases in cAMP levels only (Wedner et al., 1975) after addition of mitogen. Addition of exogenous cAMP at low concentrations was mitogenic for lymphocytes (McCrery and Rigby, 1972), but in other experiments the addition of cAMP to lymphocytes did not mimic the effects of mitogen (Novogrodsky and Katchalski, 1970).

In addition to such conflicting conclusions, the biological significance of these increases must be questioned since cAMP, but not cGMP,

also increases after addition of a non-mitogenic mushroom lectin (Burleson and Sage, 1976).

Ca^{++} and Lymphocyte Activation

Ca^{++} ions in the extracellular medium are needed for mitogen-induced lymphocyte proliferation and it has been postulated that Ca^{++} ions play a primary role in lymphocyte triggering. Increased $^{45}Ca^{++}$ uptake has been reported in human peripheral blood lymphocytes minutes after phytohemagglutinin (PHA) stimulation (Whitney and Sutherland, 1973). Freedman et al. (1974) reported ConA induced $^{45}Ca^{++}$ uptake in murine T cells within 45 seconds after addition of the lectin. However, B cells exposed to ConA or stimulated by lipopolysaccharide or pokeweed mitogen did not show this rapid early Ca^{++} influx. Maino et al. (1974) have shown pig lymphocytes can be triggered to undergo DNA synthesis by the ionophore A23187, a carrier molecule which increases Ca^{++} transport into the cell. This suggested that Ca^{++} influx was a sufficient signal for proliferation. The action of Ca^{++}once it enters the cell is speculative, although Ca^{++} activates AMP phosphodiesterase and cGMP formation (Posternak, 1974). But rapid Ca^{++} influx cannot be considered a sufficient and irreversible signal for activation; if Ca^{++} is removed 12-36 hours after PHA stimulation, DNA synthesis is inhibited (Whitney and Sutherland, 1972; Diamanstein and Ulmer, 1975).

Microfilaments and Microtubules in Lymphocyte Activation

The submembranous cytoplasmic structures, microfilaments and microtubules, have been implicated in lymphocyte triggering. Drugs which

interfere with microfilament and microtubule function suggest that these structures play a role in modulating cell surface components, although the mechanisms remain unclear. The fungal metabolite, cytochalasin B, blocks the actin-like contractile microfilament system (Wessels et al., 1971) and colchicine binds to microtubules and prevents tubulin polymerization (Margulis, 1973). Both drugs inhibit capping of anti-Ig--Ig complexes (Schreiner and Unanue, 1976) and Roberts and LaVia (1975) have reported that antigen activation of B lymphocytes (measured by antibody production) is inhibited if cytochalsin B is present at nontoxic doses during the initial six hours of a four-day in vitro culture. However, since colchicine and cytochalasin B affect other biological systems such as nucleoside uptake and glucose transport, it isn't clear if inhibition of capping and antigen activation results from the direct effect on microfilaments and microtubules (discussed in Schreiner and Unanue, 1976).

Although most investigations focus upon one mechanism for the coupling of membrane signals to cytoplasmic and nuclear events, the phenomena which have been implicated are probably interrelated. For example, Ca^{++} ions regulate cAMP and cGMP levels by inhibiting adenylate cyclase and enhancing guanylate cyclase activity (Posternak, 1974); elevation of cGMP antagonizes microtubule stability (Oliver, 1975); changes in cAMP or cGMP can lead to altered protein kinase activity which in turn may result in increased phospholipid metabolism and alteration of membrane transport sites and enzyme activation (Cooper, 1975).

Despite all investigations the mechanism(s) of generation and translation of activating signals delivered at the lymphocyte surface is not known.

CONCEPTS OF B LYMPHOCYTE ACTIVATION

The initiating event in lymphocyte stimulation is thought to be the interaction of antigen or mitogen with a specific receptor on the lymphocyte surface. The mechanism by which ligand binding initiates this series of events is not known, but there are three major proposals of ligand-receptor interaction. The following summary of the proposals is taken from Feldmann et al. (1975):

Feature	Model A	Model B	Model C
	(Diener and Feldmann, 1972)	(Bretscher and Cohn, 1970)	(Coutinho and Möller, 1975)
Stimulation	Via multivalent binding to Ig receptors, spacing of matrix critical	Two necessary signals: 1) Ig receptor; 2) other signal from T cells or macrophages	One nonspecific signal (mitogenic). Ig receptors are only passive acceptors for correct specificity
Antigens			
1. Thymus dependent	Unable to bind many receptors simultaneously	No qualitative distinction: TI need less of signal (2) or are more efficient generators	Not mitogens or polyclonal activators
2. Thymus independent	Large, repeating determinants; epitope density critical		Mitogens and/or polyclonal activators
Tolerance	Excess polymeric antigen leads to lattice immobilization of receptors	Signal (1) without signal (2)	Excess of activating signal
Mitogens	Act on other receptors than do antigens	Generate signal (2) only	Act on same receptors as antigen

The data regarding these three models are less ambiguous for thymus-independent than for thymus-dependent responses, so the models concentrate on B cell triggering by thymus-independent antigens. Models A and B are based on the idea that Ig receptors deliver the signal. In model A, the signal for immunity is generated by interaction between antigen and the Ig receptors which must be aggregated to a certain extent. According to this model, thymus-dependent antigens are unable to bind a sufficient number of receptors simultaneously and thus require accessory cell help, while the repeating nature of thymus-independent antigens allows direct B cell activation. Mitogens act on receptors other than Ig receptors. There are numerous observations which suggest B cells respond preferentially to repeating determinants of antigen or mitogen. Most thymus-independent antigens are of a repeating nature and a thymus-dependent antigen, TNP-KLH, can be made thymus-independent by covalent coupling to Sepharose beads (Feldmann et al., 1974). TNP coupled to polyacrylamide beads also behaves as a thymus-independent antigen (Feldmann et al., 1974). Cross-linked PHA and ConA can stimulate B cells (Greaves and Bauminger, 1972; Andersson et al., 1972); anti-Ig antibody coupled to polyacrylamide beads stimulates murine B cells (Parker, 1976), whereas soluble anti-Ig does so poorly if at all.

Model B requires two signals for immunity; the first is the interaction between antigen and the Ig receptor which is not sufficient for triggering. A second signal is delivered by helper T cells, macrophages or by the associative recognition of antibody which can react with the

antigen bound to Ig receptors. There is no qualitative difference between thymus-dependent and thymus-independent antigens; thymus-independent antigens either need less of signal 2 or are more efficient at generating signal 2.

Model C differs dramatically from the other two in that one non-specific signal serves to activate B cells. The signal is delivered by receptors other than the Ig receptors. Ig receptors function in a passive manner to determine which specific antigen (mitogen) will be bound. Model C makes all thymus-independent antigens B cell mitogens or inducers of polyclonal antibody formation (antibody to many different antigens). Thymus-dependent antigens are not mitogens or polyclonal activators and thus require accessory cells for antibody production. In model C, mitogens and antigens act on the same receptor.

The one nonspecific signal theory was based on the claim that all thymus-independent antigens are nonspecific polyclonal activators directly activating either polyclonal antibody synthesis and/or DNA synthesis in B cell populations (Gronowicz and Coutinho, 1975). Haptens coupled to polyclonal activators induced a specific anti-hapten response when added in low concentrations, but when added in high concentrations induced a polyclonal antibody response with paralysis of the specific anti-hapten response (Coutinho et al., 1975). Specific induction is explained by Ig receptors passively focusing the hapten-conjugate and allowing specific cells to bind larger amounts of the activator necessary for triggering. High concentrations of hapten conjugate allow B cells

of different specificities to bind the carrier-mitogen portion of the conjugate (specific anti-hapten Ig receptors are not needed) and be activated. Specific anti-hapten cells bind so much hapten-conjugate they are tolerized.

There are data supporting all three proposed models and it is reasonable that any of the three pathways can operate under certain circumstances. Furthermore, as pointed out by Feldmann et al. (1975), in addition to these proposed pathways the nature of the antigen and the state of the B cell can affect antigen-B cell interaction. Epitope density, concentration, chemical nature (charge, hydrophobicity), degradability, and mitogenicity of the antigen would all influence an immune response. In addition, B cell responses to antigen could be modified by changes in affinity, number, valency and distribution of Ig receptors and possible involvement of non-Ig receptors which have also been proposed as regulatory receptors (Fc, C3, H-2, Ia; for review see Möller, 1975). The metabolic state of the B cell may also be a critical factor in B cell responsiveness.

Attempting to invoke pathways for B lymphocyte triggering is complicated by increasing evidence which suggests there are B cell subpopulations (for review see Möller, 1975A). It is not known how many subpopulations exist and if those subpopulations so far defined are triggered by the same signals. In addition to heterogeneity in antigen specificity, subpopulations of B cells have been defined by organ distribution, sensitivity to mitogens, Ig synthesis and response to thymus-independent and thymus-dependent antigens.

T LYMPHOCYTE ACTIVATION:

THE NATURE OF THE ANTIGEN RECEPTOR ON T LYMPHOCYTES

Although the surface receptors for antigen on B lymphocytes are generally accepted to be immunoglobulin-like molecules, the nature of the surface receptors for antigen on T lymphocytes is highly controversial. Most researchers have failed to find conventional Ig on or in T lymphocytes (reviewed by Playfair, 1974), but this has been disputed by others who claim to detect Ig on T cells (reviewed by Marchalonis, 1975). Immune response genes linked to the major histocompatibility locus play a role in T cell function and it has been hypothesized that the immune response gene products may function as antigen binding receptors on T cells as opposed to conventional Ig molecules which function as antigen receptors on B cells (reviewed by Katz and Benacerraf, 1975). T cells can bind specific antigen: helper T cells and T cells participating in delayed hypersensitivity reactions can be "suicided" by binding heavily radiolabelled antigen (Basten et al., 1972; Cooper and Ada, 1972); T cells can also be induced to tolerance by specific carrier protein (Rittenberg and Bullock, 1972). Direct binding studies with radiolabelled antigen indicate T cells bind less antigen than B cells (Roelants et al., 1973; Engers and Unanue, 1974) and that different metabolic activities may be required for T cell binding of antigen (Hämmerling and McDevitt, 1972; Roelants et al., 1973).

T and B cells show differences in binding specificities. In responses to hapten-protein conjugates, helper T cells normally do not show specificity for the hapten as do B cells, but rather respond to

carrier or conjugate specific determinants (Paul, 1970). Specificity differences would suggest distinct T and B cell antigen receptors, but hapten-specific T cell responses do occur, although infrequently detected (Leskowitz et al., 1966; Alkan et al., 1972), which could reflect a lower intrinsic binding affinity of T cell receptors rather than a different set of receptors. Binz and Wigzell (1975) have found that B and T lymphocytes express shared idiotypic determinants on receptors for the same antigen, although no constant region Ig markers were found on T cells. This suggests antigen receptors on T and B cells share structural similarities which are probably variable region gene products.

A biochemical approach to identifying Ig determinants on T cells has been to label T cell surfaces in situ with radioiodine and to solubilize and characterize surface materials. By this approach some investigators find IgM-like molecules on thymus and thymus-derived cells in the same quantity as is found on B cells (Marchalonis, 1975), while others detect no Ig (Vitetta et al., 1972; Grey et al., 1972). These conflicting results using the same methodology have been attributed to the susceptibility of T cell Ig to proteolytic digestion and subsequent loss of such "IgT" during isolation (Moroz and Hahn, 1973). However, the conflict has not been resolved even when care is taken to protect against proteolysis.

Immunological approaches have also been used to attempt to identify T cell Ig. Feldmann has evidence for release of antigen-"IgT" complexes from thymus-derived lymphocytes (reviewed in Feldmann et al., 1975A). The complexes served as specific collaborative factors in the immune

response by adsorbing to macrophages and allowing for B cell responses; however, "IgT" has not been well characterized and need not be Ig at all. Radiolabelled or fluorescein-conjugated anti-Ig sera have been used to detect Ig on T cells, again with conflicting results. Some investigators find Ig on T cells (Nossal et al., 1972; Bankhurst et al., 1971; Roelants et al., 1974), but other experiments suggest this may have been passively acquired from B cells (Hudson et al., 1974). However, others have failed to detect Ig on T cells by either radiolabelling or fluorescent immune techniques (Pernis et al., 1970; Raff et al., 1970).

Anti-Ig sera have been used as blocking reagents to inhibit T cell function. Hogg and Greaves (1972) showed immune T cells could bind sheep erythrocytes to form rosettes and that this could be blocked by anti-light chain or anti-u chain sera, but only a few sera would work. Graft-versus-host reactions and delayed hypersensitivity reactions (Mason and Warner, 1970; Greaves, 1969), mixed lymphocyte reactions (Greaves, 1969) and helper T cell function (Lesley et al., 1971) could be blocked by some anti-Ig antisera. However, Crone et al. (1972) were unsuccessful in blocking graft-versus-host reactions in the chicken by anti-Ig and Wekerle et al. (1975) could not block either the sensitization or the effector functions of cytotoxic T cells.

In general, T cells may have Ig, but in far smaller amounts than that found on B cells. Whether the molecules found on T cells are synthesized by them or merely passively acquired from B cells is still unresolved, as is the role of such molecules in T cell activation.

STATEMENT OF THE PROBLEM

As indicated above, lymphocyte activation is probably initiated at the cell surface. Since surface components continually turn over either by shedding or by pinocytosis (Landovlis et al., 1975), lymphocyte activation probably begins early after binding, although the precise chronological sequence has not been defined. There are many studies which deal with early events in mitogen-induced lymphocyte activation. Mitogens rather than antigens have been used since high concentrations of mitogens nonspecifically activate a large percentage of lymphocytes, whereas similar concentrations of antigen activate antigen-sensitive lymphocytes which only comprise a small percentage ($\sim$ 0.1%) of the unprimed population. Since purification and enrichment of antigen-specific cells is fraught with difficulties, most investigators have used mitogen rather than antigen as the activator to obtain a sufficient number of activated lymphocytes.

However, it is not known if mitogen and antigen trigger cells by the same mechanism(s). In fact, as discussed above, some investigators believe that all thymus-independent antigens are mitogens (Coutinho and Möller, 1975). However, when used at mitogenic doses, these materials may mask the antigen-related fine specificity and discrimination which characterize the immune response. Here I use a thymus-independent antigen and a thymus-dependent carrier, both of which are reported to be mitogens (Coutinho et al., 1974), although by the criterion of polyclonal antibody activation (Coutinho and Möller, 1975), they were not mitogenic in my hands. I have used these materials to study early "signals" in the activation of antigen-specific lymphocytes.

The doses of antigen required to induce these early signals are much lower than those reported to be mitogenic (Coutinho and Möller, 1975; Coutinho et al., 1974). Therefore, the question of mitogenicity is not pertinent because either Coutinho and Möller are correct and all antigens work through a second nonspecific mitogenic stimulus, or at the low doses used the mitogenic effects have been diluted out and I am dealing only with the antigen-specific properties of these materials.

This thesis concentrates on activation of antigen-specific thymus-derived (T) cells and bone marrow-derived (B) cells. I devised in vitro experiments which allowed me to detect early "signals" delivered to antigen-sensitive lymphocytes. I found that both T and B lymphocytes could be given negative signals by unusually low doses of antigen. These findings are used to describe a model for T and B cell discrimination between nonspecific self-antigenic "noise" and acute foreign antigenic insult.

MATERIALS AND METHODS

ANIMALS

Female or male adult Balb/c and C3H mice were obtained from Simonsen Laboratories, Inc., Gilroy, California and Texas Inbred Laboratories, Houston, Texas. Homozygous nude mice bred on a Balb/c genetic background were purchased from Washington State University, Pullman, Washington. New Zealand white rabbits were obtained from Art Kessler, Hillsboro, Oregon.

BUFFERS

1. Modified Barbital Buffer (MBB), 0.012 M, pH 7.3 to 7.4. (Campbell et al., 1970)

   2.8750 gm 5,5-Diethylbarbituric acid

   1.8750 gm Sodium 5,5-diethylbarbiturate

   0.0830 gm $CaCl_2$, anhydrous

   0.2380 gm $MgCl_2$, anhydrous

   42.500 gm NaCl

   Distilled water to 1 liter.

   For use, dilute 1 part of this stock solution with 4 parts of distilled water.

2. Cacodylate Buffer (CAC-buffer), 0.28 M, pH 7.0 (Rittenberg and Amkraut, 1966)

   38.2 gm Cacodylate acid (hydroxydimethylarsine oxide)

   9.0 gm NaOH pellets

   Distilled water to 1 liter.

Cacodylate saline (CAC-saline), the same buffer diluted 1:10 in 0.15 M NaCl.

3. Borate Buffer, 0.08 M, pH 8.5 (Campbell et al., 1970)

   6.184 gm Boric acid

   9.536 gm Sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$)

   4.384 gm NaCl

   Distilled water to 1 liter.

   Borate saline, the same buffer diluted 1:20 in 0.15 M NaCl.

4. 0.1 M Tris-HCl, pH 7.4 (Weir, 1973)

   0.1 M Tris (2-amino-2[hydroxymethyl]-1,3-propanediol)

   0.2 M NaCl

   2.0 mM EDTA Na_2

5. 0.1 M Sodium Acetate, pH 4.5 (Weir, 1973)

   0.1 M $NaC_2H_3O_2$; pH to 4.5 with 0.1 M acetic acid.

6. Borate Buffer, 0.016 M, pH 8.2 (Benedict, 1971)

   10.3 gm H_3BO_3

   1.1 gm NaOH

   7.85 gm NaCl

   1000 ml distilled water.

7. 0.01 M Phosphate, pH 7.2 (The and Feltkamp, 1970

   Solution A: NaH_2PO_4 (0.2 M)

   Solution B: Na_2HPO_4 (0.2 M)

   For one liter: 16.5 ml A

   33.5 ml B

   4.7 gm NaCl

   950.0 ml Distilled water

8. Tris-HCl, pH 8.0 (Williams and Chase, 1968)

0.1 M Tris (2-amino-2[hydroxymethyl]-1,3-propanediol)

0.2 M NaCl

9. Locke's Buffer, pH 7.4 (Reif and Allen, 1963)

39.8 gm NaCl

1.92 gm KCl

0.70 gm sodium bicarbonate

3.52 gm dextrose

3.62 gm sodium citrate·$2H_2O$

Dissolve in 800 ml distilled water. To this add 2.5 ml of a stock solution of:

10.17 gm $MgCl_2$

2.20 gm $CaCl_2$

50 ml Distilled water

Bring volume to 1000 ml. Isotonic buffer is obtained by diluting concentrated buffer 1:5 with distilled water.

10. Barbital B-2 Veronal Buffer, pH 8.6

2.76 gm Diethyl barbituric acid

15.40 gm Sodium diethyl barbiturate

1000 ml Distilled water

Dilute 1:2 with distilled water for electrophoresis buffer.

OTHER REAGENTS

1. Trypan Blue Reagent for Cytograf Analysis, pH 7.2

   0.2 % Trypan blue

   0.145 M NaCl

   0.1 M PO_4

   Dilute 1:4 with normal saline and filter immediately prior to use.

2. Alsever's Solution (Campbell et al., 1970)

   20.5 gm Dextrose

   8.0 gm Sodium citrate (dihydrate)

   0.55 gm Citric acid (monohydrate)

   4.2 gm NaCl

   1000 ml Distilled water

3. Ficoll-Isopaque (Terasaki, 1970)

   9.0 gm of Ficoll (Pharmacia) was dissolved in 100 ml of water. 20 ml of sodium metrizoate (75%) (Isopaque) was added to 44 ml water to make a 33.9% solution of Isopaque. For use, 24 parts Ficoll was mixed with 10 parts 33.97% Isopaque. Reagents containing Isopaque were stored in the dark at 4° C.

4. Minimal Essential Medium (MEM)

   Eagle's powdered minimal essential medium for suspension cultures, without L-glutamine, catalogue #F-14, from Grand Island Biological Co., Grand Island, New York, was dissolved in distilled water. A 1X solution was prepared with 1.07 gm/100 ml water. The pH was adjusted to 7.2 with NaOH and the solution sterilized by autoclaving.

5. Hepes-Buffered MEM, pH 7.2

4.77 gm Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid)

5.35 gm Eagle's powdered minimal essential medium for suspension cultures

500 ml Distilled water

6. Tris-Buffered Ammonium Chloride (Tris-NH_4 Cl) for erythrocyte lysis, pH 7.2 (Boyle, 1968)

9 volumes 0.83% aqueous NH_4Cl added to 1 volume Tris buffer. Store at 4° for stability and warm to 37° immediately prior to use.

Tris buffer: 20.594 gm Tris (2-amino-2[hydroxymethyl]-1,3-propanediol) per 1000 ml distilled water; pH to 7.65 with HCl.

TISSUE CULTURE

The Mishell-Dutton tissue culture system (Mishell and Dutton, 1967) or a micromodification (Kappler, 1974) was used. Balb/c mice were killed by cervical dislocation and spleens were removed aseptically and placed in a 60 mm petri dish with 5 to 8 ml of MEM supplemented with 10% fetal calf serum (F-MEM). The spleens were disrupted either with sterile forceps or by forcing through a wire screen. Cells were dispersed with a capillary pipet and transferred to a tube and put at 4° for 3 to 5 minutes to allow debris to settle. Cells remaining in suspension were transferred to another tube and centrifuged 630 x g for 15 minutes. The supernatant fluid was discarded and the pellet was resuspended in F-MEM and again centrifuged. The pellet was resuspended in tissue culture

medium and brought to 0.9 to 1.1 x 10^7 nucleated spleen cells/ml. Cells were counted on a Coulter counter, model F, or a Cytograf cell counter, Model 6300A. For standard Mishell-Dutton cultures, cells were cultured in 1.0 ml volumes in 35 mm culture dishes; in the micromodification, cells (0.1 ml) were cultured in flat bottom microtiter plates with lids (Falcon Microtiter II, catalogue #3004). Cells were cultured for 5 days in plastic boxes at 37° in an atmosphere of 7% oxygen, 83% nitrogen and 10% CO_2 with rocking at 7 to 8 cycles per minute.

Each day cultures were fed with a 2:1 mixture of nutritional cocktail and fetal calf serum and regassed. Cells cultured in 1.0 ml volumes were fed with 3 drops from a capillary pipet. Each microculture was fed with 1 drop delivered from a 1 cc syringe with a 25 gauge needle.

Tissue Culture Medium

Ingredients for 50 ml:

1. Minimal essential medium (MEM) (Eagle) for suspension cultures, without L-glutamine, Microbiological associates (MBA), Albany, California (43.0 ml).
2. L-glutamine 200 mM, MBA (0.5 ml).
3. Sodium pyruvate 100 mM, MBA (0.5 ml).
4. Non-essential amino acids mixture--supplement for Eagle's MEM, 100X concentrate, MBA (0.5 ml).
5. Penicillin-streptomycin mixture, 5000 units/ ml, MBA (0.5 ml).
6. 2-mercaptoethanol, Eastman Organic Chemical Co., Rochester, New York; stock 0.1 M and diluted 1:20 with MEM prior to use (0.5 ml) (Click, Bennet, and Alter, 1972).

7. Fetal calf serum, Gray Industries, Inc., Fort Lauderdale, Florida, lot #1136 or Reheis Chemical Co., Chicago, Illinois, lot #M27704 (5.0 ml).

Nutritional Cocktail

Ingredients for 227 ml:

1. Autoclaved MEM, 1X solution (140 ml).
2. Essential amino acids--amino acid mixture for Eagle's MEM, 50X concentrate, MBA (20 ml).
3. Non-essential amino acids, as used in tissue culture medium (10 ml).
4. Dextrose, reagent grade, Merck and Co., Rahway, New Jersey. 200 mg/ml solution was prepared in double-distilled water and sterilized by filtration through a 0.45 μ Millipore membrane (10 ml).
5. L-glutamine, as in tissue culture medium (10 ml).
6. Penicillin-streptomycin as in tissue culture medium (3.6 ml). The above cocktail was adjusted to pH 7.2 with sterile 1.0 N sodium hydroxide.
7. Sodium bicarbonate solution, 7.5%, was added to the pH-adjusted cocktail, 30 ml.

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

Harvesting Cell Cultures

Cells cultured by the regular Mishell-Dutton system were scraped from the culture dishes with a rubber policeman, placed in plastic tubes and centrifuged at 630 x g for 15 minutes. Cells were washed by resuspending in 2.0 ml of MEM, recentrifuged and then resuspended in MEM for assay.

Cells from microcultures were harvested by adding 1 drop of Hepes-buffered MEM to each well. The mixture was gently aspirated 10 to 15 times with a Pasteur pipet and the cells were placed in a test tube. Each well was rinsed with 1 drop of Hepes-MEM and this was added to the first collection. Cells were centrifuged and washed as above.

TNP-Plaque Assay

Cells synthesizing anti-trinitrophenyl (TNP) antibody were detected by plaque assay using TNP-conjugated sheep erythrocytes (TNP-SRBC) (Rittenberg and Pratt, 1969) and the slide technique of Cunningham and Szenberg (1968). For detection of IgM plaque-forming cells (PFC), 3 drops MEM, 1 drop TNP-SRBC, 1 drop complement (guinea pig serum [C]) and 100 µl suitably diluted spleen cells were added to each well. IgG PFC were detected by substituting 1 drop of anti-mouse IgG for 1 drop of MEM. The anti-globulin reagent and C were absorbed with SRBC prior to use (Kabat and Mayer, 1963) and were diluted in MEM. After incubation for 1 hour, slide chambers were examined using a stereo dissecting microscope and scored for anti-TNP PFC. In some instances, unconjugated sheep erythrocytes (SRBC) were used as indicator cells in place of TNP-SRBC.

HEMOCYANIN

Hemocyanin (KLH) was obtained from the keyhole limpet, Megathura crenulata, purchased from Pacific Bio-Marine Supply Co., Venice, California. The animals were bled and the hemocyanin prepared by the method of Campbell et al. (1970). After dialysis KLH was concentrated by centrifugation at 1400 x g for 30 minutes, resuspended in 0.15 M sodium chloride and sterilized by filtration through a 0.45 μ Millipore membrane and stored at 4°.

HAPTEN REAGENT

(Rittenberg and Amkraut, 1966)

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

TRINITROPHENYL HEMOCYANIN (TNP-KLH)

General procedure: 450 mg of KLH were brought to 10 ml with cacodylate buffer (CAC-buffer) and placed in a foil-covered 50 ml flask at 37°. TNBS, 346 mg in 10 ml of CAC-buffer was added dropwise to the slowly stirred KLH solution. Stirring was continued for 60 minutes, the solution was chilled and then concentrated by centrifugation for 90 minutes at 36,000 rpm using a fixed angle Type 60 rotor in a refrigerated Beckman model L2-65 centrifuge. The supernatant fluid was discarded and the amber gelatinous pellets were dissolved in 10 ml of CAC-buffer. The

small amount of insoluble denatured KLH was discarded by centrifuging at 630 x g for 10 minutes. The clear amber supernatant fluid was freed of unconjugated TNBS by passage through a G-50 Sephadex column (2.5 cm x 42 cm) equilibrated with CAC-saline. Throughout the procedure foil was used to protect the preparation from photodecomposition (Okuyama and Satake, 1960). O.D. readings at 350 and 280 nm were taken and the TNP-KLH recycled through the G-50 column. If the 350/280 O.D. ratio remained unchanged, it was assumed that the conjugate was free of TNBS. TNP-KLH preparations used in vitro were dialyzed with three changes of saline (12 hr each) to remove cacodylate which contains arsenic. The degree of hapten conjugation was estimated by spectrophotometric measurement at 350 nm. Protein was determined by the method of Lowry (Lichstein and Oginsky, 1965). This procedure usually produces 850 to 1000 moles of TNP groups per mole of KLH, assuming a molecular weight of 8×10^6 for KLH (Rittenberg and Amkraut, 1966).

CGG-BENTONITE (CGG-B), KLH-BENTONITE (KLH-B) AND TNP-KLH-BENTONITE (T-K-B)

KLH and TNP-KLH were adsorbed onto bentonite using the method of Gallily and Garvey (1968) as modified by Rittenberg and Pratt (1969). The bentonite pellet obtained after centrifugation of 40 ml of stock bentonite solution was resuspended in 2 ml CAC-buffer containing 5 mg of KLH or TNP-KLH/ml. The suspension was allowed to stand at room temperature for 1 hour with occasional shaking. After centrifugation, particles were washed 3 times in CAC-saline. The amount of protein coated on the

particles was determined by the Lowry method (Lichstein and Oginsky, 1965).

BENTONITE

(Gallily and Garvey, 1968)

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

SHEEP ERYTHROCYTES (SRBC)

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

TNP-SHEEP RED BLOOD CELLS (TNP-SRBC)

(Rittenberg and Pratt, 1969)

Sixty mg of TNBS were dissolved in 21 ml of CAC-buffer in a foil-covered 50 ml flask. Three ml of wet-packed SRBC, washed 3 times with cold modified barbital buffer (MBB) were added dropwise with stirring.

The mixture was stirred slowly at room temperature for 15 min. Reacted cells were brought to 40 ml with cold MBB and centrifuged for 10 min at 1230 x g. The resulting TNP-SRBC pellet was resuspended in 35 ml of cold MBB containing 22 mg of glycyl-glycine (Nutritional Biochemicals Corp., Cleveland, Ohio). Glycyl-glycine reacts with residual TNBS. This suspension of TNP-SRBC was recentrifuged. The supernatant was bright yellow due to the formation of TNP-glycylglycine. The TNP-SRBC pellet was washed twice more with cold MBB and stored at 4° until used. For use in the plaque assay, cells were washed in cold MBB until the supernatant was free of lysis (about 3 washes). Cells were used at 30% suspension in MBB.

IMMUNIZATIONS

(Rittenberg and Pratt, 1969)

Mice were given intraperitoneal (ip) injections of KLH-bentonite every week for a total of 3 injections. TNP-KLH-bentonite primed mice were given TNP-KLH-bentonite every 2 weeks for a total of 3 injections. In some experiments, TNP-KLH-bentonite primed mice were boosted with TNP-KLH-bentonite four days prior to use. In all cases each injection contained 100 µg protein in 0.5 ml saline.

Mice injected with sheep erythrocytes received 0.5 ml of a 20% SRBC suspension (in saline) ip 4 days prior to use.

ADDITIONAL ANTIGENS AND MITOGENS

Dinitrophenylated chicken gamma globulin (DNP-CGG) was a gift of Dr. N. A. Mitchison. The degree of hapten conjugation was 6.6 DNP groups

per molecule of CGG. Dr. Mitchison also supplied me with one batch of CGG.

Dinitrophenylated-dextran B1299 (DNP-D) was a gift of Drs. C. Desaymard and M. Feldmann. Preparation and characterization has been previously described (Desaymard and Feldmann, 1975). The conjugation ratio was 2.8 DNP groups per 40,000 daltons of dextran. The molecular weight of dextran B1299, a branched molecule, is about 4×10^7 daltons.

Trinitrophenylated-bacteriophage T4 was a gift of Dr. John Jennings. It was prepared according to the method of Jennings et al. (1975).

Lipopolysaccharide (LPS) from E. coli 055-B5 was a gift of Dr. Wesley Bullock. It was prepared by the method of Andersson et al. (1972A) and had a molecular weight of about 1.5×10^6 daltons.

PREPARATION OF CHICKEN GAMMA GLOBULIN (CGG)

CGG was isolated by the method of Benedict (1971). Fresh chicken blood (Hunt's Poultry Co., Portland, Oregon) was allowed to clot at room temperature for 3 hours. Serum was collected by centrifugation and globulins were fractionated by the sodium sulfate (Na_2SO_4) method using consecutive fractionations at 18, 14 and 14%. Following centrifugation at 1230 x g after the third fractionation, the precipitate was dissolved in 40 ml cold borate buffer, 0.016 M, pH 8.2, and dialyzed extensively against borate buffer, pH 8.2. The protein concentration was 47.4 mg protein/ml as determined by the Lowry method. The gamma globulin preparation was stored at 4° C. CGG was further purified by Sephadex G200 (Pharmacia) gel filtration using standard upward flow technique (64

x 2.5 cm column; flow rate 20-24 ml/hr; 4 ml fractions collected). The equilibrating and running buffer was borate buffer, pH 8.2. All preparations were loaded in 10% sucrose (final concentration) and chased with an equal volume of 20% sucrose. Protein concentration loaded ranged from 213 mg protein (ml total) to 119 mg protein (5 ml total). The initial protein peaks (OD_{280} absorbing material) eluted from the void volume from all runs were pooled and concentrated. The pool was refiltered on G-200 under similar conditions. The IgG peaks were pooled and concentrated. The concentrated pool was tested by immunoelectrophoresis against a rabbit anti-whole chicken serum, a gift of Dr. Gerrie Leslie of our department; and the protein in the pool was identified as gamma globulin. The CGG was stored at 4° C.

PREPARATION OF NORMAL MOUSE GLOBULIN IMMUNOABSORBENT

Normal mouse blood was obtained from (Balb/c x C3H)F_1 (CC_3F_1) mice by bleeding from the brachial artery and the serum was collected by centrifugation. Mouse globulin was partially purified by an initial 50% saturated ammonium sulfate (SAS) precipitation. The suspension was stirred overnight at 4°, centrifuged at 2510 x g for 30 minutes at 4°, and the pellet resuspended in normal saline. The suspension was refractionated by an additional 40% SAS precipitation performed as above. After stirring overnight and centrifugation the pellet was dissolved in normal saline, dialyzed against borate saline for two days, and frozen at -20°. The protein concentration was 31.0 mg protein/ml.

Sepharose 4B (Pharmacia) was washed extensively with distilled water using a sintered glass filter. 30 gm of Sepharose (wet weight

after filtering) was allowed to pack by 1 g sedimentation overnight at 4°. The packed Sepharose (30 ml) was added to 30 ml distilled water; 3.0 gm CnBr dissolved in 30 ml distilled water was added. The pH of the mixture was immediately brought to pH 11.0 using 4 N NaOH, and maintained at pH 11.0 by NaOH until the pH remained constant, about 8 to 10 minutes. 500 ml cold distilled water was added and the mixture transferred to a sintered glass filter and washed with 1 liter of cold distilled water, followed by 1 liter of 0.5 M $NaHCO_3$, pH 8.0. The activated resin was transferred to a beaker and an equal volume of 0.01 M phosphate buffered saline (PBS), pH 7.2, was added.

2 ml of mouse globulin (31.0 mg protein/ml) was added and the mixture was stirred for 12 hours in the cold. The resin was washed with 0.01 PBS until the PBS washes showed baseline absorbance at 280 nm. The amount of bound protein, determined by absorbancy of the washes, was 1.2 mg protein per mg beads (37 mg total bound). Elution conditions were simulated to test for spontaneous elution by washing with 3 M NaCSN, the eluting buffer. No protein was eluted under these conditions. The column was washed extensively with 0.01 M PBS.

Sheep 144 anti-θ serum was passed over the immunoabsorbent column and unbound protein was eluted with 0.01 M PBS and concentrated to its original serum volume. Bound protein was eluted using 3 M NaSCN, then dialyzed extensively against 0.01 M PBS and concentrated. The column was regenerated for additional runs by extensive washing with 0.01 M PBS.

FLUORESCENT STAINING OF LYMPHOCYTES

Spleen or thymus cells were prepared by teasing the tissue in 5% heat-inactivated FCS in MEM (FCS-MEM). The cells were washed once and the red cells were lysed by Tris-buffered ammonium chloride (Boyle, 1968). The remaining cells were washed twice and brought to a concentration of 10^7 cells/ml in FCS-MEM. For indirect staining, 0.1 ml cells and 0.1 ml appropriately diluted antiserum were mixed, incubated for 60 minutes at 4° and washed once with 5% FCS-MEM. The pellet was resuspended in 0.1 ml MEM; 0.1 ml of a 1:16 dilution in MEM of fluoresceinated goat anti-rabbit Ig antiserum (lot #2?32V002A1, Hyland Laboratories) was added to the tubes, mixed and incubated for 60 minutes at 4°. The cells were washed three times in MEM containing 3 x 10^{-2} M sodium azide (NaN_3-MEM) to prevent cell membrane rearrangements (Taylor et al., 1971) and resuspended in 0.25 ml NaN_3-MEM. One hundred cells were examined for staining using a Zeiss microscope with an integral illuminator 6V 15W. For direct staining, 0.1 ml cells and 0.1 ml of a 1:8 dilution in MEM of fluoresceinated rabbit anti-mouse Ig antiserum (lot #14454, MBA) were mixed and incubated 60 minutes at 4°. The cells were washed with NaN_3-MEM as described above and examined.

TISSUE ABSORPTION OF SERUM

All serum absorptions were performed at 4° for 60 minutes unless indicated otherwise. Tissue was removed from the animal, erythrocytes were lysed using Tris-NH_4Cl solution and the tissues washed with either

MEM or saline. Serum was added to the packed tissue and after absorption was collected by repeated centrifugation at 2510 x g for 30 minutes at 4°.

MOUSE IMMUNOGLOBULIN (Ig) ABSORPTION OF SERUM

Purified mouse IgG (Pentex, Miles Laboratories) was added to the antiserum (diluted as indicated) in varying concentrations. The mixture was incubated at 37° for 30 minutes to allow antigen-antibody binding, cooled to 4° and used in cytotoxicity studies.

MACROPHAGE DEPLETION

Macrophages were depleted from spleen cell suspensions by allowing them to phagocytose carbonyl iron particles and then removing them with a strong magnet (Erb and Feldmann, 1975). 2 x 10^8 spleen cells in 10 ml of Hepes-buffered MEM supplemented with 10% FCS were incubated with 0.1 gm carbonyl-iron (sample #1-1; Particle Information Services, Grants Pass, Oregon) in a 100 x 15 cm petri dish for 1 hour at 37°. A strong magnet was passed under the bottom surface and held in place on the bottom of the dish while pouring the cells into another dish. The magnet treatment was repeated once and the cells washed in MEM supplemented with 10% FCS. This procedure removed adherent and phagocytic cells. Cell recovery after treatment was 40 to 60%. Macrophage depletion was monitored by the inability of treated cells to respond in vitro to TNP-KLH, a macrophage-dependent antigen (Feldmann et al., 1975A); loss of B cells was monitored by the response to TNP-T4, a thymus-independent antigen (Jennings et al., 1975).

PREPARATION OF RABBIT ANTI-SHEEP RED CELL ANTISERUM (aSRBC)

Rabbit 95-97 aSRBC serum was a gift from D. Benson. Rabbits were immunized by intravenous injection of 3.0 ml of a 10% suspension of SRBC on days 1, 2 and 4. Sera obtained on day 7 were pooled, heat inactivated at 56° for 30 minutes. The serum had a hemagglutination (HA) titer of 1:64; when treated with 0.01 M DTT, the HA titer was 1:32.

Rabbit H61 aSRBC serum was prepared by intravenous injection with 2.0 ml of a 10% suspension of SRBC on days 1, 2, 6, 9, 17 and 77. Serum was collected on days 7, 13, 21, 30, 36 and 86, pooled, and heat inactivated at 56° for 30 minutes. The HA titer of the serum was 1:64 and after treatment with 0.01 M DTT was reduced to 1:16.

HEMAGGLUTINATION TITERS

HA were determined in round bottom microtiter plates (Cooke Engineering Co., Los Angeles, California) using modified barbital buffer as diluent. Equal volumes of test serum dilutions and 2% SRBC were incubated at 37° for 30 minutes. For determination of DTT resistant antibodies (IgG), serum was incubated with an equal volume of 0.01 M dithiothreitol (DTT) for 15 minutes at 37° immediately prior to addition of SRBC. The hemagglutination titer is the reciprocal of the highest serum dilution giving visible agglutination.

PREPARATION OF SRBC-aSRBC MONOLAYERS FOR B CELL DEPLETION

Antigen-antibody complexes for B cell depletion were prepared by the method of Kedar, Ortez de Landazuri, and Bonavida (1974). Falcon petri

dishes (#3303, 100 x 15 mm) were treated for 45 minutes at room temperature with 7.0 ml of poly-L-lysine hydrochloride (PLL, molecular weight 195,000; Mann Research Labs) at 50 µg/ml in sterile 0.01 M PBS, pH 7.3. Plates were washed extensively with PBS and incubated at room temperature with 7.0 ml of a 1.2% solution of SRBC. At this point monolayers could be stored for up to 4 days at 4°. For final preparation of SRBC-aSRBC complexes, 7.0 ml of aSRBC serum were added to the washed monolayers and incubated at 37° for 45 minutes. Sensitized monolayers were rinsed well with PBS prior to use. Control plates were sensitized with normal rabbit serum which had been absorbed 3X with SRBC.

For spleen cell adsorption, 5.5 ml of 10^7 spleen cells/ml suspended in 10% FCS-MEM were added to each plate and incubated for 60 minutes at 37° with rocking at 5 cycles per minute for 30 minutes and holding stationary for an additional 30 minutes. Nonadherent cells were removed with a Pasteur pipet, washed twice in FCS-MEM and diluted to the desired cell concentration for further use.

PREPARATION OF NYLON WOOL COLUMNS TO SEPARATE B CELLS FROM T CELLS

The procedure of Julius et al. (1973) was used for preparation of nylon wool columns. LP-1 Leuko-Pak leukocyte filter (lot #LD5H5 and lot #YO69151; Fenwal Laboratories) were sources of nylon wool. The nylon wool was soaked in distilled water at 37° for 1 week with 3 to 4 changes of water. Excess water was squeezed from the nylon wool and it was allowed to dry at 37° for 2 to 3 days. Unless otherwise stated, 0.6 gm of the dry nylon were packed into a 12 cc plastic syringe barrel up to the 6 cc mark. The covers were replaced and the syringe was sterilized

by autoclaving. Immediately prior to use, the sterile column was rinsed with 20 ml of FCS-MEM and 1% penicillin-streptomycin. This will be termed wash medium. Excess medium was allowed to drain from the columns, the covers were sealed with tape to prevent evaporation, and the columns were incubated at 37° C for at least one hour prior to loading the cells.

Before loading the cells onto the nylon wool column, dead cells and macrophages were removed by passage over glass wool columns prepared by the method of Julius (personal communication). 0.2 gm glass wool (Pyrex, Corning Glass Works) was packed into a 3 cc syringe barrel up to the 1.5 cc mark. After replacing the covers the syringes were sterilized by autoclaving. Before use the glass wool columns were equilibrated with 10 ml of wash medium.

2 ml of cells were loaded onto the glass wool columns, which remained in a vertical position throughout the passage, and subsequently washed into the nylon wool with 0.5 to 1.0 ml of 37° wash medium. The nylon wool columns were replaced in the syringe covers, resealed and incubated for 45 minutes at 37°. The columns were washed slowly with wash medium at 37° and the first 25 ml of the effluent was collected and the cells pelleted at 630 x g for 10 minutes at 4°, washed once, and brought to the desired cell concentration in the appropriate medium. Cell viability after nylon wool pass was usually > 95% as determined by trypan blue dye exclusion.

IMMUNOELECTROPHORESIS (IEP) AND OUCHTERLONY GEL DIFFUSION ANALYSIS

These tests were performed according to the method of Campbell et al. (1970). For IEP, barbital B-2 buffer was used and the antigens were electrophoresed at 40 volts for 40 minutes.

PREPARATION OF RABBIT IgG FOR INJECTING SHEEP #93

A crude IgG fraction from whole rabbit serum was prepared by precipitation with 33% ammonium sulfate; salt ions were removed by dialysis. The IgG enriched fraction was chromatographed using DEAE-Sephadex A50 (Pharmacia) following the procedure of The and Feltkamp (1970). The resin was activated by alternate washings with 1N NaOH and 1N HCl and equilibrated with 0.01 M PBS, pH 7.2. 122 mg protein (2.5 ml) was loaded onto the equilibrated column (1.5 x 30 cm; flow rate $\sim$ 20 ml/hr). Three separate runs were made under equivalent conditions. The protein eluting in the equilibration buffer from all 3 runs was pooled, concentrated and rechromatographed. Absorbancy at 280 nm was monitored; tubes from the ascending portion of the curve and the peak tube were pooled and concentrated. When tested by IEP against sheep anti-rabbit Ig serum, only IgG was detected.

Sheep #93 was injected s.c. with 2 mg of rabbit IgG in CFA. Day 28 bleed was tested by IEP against whole rabbit serum and reacted with the IgG fraction.

SEPHADEX G-200 CHROMATOGRAPHY OF 2148 AND 2170 (ANTI-MOUSE IgG)

Gamma globulin was isolated from heat-inactivated rabbit serum by one precipitation with 33% SAS. Salts were removed by passage over a Sephadex G-25 column (27 x 2.5 cm) using Tris-HCl buffer, pH 8.0. The effluent was monitored for protein by absorbancy at 280 nm and for sulfate ions by the addition of $BaCl_2$. The gamma globulin enriched

sample was chromatographed on Sephadex G-200 (82 x 2.2 cm) equilibrated with Tris-HCl (pH 8.0) by standard upward flow technique. The IgM and IgG peaks were concentrated.

PREPARATION OF $F(ab')_2$ FRAGMENT OF HUMAN GAMMA GLOBULIN (HGG)

Gamma globulin from HGG (Immune Serum Globulin, American Red Cross, Massachusetts Public Health Biologic Labs, lot #RC70B) purified by ion exchange chromatography was a gift from K. L. Pratt. The ion exchange purified HGG (25.0 mg protein/ml) was pepsin digested according to the method of Weir (1973). 2.0 mg pepsin (Matheson, Coleman and Bell) dissolved in 0.1 M sodium acetate buffer, pH 4.5, was warmed to 37° and added to 200 mg HGG. The mixture was stirred for 24 hours at 37°. The pH was brought to 8.0 with solid Tris salt (2-amino-2-[hydroxymethyl]-1,3-propanediol; Matheson, Coleman and Bell). The digest was dialyzed against 0.1 M Tris-HCl buffer, pH 7.4 at 4°. The sample was centrifuged 2510 x g for 15 minutes at 4° to remove any precipitated material. The digest was run on a Sephadex G-150 column (2.7 x 83 cm) employing standard upward flow technique. The equilibrating and eluting buffer was 0.1 M Tris-HCl, pH 7.4.

Four separate column runs were made employing similar chromatographic conditions. The first peak from each of the runs was pooled, concentrated and rechromatographed. Two overlapping peaks were obtained, indicating possible IgG contamination. After concentration to 1.8 mg/ml the preparation was shown to contain human Fab and IgG when tested by gel diffusion. Consequently, it was used for immunization since there appeared to be sufficient Fab to insure the resultant antiserum would have both anti-IgG and anti-Fab reactivity.

CYTOTOXICITY TESTING

Cytotoxic antiserum and complement (C) treatment can be used to deplete specific cells. Antiserum (As) specificity is determined by cytotoxicity testing, which involves allowing antiserum to bind to an appropriate target cell population, adding C and determining cell lysis, death or membrane damage. Criteria used for cell death depend upon sufficient cell membrane damage to allow for supravital dye uptake (trypan blue dye exclusion test) or cell release of radiolabeled macromolecules (^{51}chromium release assay). Limited membrane damage could allow for immediate dye uptake or isotope release, but if damage were repaired, the cell could function normally. Therefore, when antiserum and C are used for depletion, functional tests must be done on the target cell population. Functional tests are the ability of treated cells to generate immune responses <u>in vitro</u> and can measure helper (T) cell or B cell function.

Initially I used the ^{51}Cr release assay, but abandoned this in favor of the trypan blue dye exclusion test, as the latter was simpler and less time consuming. Scoring of stained cells was done by light microscopy until our laboratory purchased a Cytograf cell counter (Model 6300A; Biophysics, Mahopec, N.Y.), an automatic cell counter which counts and differentiates live and dead cells simultaneously. Automated differentiation suppressed subjective bias inherent in scoring stained cells by light microscopy; simultaneous determination of cell number remaining after treatment is necessary as cell lysis can result in a disproportionately low number of cells staining.

^{51}Chromium Release Assay

Spleens or thymuses were teased apart in 10% FCS-MEM and clumps broken up by gently aspirating with a Pasteur pipet. Debris was allowed to settle for 5 minutes at 4° and the supernatant fluid was passed through a wire screen to remove clumps. 6 ml of cell suspension (1×10^7 cells/ml) were layered onto 2 ml of Ficoll-Isopaque solution and centrifuged 2800 x g for 30 minutes at 4°. The lymphocyte layer was pipetted off and the cells were washed 2X in 10% FCS-MEM. Cells were labeled according to a modification of Raff's method (1970). One ml cells (10^7 cells/ml) was incubated with 50 μCi/ml $Na^{51}CrO_4$ (New England Nuclear, El Cerrito, California) for 30 minutes at 37° with occasional mixing. Cells were washed twice with 2 ml F-MEM (280 x g/10 min/4°) and resuspended in F-MEM to 5X original volume. Cells were incubated 30 minutes/4° to allow for apontaneous rapid release of isotope, centrifuged and resuspended to 10^7 cells/ml.

One-tenth ml of cells and 0.1 ml appropriate As dilution were incubated at 37°/30 min. One ml warm MEM was added, cells were centrifuged at 280 x g/10 min and the cell pellet resuspended in 0.1 ml agarose-absorbed C (1:6 final dilution). After 30 minutes at 37° the reaction was stopped by addition of 1 ml MEM (room temperature). Cells were pelleted at 280 x g for 10 minutes and the supernatant fluid counted in a Gamma counter. A cytotoxic index (CI) was calculated by the formula:

$$CI = \frac{\text{cpm (As + C)} - \text{cpm (NS + C)}}{\text{cpm (100\% lysis)} - \text{cpm (NS + C)}}$$

Each sample was run in duplicate and the means of duplicate samples were determined. Total release (100% lysis) was determined by incubating cells as in the test system, but substituting F-MEM for As and C. After the final wash the cells were resuspended in 1 ml MEM and freeze-thawed 5X by alternate treatments with dry ice and hot water.

Trypan Blue Dye Exclusion Test

A two-step cytotoxicity test was used (Chan et al., 1970). One-tenth ml of target cells (1 x 10^7 cells/ml) was added to 0.1 ml of appropriately diluted antiserum (As). After mixing well the antiserum was allowed to bind to the cells for 60 min/4°. The cells were washed, resuspended in 0.1 ml agarose-absorbed C (1:6 final dilution) and incubated at 37° for 45 minutes. Trypan blue was added and stained cells determined by light microscopy or by use of a Cytograf cell counter, Model 6300A.

For light microscopy, 0.03 ml of 1% aqueous trypan blue was added to the cells; at least 100 cells were scored. For automated counting on the Cytograf, 1.9 ml of 0.05% trypan blue in phosphate buffered saline was added, and total cell number and percent viable cells determined.

When using the Cytograf cell counter, percent dead was calculated for each serum dilution (in the presence and absence of C) by the formula:

$$\frac{(\%\ \text{cells stained}) \times (\#\ \text{cells remaining})\ \text{As} + \text{C}}{(\%\ \text{cells stained}) \times (\#\ \text{cells remaining})\ \text{C only}}$$

This formula accounts for percent of staining cells in addition to cell

lysis by antiserum and C (C dependent lysis) or antiserum alone (C independent lysis).

Functional Testing: Generation of In Vitro Immune Response

To determine the effect of As and C treatment on the functional capacity of spleen cells, cells were treated as described for trypan blue dye exclusion testing. After incubation at 37° for 45 minutes with compelement, cells were washed 3X with 10% FCS-MEM (630 x g for 10 minutes), counted and resuspended to 1.1 x 10^7 cells/ml. Cells were cultured with thymus-dependent antigens, TNP-KLH or SRBC, or thymus-independent antigens, TNP-T4 or DNP-dextran, for 5 days in a Mishell-Dutton system. Anti-TNP or anti-SRBC PFC were measured. If helper T cells were affected by treatment, there would be no response to thymus-dependent antigens. Any effect on B cells is measured by reduced responses to thymus-independent antigens.

All sera and complement were filter-sterilized (0.45 μ Millipore filter) prior to use. Antisera were heat inactivated (56° for 30 minutes).

PREPARATION AND TESTING OF ANTISERA

See Appendix.

EXPERIMENTAL RESULTS

A. A DECISION TEST TO DETECT THE EFFECTS OF EARLY SIGNALS ON ANTIGEN-SENSITIVE B LYMPHOCYTES

I have devised a "decision test" which provides a functional means for detecting early signals delivered to antigen-specific lymphocytes. The general design of the decision test is to expose spleen cells to a biologically active substance (such as antigen or mitogen) and then give the cells a one-hour decision period in which to accept an immunogenic pulse of antigen before being placed in culture without additional antigen. The response in culture thus reflects the effect of the early signal on the cells' decision on whether or not to utilize the immunogenic pulse.

The decision test can be used to detect the effects of early signals delivered by antigen on antigen-sensitive cells. Previous studies on lymphocyte activation have generally used mitogens rather than antigens to detect early signals to lymphocytes since mitogens nonspecifically activate a large percentage of the lymphoid population, whereas antigens activate specific antigen-reactive cells which comprise less than 0.1% of the unprimed lymphoid population (Klinman and Press, 1975). As a result it is technically difficult to detect early signals to antigen-specific cells. The effects of T cell factors (Dutton, 1975), cyclic nucleotides (Watson, 1975), and mitogens (Coutinho et al., 1975) on antigen-specific cells have been examined for their effects on *in vitro* generated immune responses, but their experiments were not designed to detect early signals.

1. Design of the Decision Test

Figure 1 shows the basic experimental procedure for a decision test to detect effects of early signals by DNP-D, a thymus-independent antigen (Desaymard and Feldmann, 1975; Desaymard, personal communication). Normal spleen cells were exposed in vitro to DNP-D at 4°/60 min (signal). Excess antigen was removed by washing. Capping of membrane immunoglobulins (Ig) and extensive membrane rearrangement occur at 37° C within 15 minutes (Taylor et al., 1971), so cells were incubated at 37° to allow for any movement necessary for "signal translation". Further membrane movement and metabolism were slowed by lowering the temperature to 4°/5 min prior to adding the immunogenic pulse of DNP-D. After pulsing at 4°/60 min the cells were washed extensively to remove antigen and cultured without additional DNP-D. After 5 days direct (IgM) and indirect (IgG, IgA) antihapten plaque-forming cells (PFC) were determined using trinitrophenylated sheep red blood cells (TNP-SRBC). For detection of IgA PFC, an anti-mouse IgA antiserum was used in place of the anti-IgG facilitation serum described in Methods. Only direct IgM PFC are reported as no indirect PFC were detected. Where indicated, cells were also plated against non-haptenated SRBC for detection of polyclonal antibody responses.

2. Generation of Negative and Positive Signals by Antigen

Figure 2 shows two representative experiments demonstrating a differential effect of high and low signal doses on DNP-D on the cells' decision to respond to the immunogenic pulse. Cells incubated with subimmunogenic doses ($\ll 10^{-2}$ μg) of DNP-D during the signal stage showed a

Figure 1

Basic experimental procedure for a decision test to detect effects of early signals by DNP-D, a thymus independent antigen.

(1) Normal mouse (Balb/c) spleen cells (10^7/ml) in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin.

(2) Signal

4^0/60 min

Centrifuge through 1 ml 100% FCS at 630 x g/4^0/10 min
Wash in MEM-FCS

37^0/15 min

(3) Pulse

Immunogenic dose DNP-D (10^{-1} - 10^{-2} ug/ml)

(4) Decision period

4^0/60 min

Wash 3X in MEM-FCS

Tissue culture (10^7 cells/ml), no additional DNP-D

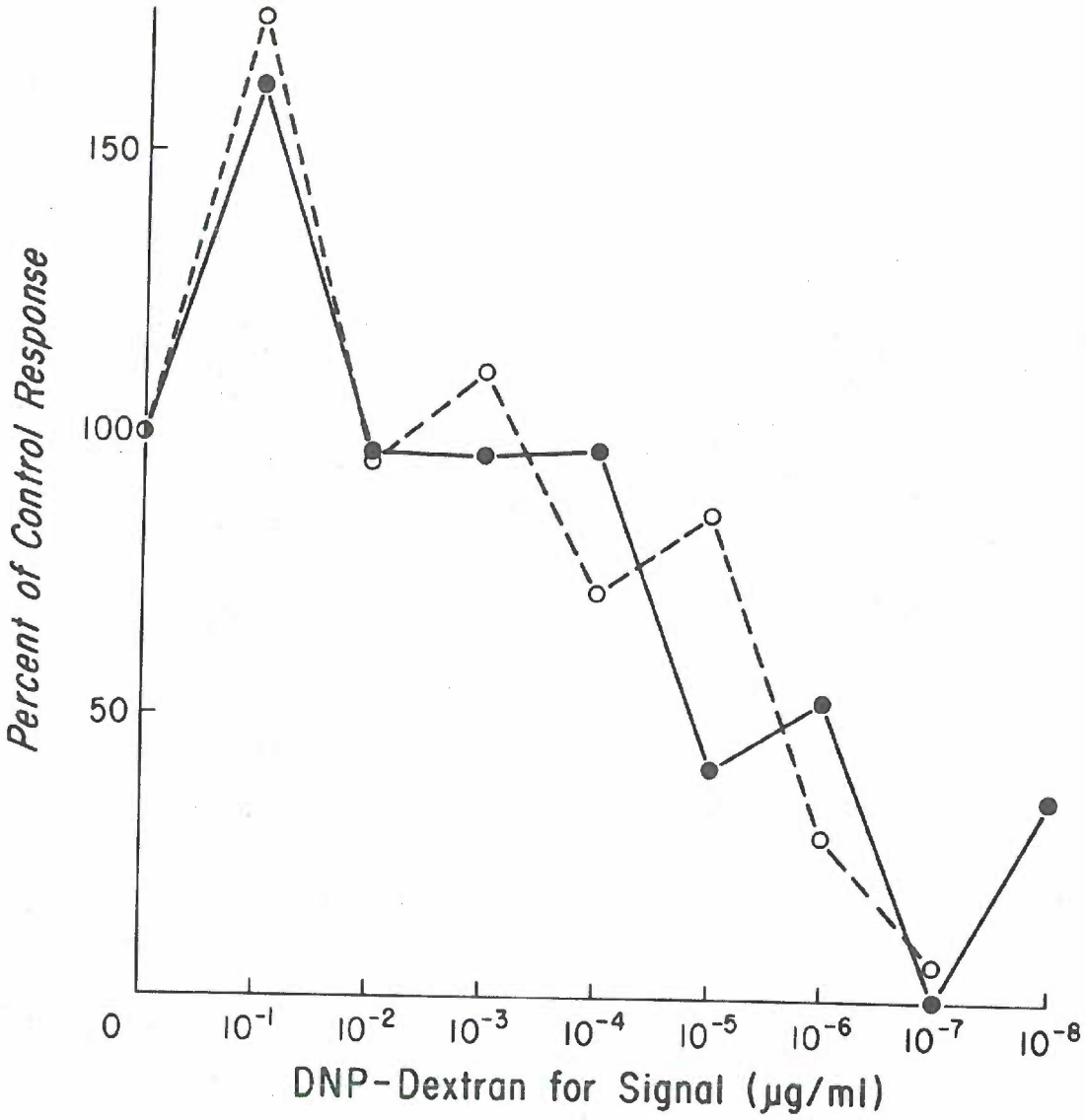
(5) Response to pulse

5 days 37^0

Assay antihapten PFC

Figure 2

Ability of the decision test to discriminate between high and low dose signals generated by DNP-D in 2 representative experiments. Each point represents a pool of 8 replicate microcultures. Results are expressed as % of control response with control cells pulsed but not signaled. Cells treated as in Figure 1. Control responses: PFC/10^6 cultured cells assayed ●—●—● 111, o--o--o 308.


Percent of Control Response
150
100
50
0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
10^{-8}
DNP-Dextran for Signal (μg/ml)

decreased response to the pulse compared to cells pulsed but not signalled with DNP-D. The dose (10^{-7} μg) delivering the most negative signal was only $1/10^5$ to $1/10^6$ of the pulse dose.

A high signal dose (10^{-1} μg) enhanced the cells' response (> 100%) to the pulse in the experiments shown in Figure 2. Although such enhancement was repeatedly observed, as seen in Figure 3, this enhancement was not statistically significant ($p > 0.4$). Nevertheless, this may represent a positive signal since, as shown below, high doses of LPS induce similar enhanced responses to the DNP-D pulse.

Signalling with low doses of DNP-D resulted in unresponsiveness to the pulse in 16/18 (89%) experiments, considering significant reduction to be 50% or greater. Figure 3 summarizes the data from nine of the positive experiments in which enough signal doses had been tested to generate the dose response curve shown. The data from each individual experiment are shown in Table 1. The data were analyzed for significance in a two-way analysis of variance (Sokal and Rohlf, 1969). Low dose signals of 10^{-6} μg, 10^{-7} μg and 10^{-8} μg decreased the response to the pulse significantly ($p < 0.001$) testing these cells as a unit against controls (cells signalled with diluent). Although the high signal dose of 10^{-1} μg appears to enhance the response to the pulse dose, this value was not statistically different from control cells ($p > 0.4$). These results suggest that a critically low dose is necessary to provide the negative signal. The dose range is broad, but this may reflect heterogeneity in the relative physiological state of the cells at the time

TABLE 1

COMPOSITE DATA ANALYZED FOR DNP-D SIGNAL-DOSE RESPONSE CURVE

		Anti-TNP PFC/10^6 Cells Experiment No.								
µg/ml DNP-D	Signal	1	2	3	4	5	10	11	13	14
(Control)	None	94	111	592	308	1549	860	733	250	454
	10^{-1}	406[a]	193	800	500	2185	1005	1006	109	722[a]
	10^{-2}	13	106	615	300	880	547	500	220	402
	10^{-3}	78	123	550	297	708	635	1246	175	427
	10^{-4}	100	80	574	300	1837	669	1029	175	545
	10^{-5}	65[a]	96	392	125	905	565	1220	60	380
	10^{-6}	5	32	245	163	738	319	1020	0	228
	10^{-7}	0[b]	7	203	0	809	135	720	20	212
	10^{-8}	23	0	287[a]	111	822	365	460	0	287

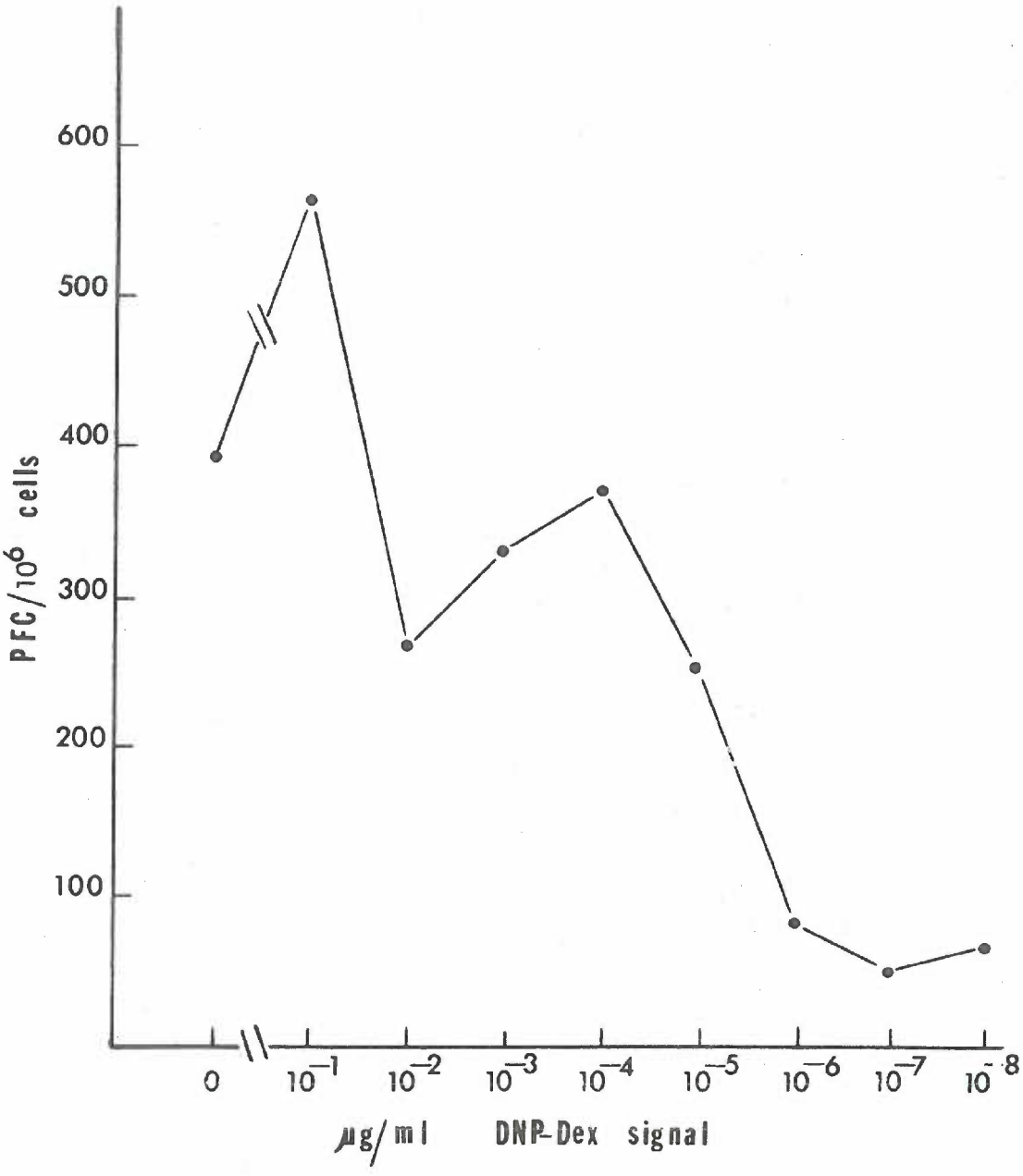
Dose response curve plotted in Figure 3.

[a]Missing data in experimental block; values were estimated by standard Yates' formula (Yates, 1933).

[b]0 PFC/10^6 cells was assigned a value of 1 PFC/10^6 cells for $\log_e$ transformation when tested for significance by two-way analysis of variance.

Figure 3

Composite data averaged to obtain a DNP-D signal-dose response curve. The 9 individual experiments are presented in Table 1. Data are expressed as mean PFC/10^6 cells for each signal level. Data were analyzed in a two-way analysis of variance for significance. Cells signaled with 10^{-6} µg, 10^{-7} µg and 10^{-8} µg DNP-D differed significantly ($p < 0.001$) when tested as a unit against controls (cells signaled with diluent) with $F_{1,64} = 22.1$. Cells signaled with 10^{-5} µg or greater DNP-D did not differ significantly from controls ($p > 0.05$).


600
500
400
300
200
100
PFC/10^6 cells
0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
μg/ml DNP-Dex signal

they are signalled. Thus, in Figure 2 it was possible to achieve greater than 90% inhibition with 10^{-7} μg; whereas in other experiments such a degree of inhibition was only achieved at 10^{-6} μg or 10^{-8} μg. At doses lower than 10^{-8} μg there was a gradual return to control values.

3. Generation of Negative and Positive Signals by Mitogen

Since some thymus-independent antigens are B cell mitogens and/or inducers of polyclonal antibody formation (Coutinho and Möller, 1975), I used the decision test to detect signals delivered to antigen-sensitive lymphocytes by LPS, which is a B cell mitogen and an inducer of polyclonal antibody production. The basic experimental procedure for the decision test was followed, except that LPS at varying doses was used as the signalling agent.

Figure 4 shows three representative experiments demonstrating a differential effect of low (Figure 4a) and high (Figure 4b) signal doses of LPS on the cells' decision to respond to the immunogenic pulse of DNP-D. At signal doses of 10^{-4} μg to 10^{-8} μg LPS there was a consistent decrease in the response to DNP-D pulse. Signalling with these low doses led to negative signals (≥ 50% reduction) in 14/16 experiments. Table 2 shows data from five such positive experiments in which enough signal doses had been tested to generate the dose response curve shown in Figure 5. The data were analyzed for significance in a two-way analysis of variance. Treatment with 10^{-4} μg, 10^{-5} μg and 10^{-6} μg LPS decreased the response to the pulse significantly ($p < 0.005$) testing these cells as a unit against controls (cells signalled with diluent).

Figures 4a and 4b

Low dose (Figure 4a) and high dose (Figure 4b) signals generated by LPS. Each point represents a pool of 8 replicate microcultures. Results are expressed as % of control response with control cells pulsed but signaled with diluent. Cells treated as in Figure 1, but signaled with LPS rather than DNP-D. Control responses: anti-TNP PFC/10^6 recovered cells assayed ●—● 832; ▲---▲ 880; □—□ 733.

FIG. 4a

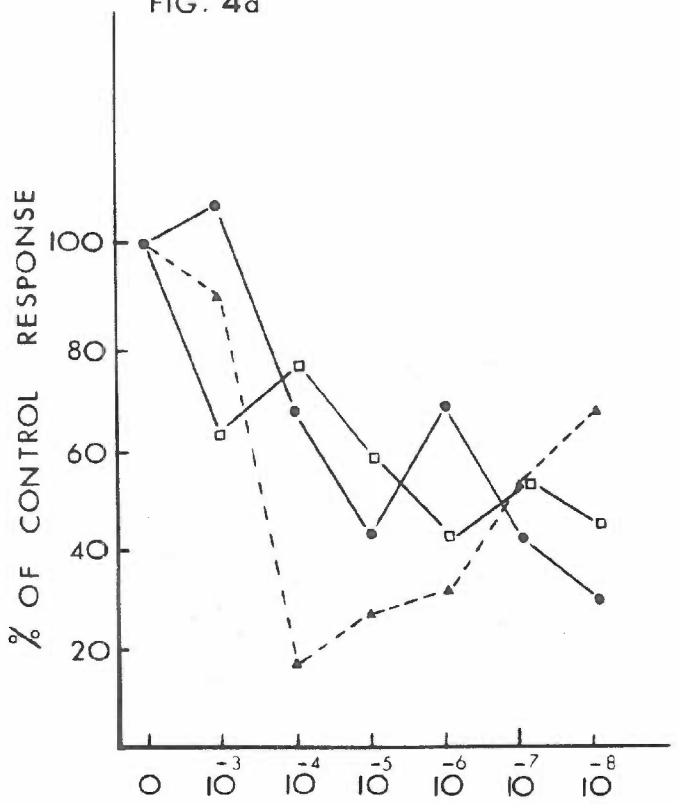
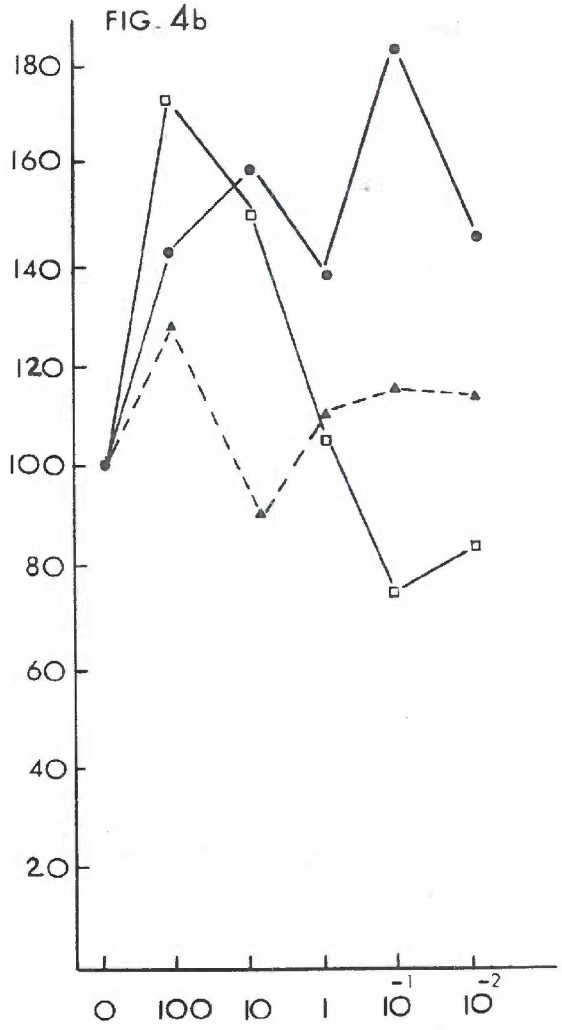


FIG. 4b



LPS SIGNAL DOSE (ug/ml)

TABLE 2

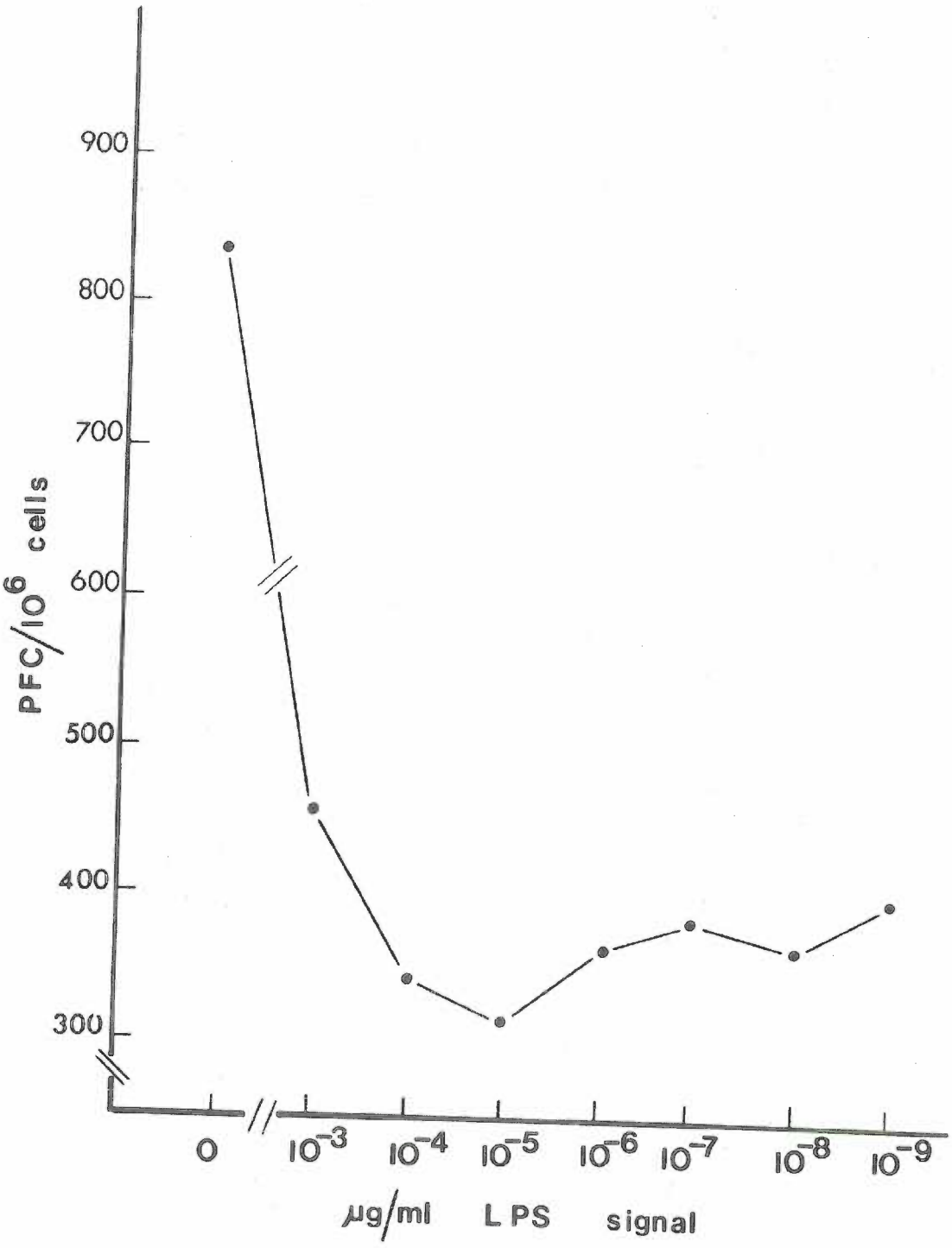
COMPOSITE DATA ANALYZED FOR LPS SIGNAL-DOSE RESPONSE CURVE

		Anti-TNP PFC/10^6 Cells Experiment No.				
µg/ml LPS	Signal	21	22	23	24	38
(control)	None	832	880	1315	579	733
	10^{-3}	893	791	168	379	442
	10^{-4}	546	140	280	395	554
	10^{-5}	331	231	546	206	370
	10^{-6}	603	262	333	428	292
	10^{-7}	334	456	448	324	382
	10^{-8}	278	580	292	296	490
	10^{-9}	830	323	115	490	733

Data analyzed by two-way analysis of variance and plotted in Figure 5. Data were transformed to $\log_e$ for analysis.

Figure 5

Composite data analyzed to obtain an LPS signal-dose response curve. The 5 individual experiments are presented in Table 2. Data are expressed as mean PFC/10^6 cells for each signal level. Cells were treated as in Figure 1, but signaled with LPS rather than DNP-D. Data were analyzed for significance in a two-way analysis of variance. Cells signaled with 10^{-4} µg, 10^{-5} µg and 10^{-6} µg LPS differed significantly ($p < 0.005$) when tested as a unit against control (cells signaled with diluent) with $F_{1,28} = 12.98$.


900
800
700
600
500
400
300
PFC/10^6 cells
0
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
10^-9
µg/ml LPS signal

Signalling with high doses of LPS (100 μg, 10 μg and 1 μg) enhanced the response to the DNP-D pulse in all eight experiments where cells were signalled with high doses of LPS. Data from five of these experiments in which all three high signal doses were used are shown in Table 3 and the composite high signal dose response is presented in Figure 6. The data were analyzed for significance in a two-way analysis of variance. Cells treated with 100 μg, 10 μg and 1 μg LPS differed significantly ($p < 0.05$) from controls when tested as a unit against control cells. Thus, using DNP-D as the immunogenic pulse, the LPS signal-dose response curve appears to mimic the DNP-D signal-dose response curve; whereas low signal doses result in unresponsiveness to the pulse, high-signal doses enhance the response to the pulse, although enhancement was statistically significant only with LPS and not with DNP-D.

4. Mechanism of Negative Signal Generation

a. Induction of Polyclonal Antibody (PAB) Responses

Since some B cell mitogens induce polyclonal antibody responses (PAB), I measured PAB responses by assaying cells against SRBC and TNP-SRBC. Representative data from one of four experiments are shown in Table 4. Column A shows PAB responses generated by signalling with LPS or DNP-D; no background PFC's have been subtracted. Cells signalled with high doses LPS have increased numbers of anti-SRBC and anti-TNP-SRBC PFC compared to cells receiving no LPS signal. This constitutes a PAB response. Lower doses of LPS and low doses of DNP-D give an inconsistent PAB response. Cells pulsed with DNP-D (column B) show sporadic

TABLE 3

COMPOSITE DATA ANALYZED FOR LPS HIGH-SIGNAL DOSE CURVE

	Anti-TNP PFC/10^6 Cells Experiment No.				
µg/ml LPS Signal	21	22	37	38	39
None	832	880	967	733	167
100	1182	1111	1271	1256	175
10	1321	795	949	1078	375
1	1139	970	1259	775	214

Data analyzed by two-way analysis of variance and plotted in Figure 6. Data were transformed to $\log_e$ for analysis.

Figure 6

Composite data averaged to obtain an LPS high-signal dose response curve. The 5 individual experiments are presented in Table 3. Data are expressed as mean PFC/10 cells for each signal level. Cells were treated as in Figure 1, but signaled with LPS rather than DNP-D. Data were analyzed for significance in a two-way analysis of variance. Cells signaled with 100 μg, 10 μg and 1 μg LPS differed significantly ($p < 0.05$) when tested as a unit against controls) (cells signaled with diluent) with $F_{1,12} = 5.43$.

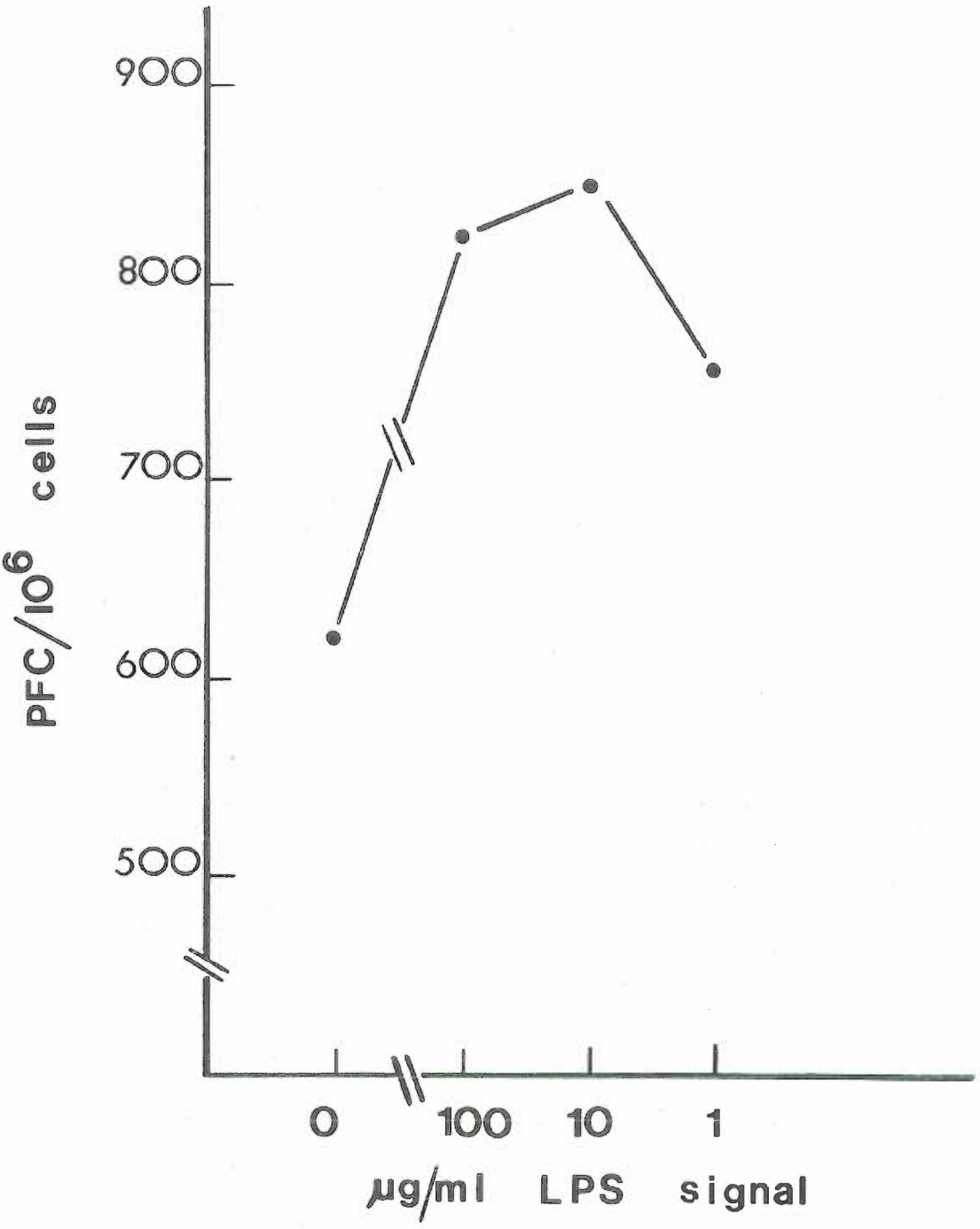

900
800
700
600
500
PFC/10^6 cells
0
100
10
1
µg/ml LPS signal

TABLE 4

INDUCTION OF POLYCLONAL ANTIBODY RESPONSES

Signal Dose	A No Pulse		B DNP-D Pulse	
	Anti-TNP-SRBC	Anti-SRBC	Anti-TNP-SRBC	Anti-SRBC
None	222	72	1054	0
µg/ml DNP-D				
10^{-6}	243	22	1129	17
10^{-7}	125	5	981	4
10^{-8}	286	114	771	75
µg/ml LPS				
100	289	115	1404	4
10	317	163	1543	9
1	232	60	1361	42
10^{-1}	20	5	1743	0
10^{-2}	100	38	1429	10
10^{-3}	123	85	935	9
10^{-4}	21	0	768	0
10^{-5}	270	74	553	0
10^{-6}	308	158	825	0
10^{-7}	191	0	556	0
10^{-8}	73	7	500	0
10^{-9}	78	26	1052	0
10^{-10}	276	19	1579	0
10^{-11}	77	55	1126	0
10^{-12}	110	20	978	33
10^{-13}	31	4	1243	5

Data are expressed as PFC/10^6 recovered cells assayed. Each value represents a pool of 8 replicate microcultures. Cells treated as in Figure 1. Column A, no pulse of DNP-D; Column B, pulsed with 10^{-2} µg DNP-D. Cells were assayed against SRBC and TNP-SRBC.

anti-SRBC responses; the response to TNP-SRBC is about five times greater than compared to non-pulsed cells. Although I can detect PAB responses, such responses are not unique to doses which generate negative or positive signals.

b. Effect of Temperature on Signal Translation

Initial experiments as diagrammed in Figure 1 included a 15-minute incubation step at 37° after the signal administration to allow for necessary membrane rearrangements. However, subsequent experiments showed this 37° incubation step was not necessary for generation of negative signals. The response of cells signalled with low doses of DNP-D (10^{-6} μg to 10^{-8} μg) and incubated at 4° was compared to the response of cells signalled in a similar manner but incubated at 37°. When tested by Student's t test the two groups were not statistically different ($p > 0.1$). Since the 37° incubation step after the signal was not necessary for generation of negative or positive signals delivered by LPS, all subsequent experiments were carried out entirely at 4°. That positive and negative signals were generated at 4° suggested that if cell metabolic activity is required, it must be minimal.

c. Reversibility of the Negative Signal: The Effect of Temperature and Time

In two experiments, cells were signalled with LPS or with DNP-D at 37° for 60 minutes rather than at 4° prior to pulsing with DNP-D. Negative signals were not generated at any signal-dose of LPS (100 μg to 10^{-8} μg) or of DNP-D (10^{-2} μg to 10^{-9} μg) if the signal was provided at

37° for 60 minutes. Since negative signals generated at 4° are still detectable after 15 minutes at 37°, these results suggest the negative signal is reversible, but that 15 minutes at 37° is inadequate and that reversion requires more than 15 minutes to be detected. From the two experiments it isn't clear if positive signals are also reversed at 37° for 60 minutes. The negative signals could also be reversed if the signal time at 4° was increased to 3 hours, suggesting that reversal is a metabolic process which is slowed but not blocked at 4°. Additional support for reversibility of the negative signal comes from experiments where cells were signalled (4°/60 min), pulsed and cultured for five days in the continuous presence of an immunogenic dose of DNP-D; signalling had no consistent effect on the response to antigen in culture.

d. The Negative Signal Is Independent of Mitogenic Properties of FCS

The dose response data suggest that the negative signal is due only to the antigen dose and not to nonspecific factors since all cells were treated the same except for the signal dose. However, since fetal calf serum (FCS) has been reported to be mitogenic (Coutinho et al., 1973), experiments were done to determine if FCS was in some way causing the negative signal. In some experiments FCS was omitted from all steps prior to placing the cells in final culture. In other experiments the cells were not washed through 100% FCS after signalling. Omitting FCS had no effect on generation of the negative signal.

e. Effect of T Cell and Macrophage Depletion on Generation of Negative Signals

It is possible that either T cells or macrophages could mediate the generation of negative signals. These possibilities were tested by depleting cell suspensions of either cell type prior to signalling and culture. T cells were depleted by treating KLH primed spleen cells with anti-T cell serum and C; residual cells (B cells and macrophages) were signalled with varying doses of LPS and pulsed with DNP-D. T cell depletion was monitored by the response to TNP-KLH which is a thymus-dependent antigen (Jennings et al., 1975). As seen in Table 5, T cell depletion did not affect negative or positive signals generated by LPS.

Macrophages were depleted from spleen cells by carbonyl iron and magnet treatment. Depletion did not inhibit generation of negative signals (Figure 7); in some cases macrophage removal intensified the negative signal. The success of macrophage depletion was assessed by determining the *in vitro* response to TNP-KLH, which requires macrophages, and to TNP-T4, a thymus-independent antigen. Depletion reduced the TNP-KLH response by 100% while reducing the response to TNP-T4 by only 21%, indicating macrophage depletion with minimal B cell loss.

These depletion experiments suggest that negative signals can be generated in spleen cells substantially depleted of T cells or macrophages.

TABLE 5

GENERATION OF NEGATIVE SIGNALS AFTER T CELL DEPLETION[a]

BY ANTI-T CELL SERUM[b] AND C TREATMENT

Signal Dose	Anti-TNP PFC/10^6 Recovered Cells Assayed	% of Control Response[c]
(Control) None	561	100
µg/ml LPS		
100	1537	274
10	906	161
1	618	110
10^{-1}	775	138
10^{-2}	236	42
10^{-3}	136	24
10^{-4}	106	19
10^{-5}	450	80
10^{-6}	765	136

[a]Depletion controls: response of KLH-primed cells cultured for 5 days with 0.02 µg TNP-KLH: T cell depleted cultures: 0 PFC/10^6 cells; whole spleen cells: 805 PFC/10^6 cells.

[b]Sheep anti-T cell serum, a gift from Dr. M. Feldmann, used at 1:30 final dilution.

[c]Control response is response of T cell depleted spleens pulsed but not signaled. All cells pulsed with 10^{-2} µg DNP-D. Control response = 561 PFC/10^6 recovered cells assayed.

Figure 7

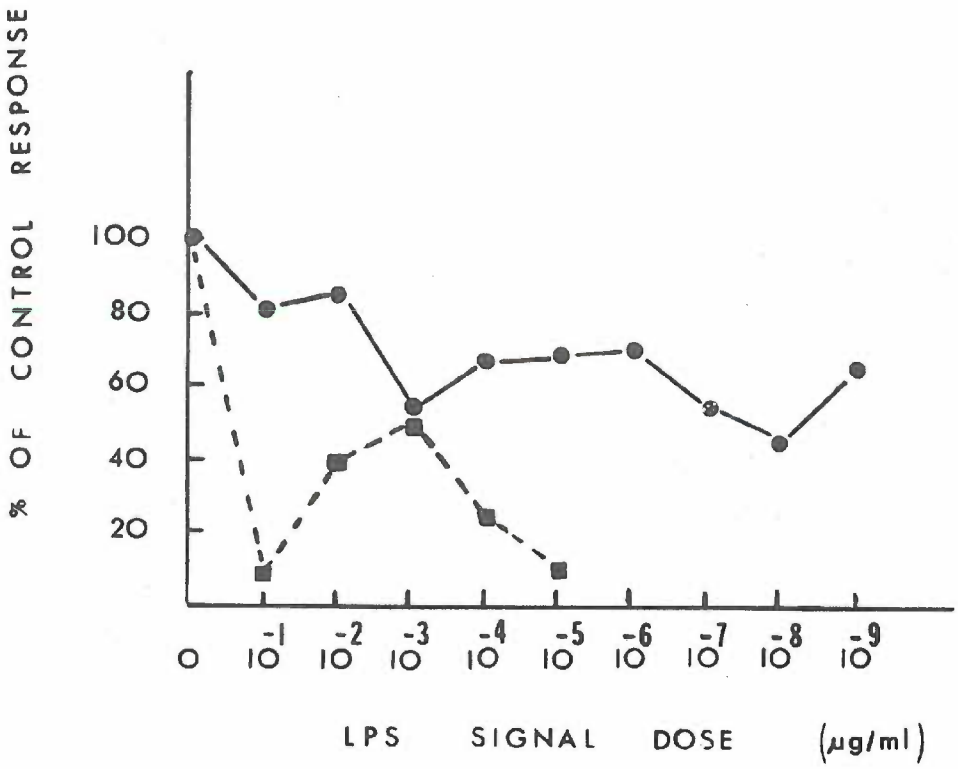
Effect of macrophage depletion on generation of negative signals. Macrophages were depleted by treatment of KLH-primed spleen cells with carbonyl iron and a magnet. Depleted cells were signaled and pulsed as previously described.

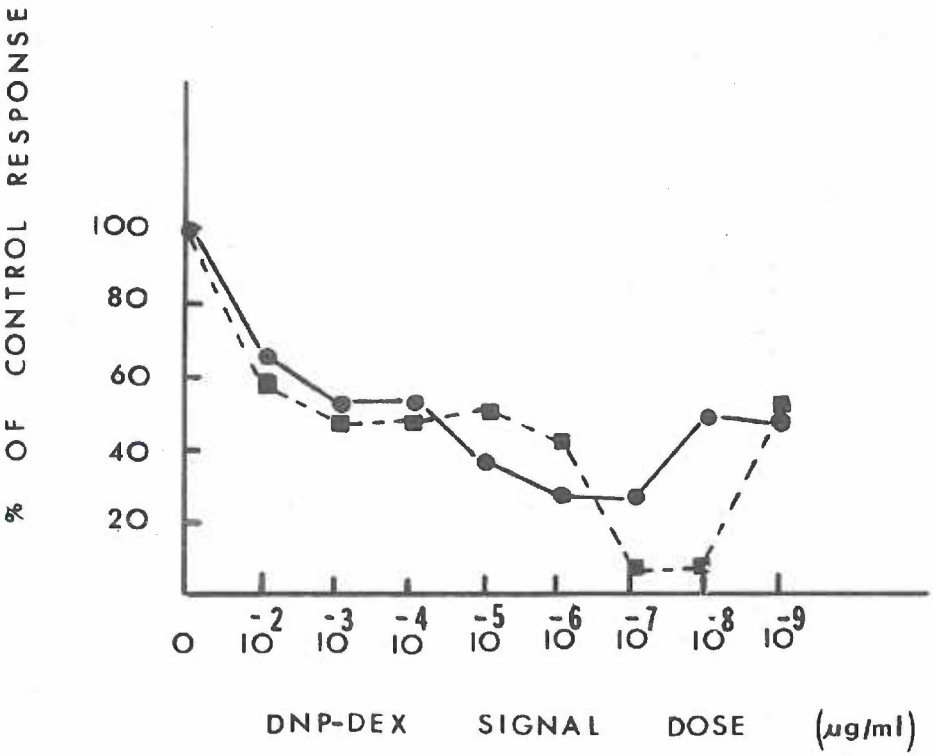
Depletion controls: response of KLH-primed cells cultured for 5 days with 0.02 μg TNP-KLH or 3×10^7 PFU TNP-T4.

	PFC/10^6 cells	
	TNP-KLH	TNP-T4
Macrophage depleted spleen cells	0	522
Whole spleen cells	469	661

Control response is response of depleted cells pulsed but not signaled. All cells pulsed with 10^{-2} μg DNP-D.

Depleted spleen cells: ■···■···■ ; whole spleen cells: ●—●—● .


% OF CONTROL RESPONSE
100
80
60
40
20
0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
10^-9
LPS SIGNAL DOSE (μg/ml)


% OF CONTROL RESPONSE
100
80
60
40
20
0
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
10^-9
DNP-DEX SIGNAL DOSE (μg/ml)

f. Effect of Colchicine

Microtubules play a role in modulating cell surface molecules. Low temperatures (4°) and the drug colchicine disrupt microtubules (Margulis, 1973). Since generation of negative signals occurs at 4° and not at 37° (Part c, above), microtubule disruption might be required for negative signal generation. To test this, cells were pretreated with 10^{-4} M colchicine (Calbiochem, A grade; lot #501167) for 30 minutes at 37° to disrupt microtubules (Yahara and Edelman, 1975). Cells were signaled with LPS or DNP-D for 60 minutes at 37° or 4°. Signaling at 37° was necessary in order to observe the effect of colchicine since microtubules are disrupted at 4°. Cells were washed to remove colchicine and then pulsed with 10^{-2} μg DNP-D in the usual manner. Cells pretreated with colchicine were unable to utilize the pulse; this occurred in both signaled and non-signaled (control) cells. Colchicine treatment was not toxic as colchicine treated and non-treated cells cultured for five days in the continuous presence of DNP-D responded equally well. Although this experiment could not be used to assess the role of microtubules in signal generation, it does suggest a role for microtubules in immunogenic pulse utilization by by cells.

g. Inhibition of the Negative Signal by Cytochalasin B (CB)

Cytochalasin B, a fungal metabolite, affects various cell functions such as blocking the activity of the microfilament-related contractile system (Wessells et al., 1971). Microfilaments may play a role in the movement of molecules within the plane of the membrane such as in the

translocation of patches to caps, and in cell movement (Unanue and Karnovsky, 1974).

In three different experiments cells were treated with CB during the LPS or DNP-D signal to determine if generation of low-dose negative signals involves the microfilament system. CB (Aldrich Chemical Co., Inc.; lot #PH/2668/77K) was dissolved in pure dimethylsulfoxide (DMSO), a gift of Dr. S. Jacob, at a concentration of 10 mg CB/ml. CB (1 μg/ml) or DMSO (0.01%) was added to the cells during the signal step. Cells were signaled for 60 minutes at 4°, washed and pulsed with DNP-D (10^{-2} μg) for one hour at 4°.

In one of the three experiments, if cells were exposed to low signal doses of LPS (10^{-3} μg to 10^{-8} μg) or DNP-D (10^{-6} μg to 10^{-8} μg) in the presence of CB, there was no negative signal. However, there were variable effects in the other two experiments. In all three experiments negative signals generated in the presence of DMSO were not as great as in previous experiments (≥ 50% reduction), suggesting DMSO by itself may partially inhibit the generation of the negative signal. Consequently I cannot draw any conclusions about the role of microfilaments in generation of the negative signal.

5. Discussion

The decision test can be used to detect early signals delivered to antigen-sensitive cells by antigen or mitogen. Both positive and negative signals are generated depending on signal dose; the mechanism for generation of negative signals was investigated in greater detail and will be discussed here.

Low dose negative signals generated by mitogen and antigen appear similar since experiments showed the following:

1. Both signals were generated at 4°, suggesting that if cell metabolic activity is required, it is minimal.

2. Low dose negative signals were not generated at 37° after 60 minutes.

3. Negative signals were generated after substantial T cell or macrophage depletion, suggesting the signal is acting directly on B cells. DNP-D is T cell and macrophage independent and LPS stimulates B cells directly; however, negative signals could be mediated by macrophages or T cells. Even though the depletion procedures used gave "substantial" rather than 100% depletion, negative signals generated in depleted cell populations were comparable to those in non-depleted populations. This suggests that macrophages or T cells are not involved, although the possibility cannot be ruled out that a few remaining accessory cells provide all of the negative signal.

4. The optimum negative signal dose is low; in the case of DNP-D it was 10^{-5} to 10^{-6} of the pulse dose. Higher signal doses of both LPS and DNP-D were less effective, with high doses generating positive signals. This suggests that the signal does not involve blocking of cell receptors or of antibody since under those circumstances, higher signal doses should be as or more effective.

I cannot draw any conclusions about the role of microfilaments in generation of the negative signal. However, cytochalasin B at doses

used in my experiments is not toxic and does not affect antigen binding to macrophages (Rosenthal et al., 1975) or to thymus-derived lymphocytes (Yoshinaga et al., 1972). Roberts and LaVia (1975) have shown cytochalasin B can inhibit B cell activation and subsequent antibody production if present for only the first 6 hours of a 4-day *in vitro* culture. These findings suggest a role for microfilaments in translation of signals leading to B cell activation, but it should be kept in mind that cytochalasin B affects other biological functions including glucose transport, cell-to-cell contact and cell movement, which may account for these observations.

The effect of such signals on B lymphocytes is T cell and macrophage independent. Extensive membrane rearrangement is not involved. The signals are transient and may represent an initial reversible step in low zone tolerance induction which requires a second irreversible event not supplied by the *in vitro* conditions used. Until recently it was believed that thymus-independent antigens induce unresponsiveness only in high concentrations (Howard and Mitchison, 1975). However, Waldmann and Pope (1975) recently reported that very low doses of LPS, a B cell mitogen and a thymus-independent antigen, rendered mice unresponsive to challenge with TNP-LPS. The low zone unresponsiveness was specific and required no obvious T cell mediated suppressor mechanisms. As seen in Figure 3, 10^{-7} μg DNP-D provides optimal negative signals. With such low doses the lymphocyte could be expected to encounter very few molecules of antigen or mitogen during the one-hour signal period.

Furthermore, interaction with this limited number of antigen or mitogen molecules appears to provide only a transient signal for turn-off of antigen-specific cells. Although there is no direct proof, the cells' unresponsiveness to the pulse dose probably results from an inability to bind the immunogenic pulse of antigen and carry it over into tissue culture. Thus, within the defined one-hour decision period the antigen-binding receptors of these cells are non-functional. Although the mechanism is not clear, it is possible to speculate as follows: control and test cells are signaled at 4°; control cells can utilize the pulse, whereas negatively signaled cells cannot. Patching but not extensive membrane rearrangements can occur at 4° (dePetris and Raff, 1973), suggesting that patching or a limited degree of receptor aggregation may be necessary for pulse "binding". According to this hypothesis the negative signal delivered by a limited number of antigen or mitogen molecules would interfere with this aggregation. This is supported by the observation that the negative signal can be reversed by increasing the signal time or temperature which may allow for appropriate membrane receptor redistribution.

Although there are controversies regarding the role of mitogens in B cell activation, it is clear that the mitogen receptor is distinct from the antigen-specific Ig receptor (Coutinho et al., 1975). My observations suggest that a limited number of mitogenic or antigenic hits result in antigenic unresponsiveness, but it isn't known if the mechanisms are the same. These experiments do not delineate the role of

mitogens in B cell activation; they do suggest that low numbers of antigen or mitogen molecules interacting with antigen-specific cells can render these cells transiently unresponsive to an antigenic pulse. The implications and importance of cell turn-off by a limited number of "hits" will be discussed in the Hypothesis in conjunction with modulation of T cell membranes by a similar mechanism.

B. EARLY SIGNALS DELIVERED TO T CELLS BY ANTIGEN

1. Experimental Design

The nature of antigen receptors on T cells is controversial and receptors have been identified as Ig-like and non-Ig like. All biochemical and immunological approaches pursued have led to conflicting ambiguous results. I reasoned that if Ig molecules were present on T cells they must be generally inaccessible because they are either buried deeply in the cell surface or so pauci-distributed as to make detection an infrequent and difficult event. Nevertheless, it has been shown that T cells can bind antigen: heavily radiolabelled antigen can cause specific "suicide" of T helper cells (Basten et al., 1972; Cooper and Ada, 1972), and that antigen-specific tolerance can be induced in T helper cells (Rittenberg and Bullock, 1972). These observations suggested to me that, if Ig-like molecules were present on T cells but generally inaccessible, they might become accessible in the presence of antigen, as was suggested by Greaves and Hogg (1971). I devised a series of experiments to ask if antigen could induce Ig on specific helper T cells. Induced Ig would be detected by the failure of these cells to cooperate as helper cells in a specific anti-hapten response following treatment with anti-Ig serum and complement. This strategy utilizes the loss of specific helper function as a positive indication of Ig rather than direct detection of Ig on helper cell surfaces, since the latter procedure has led to such contradictory results in the past.

In accord with this reasoning I based my experiments upon the following strategy:

1. Employ an immune response *in vitro* which requires the cooperation of T cells and B cells. The thymus-dependent responses I used were anti-hapten responses generated by the conjugates TNP-KLH and DNP-CGG.

2. Use mouse spleen cells previously primed to the carrier (KLH or CGG) *in vivo* as a source of helper T cells.

3. Expose the helper cells to the specific carrier for a brief period (one hour) in the cold to allow carrier to bind. Wash out unbound carrier and warm the cells to 30° or 37° for various intervals to allow "induction" of surface Ig on T cells. Return the cells to 4° to slow down metabolic processes (this step retards translational movement in the cell membrane and capping and pinocytosis of surface Ig on B lymphocytes [Taylor et al., 1971]).

4. Treat the cells with anti-mouse Ig serum and C to inactivate cells bearing surface Ig. This would include most B cells and helper T cells which had been induced to expose Ig by the prior treatment.

5. Add back normal unprimed B cells to replenish those inactivated by anti-Ig and C.

6. Add the intact antigen (hapten-carrier conjugate) to the cell suspension and incubate in a Mishell-Dutton culture system to develop an *in vitro* anti-hapten response.

7. If Ig "induction" on T cells had occurred the specific helper cells would be inactivated after anti-Ig and C treatment and no anti-

hapten response would occur. If "induction" had not occurred the helper cells would still be present and a normal anti-hapten response would develop.

8. As an antigen specificity control, two kinds of helper cells to different carriers would be used in the experiment, but only one of the two carrier molecules would be used as an "inducer". If the model worked, only the anti-hapten response to the "inducer" hapten-carrier$_1$ conjugate would be reduced as carrier$_2$-specific helper cells would be able to cooperate in the anti-hapten immune response to hapten-carrier$_2$ conjugate.

The specific experimental protocol is as follows. Mice were primed with the protein carriers keyhole limpet hemocyanin (KLH) or chicken gamma globulin (CGG) and their spleens used one to three months later. KLH-primed spleen cells or a mixture of 50% KLH-primed and 50% CGG-primed spleen cells were used where indicated. Spleen cells were incubated without KLH (control cells) or with varying doses of KLH for 60 minutes at 4° (signal). Cells were centrifuged through FCS and washed in the cold to remove unbound KLH and incubated at either 30° or 37° to allow for any membrane movement or metabolism necessary for the signal translation (induction). The temperature was lowered to 4° and rabbit anti-mouse Ig antiserum was added and allowed to bind at 4° for 60 minutes (direct technique). Cells were washed free of unbound anti-Ig. In some experiments the effect of the rabbit anti-mouse Ig was amplified or enhanced by adding an anti-rabbit Ig serum (indirect technique)

before adding C. Presumably, as in the enhancement of IgG plaques (Dresser and Wortis, 1969), this step provides additional binding sites for C and allows for more effective killing.

If an amplifying serum was used it was allowed to bind at 4° for 60 minutes and the cells were then washed. Agarose absorbed guinea pig serum was added as a C source and cells were incubated at 37° for 30 or 45 minutes. The cells were washed three times. B cells inactivated by the treatment were replenished by adding anti-Bø and C treated normal spleen cells or nontreated normal spleen cells. Since T cells must be primed to the carrier for helper cell function, T cells contaminating the normal spleen preparations could not function as helper cells. Cells were then cultured with optimal doses of antigen or with no antigen in the Mishell-Dutton culture system. Direct anti-TNP PFC were measured on day 5 of culture. Background (no antigen) plaques were subtracted from all responses.

Sera Used

Complement:

Guinea pig serum (Rockland, Gilbertsville, Pa.) absorbed with agarose was used as a source of C at 1:6 final dilution.

Antisera:

All sera are described in detail in the Appendix.

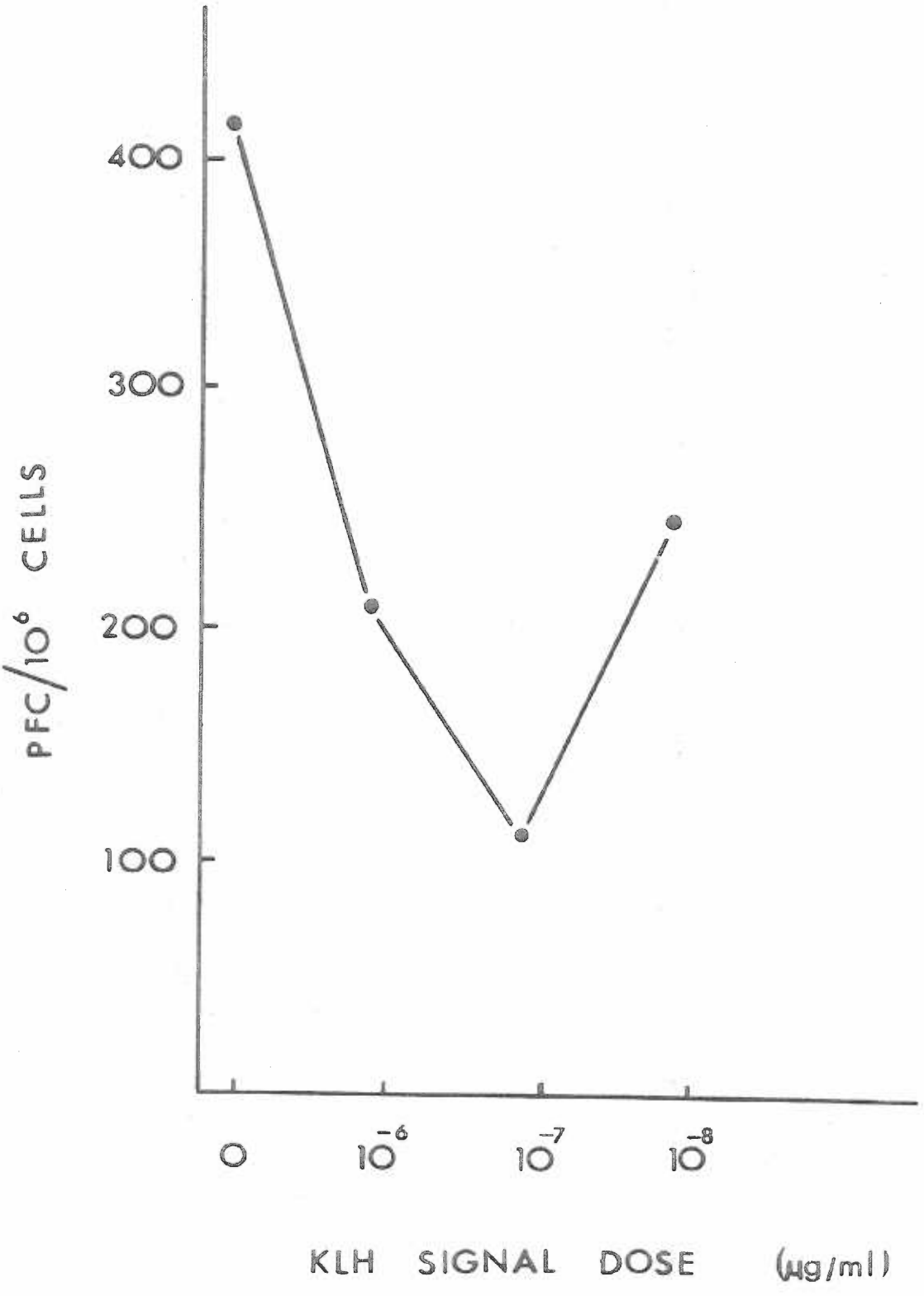
#1: Rabbit anti-mouse #67-69.

At 1:75 dilution it killed 32% spleen cells and 15% thymocytes. Cytotoxicity could be absorbed out with purified mouse IgG (73 µg/ml serum; Pentex, Miles Laboratories).

Figure 8

Ability of low-signal doses of KLH to induce helper cell susceptibility to anti-Ig and C. Data are from Experiment 2, Table 6. Each point represents the mean PFC/10^6 recovered cells assayed of triplicate cultures.

KLH-primed spleen cells were signaled with KLH (10^{-6} µg to 10^{-8} µg) or with diluent (control cells) and induced at 37° for 15 minutes. Cells were treated with anti-Ig and C by the indirect technique. Normal spleen cells were used to replenish B cells. 0.02 µg TNP-KLH was used in culture and cells were assayed on day 5 of culture against TNP-SRBC. Data were analyzed for significance in analysis of variance. Responses of cells signaled with 10^{-6} µg, 10^{-7} µg and 10^{-8} µg KLH differed significantly ($p < 0.001$) when tested as a unit against controls with $F_{1,8} = 27.0$.


400
300
200
100
PFC/10⁶ CELLS
0
10⁻⁶
10⁻⁷
10⁻⁸
KLH SIGNAL DOSE (μg/ml)

#2: Rabbit anti-MIgG2bk.

At 1:5 dilution the serum killed 11% thymocytes and 21% spleen cells in a C dependent cytotoxicity test assayed by trypan blue dye exclusion.

#3: Rabbit anti-MIgG #2148.

At 1:5 dilution it killed 14% thymus cells and 44% spleen cells. Cytotoxicity could be removed by absorption with mouse IgG (250 µg/ml serum; Pentex).

#4: Rabbit anti-kappa chain, pool 1:

At 1:5 dilution it killed < 10% thymus cells and 40% spleen cells. Cytotoxicity could be removed by absorption with mouse IgG (250 µg/ml serum; Pentex).

2. Helper Cell Susceptibility to Anti-Ig and C After Signaling With Low Doses of Carrier Antigen

Figure 8 shows data from experiment #2 (Table 6) which is representative of initial experiments which suggested that very low signal doses of KLH were effective in inducing susceptibility to anti-Ig and C. Cells signaled with 10^{-6} µg, 10^{-7} µg and 10^{-8} µg KLH and treated by the indirect anti-Ig technique generated responses equal to 50, 27 and 59% respectively of control responses (cells not signaled but treated with anti-Ig and C). The data were analyzed for significance by analysis of variance. The response of signaled cells differed significantly ($p < 0.001$) when tested as a unit against controls. In this experiment the 10^{-7} µg KLH signal was most effective as an inducer of susceptibility

TABLE 6

COMPILED DATA DEMONSTRATING KLH HELPER CELL INACTIVATION

BY ANTI-Ig AND C AFTER SIGNALING WITH LOW DOSES KLH

Serum and Dilution	Exp. #	% of Control Response[a] μg/ml KLH Signal 10^{-5}	10^{-6}	10^{-7}	10^{-8}
1: Rabbit anti-MIg 67-69 (1:25)	7			40	
	8			105	
	9		13		
	10		21		
2: Rabbit anti-MIg$\gamma G_{2_b}\kappa$ (1:20)	2		77	76	88
	3		36	65	
	4			77	64
#2 and Facilitating Serum: goat anti-MIg #A (1:10)	2		50	27	59
	3		48	80	
Goat anti-rabbit Ig #B (1:25)	4		83	100	91
3: Rabbit anti-MIgG #2148 (1:5)	5	22	47	47	
#3 and Facilitating Serum #A (1:10)	5	84	47	33	
4: Rabbit anti-Kappa Pool 1 (1:5)	30		69	104	103
	31		75	53	85
#4 and Facilitating Serum #B (1:25)	30		60	37	88
	31		123	51	41

[a]Control response: cells signaled with diluent and treated with anti-Ig and C.

TABLE 7

HELPER CELL INACTIVATION BY ANTI-Ig AND C

AFTER SIGNALING WITH LOW DOSES OF KLH

Exp. #	Anti-Ig and C Treatment	PFC/10^6 Cells µg/ml KLH Signal 0	10^{-6}	10^{-7}	10^{-8}
2	Indirect	416	207	112	246
	Direct	258	198	195	226
31	Indirect	746	916	384	303
	Direct	452	337	239	383
30	Indirect	533	313	197	469
	Direct	478	331	498	491

See text for detailed description of procedure. Data are presented as PFC/10^6 recovered cells assayed. Three experiments are shown in which cells were signaled with diluent or with 10^{-6} µg to 10^{-8} µg KLH and then treated by the indirect or the direct anti-Ig technique. Cells were cultured with 0.02 µg TNP-KLH; anti-TNP PFC were measured on day 5. Each value represents the pool of 8 replicate cultures. Using the indirect anti-Ig technique, the response of cells signaled with low doses of KLH was statistically different from cells signaled with diluent ($p < 0.001$). Using the direct anti-Ig technique, there was no statistical difference between control and signaled cells ($p > 0.1$). Data were tested for significance in a two-way analysis of variance.

to anti-Ig and C (73% reduction in the response). Both 10^{-6} µg and 10^{-8} µg induced less well, suggesting a critical signal-dose range. There was only slight inhibition of the response using the direct anti-Ig technique (see Table 6).

Four different cytotoxic anti-mouse Ig sera were used in ten signal experiments. Signaling with 10^{-5}, 10^{-6}, 10^{-7} or 10^{-8} µg KLH prior to anti-Ig and C treatment resulted in loss of helper cell function in 8/10 experiments, considering significant reduction to be 50% or greater. The results from the ten experiments are given in Table 6. Experiments 8 and 4 were the two experiments in which a significant reduction in the response was not observed with at least one antiserum. Even though all anti-Ig sera were cytotoxic in the presence of C, use of the direct anti-Ig technique was not always sufficient for depletion of helper cell function. Use of a facilitating serum (indirect anti-Ig technique) in experiments 2, 30 and 31 resulted in loss of helper cell function. The data from the individual experiments are presented in Table 7. The data were analyzed for significance in a two-way analysis of variance. The response of cells signaled with KLH differed significantly ($p < 0.001$) when tested as a unit against controls (cells signaled with diluent). In these three experiments the response of signaled cells treated by the direct technique was not statistically different from control cells ($p > 0.1$).

However, the direct anti-Ig technique depleted helper function in experiments 7, 9, 10, 3 and 5, indicating that some anti-Ig sera could

bind sufficiently without a facilitating serum. In these experiments the response of cells signaled with 10^{-6} µg KLH was statistically different from the response of control cells ($p < 0.001$). The enhancing effect of the indirect anti-Ig technique suggests that even though antigen induced cells might be able to bind anti-mouse Ig antibodies, there still may be a sparse distribution of such Ig-like molecules on the carrier-induced T cells, perhaps because such distributions are not permanent. The inability to decrease the response by 100% may represent physiological differences of the helper cells at the time they are induced.

Since the cytotoxic activity of the sera (serum #2 not tested) was inhibited by purified mouse IgG, I refer to these reagents as anti-Ig; however, I can't exclude the possibility that these sera possess some anti-membrane activity and that it is the latter specificity which is induced by signaling with low doses of carrier. Nevertheless, the important point from these experiments rests on the observation that the cells only become sensitive to these reagents after brief exposure to picogram quantities of carrier protein. I shall continue to refer to these reagents as anti-Ig, although the limitations discussed above should be kept in mind.

3. Specificity of Induction

A crucial question was to determine the specificity of induction by KLH. That is, are all helper T cells affected by signaling with KLH or are only KLH-specific helper T cells affected? To answer this question,

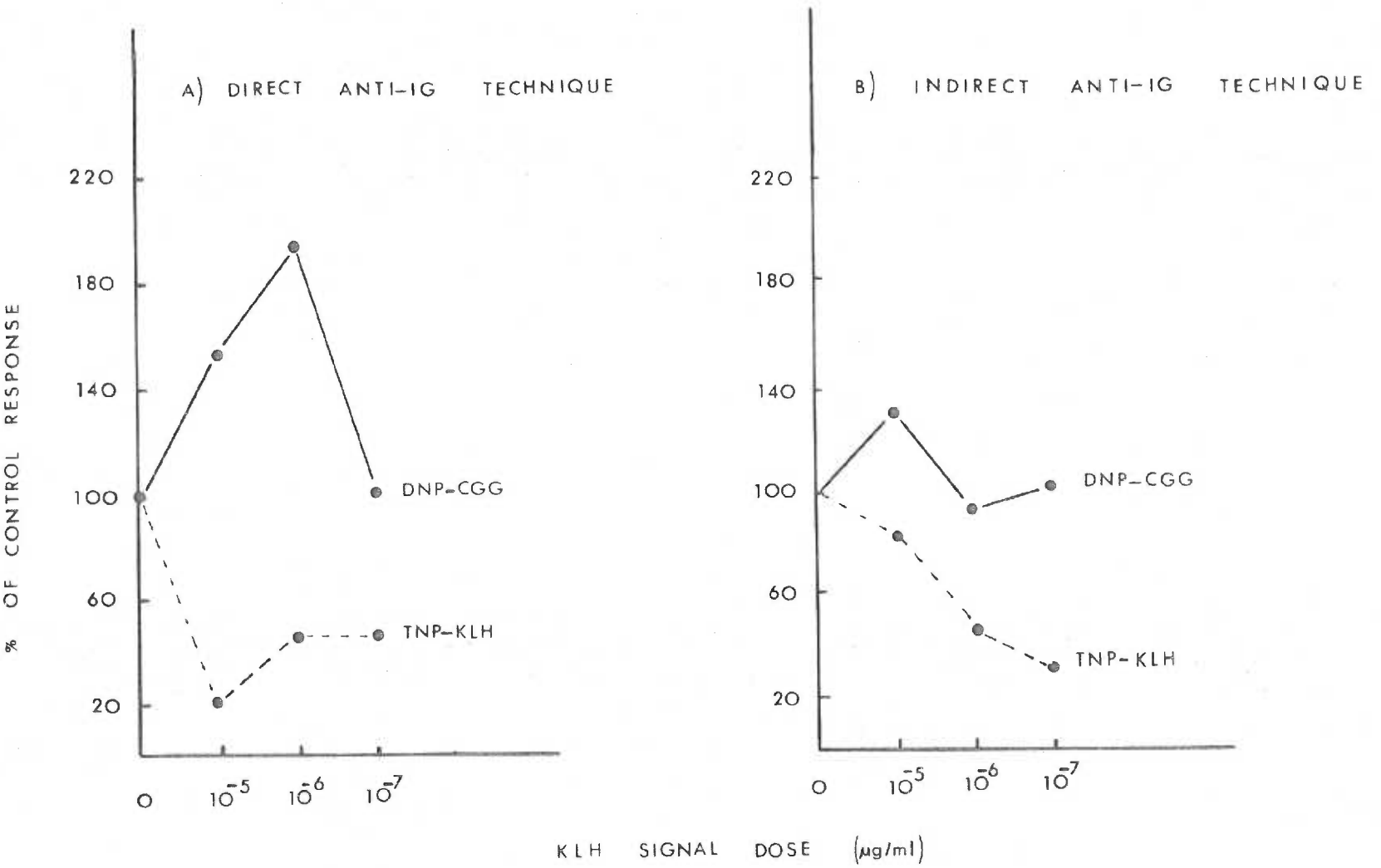
in three experiments I used a mixture of 50% KLH-primed spleen cells and 50% CGG-primed spleen cells. In the experiment shown in Figure 9, cells received no signal (control response) or 10^{-5} µg to 10^{-8} µg KLH as signal dose. Cells were induced at 37° for 15 minutes after signaling, treated with anti-Ig (direct and indirect techniques) and C. Anti-Baθ and C treated normal spleen cells were used to replenish B cells prior to culture without antigen or with 0.02 µg TNP-KLH or 0.2 µg DNP-CGG. In this experiment only KLH-specific helper cells were depleted, while CGG helper cells were unaffected. This specificity is indicated by the data which show that the anti-DNP responses remained at or above control values, while the anti-TNP response to TNP-KLH was reduced as much as 78% when 10^{-7} µg of KLH was used as inducer. The increased response to DNP-CGG could reflect enrichment for CGG helper cells after depletion of KLH helper cells.

This experiment suggested carrier specificity in the induction process and argues against a nonspecific effect on B cells or macrophages since DNP-CGG, like TNP-KLH, is macrophage-dependent (Feldmann et al., 1975A). However, in several additional specificity experiments I could not obtain a consistent response to DNP-CGG in control (non-signaled) cells. In standard dose-response titrations, DNP-CGG was often a weak immunogen for generating primary in vitro responses; frequently there was no net response after background (no antigen in vitro) PFC were subtracted. Therefore, the question of antigen specificity in the induction process should be considered tentative as these experiments were not repeated.

Figure 9

Specificity of induction of helper cell susceptibility to anti-Ig and C after signaling with low doses of KLH.

A mixture of CGG and KLH primed spleen cells were signaled with KLH prior to anti-Ig and C treatment. B cells were used to replenish cultures were normal spleen cells treated with anti-Baθ (Rabbit D57, pool A, 1:4 dilution) and C. Cells were cultured without antigen or with 0.2 µg DNP-CGG ●—●—● or 0.02 µg TNP-KLH ●···●···● for 5 days. Background PFC were subtracted from antigen stimulated cultures. Results are expressed as % of control response with control cells not signaled but treated with anti-Ig and C. Both direct anti-Ig technique (9A) and indirect anti-Ig technique (9B) were used. Each point represents a pool of 8 replicate microcultures. Cells were assayed against TNP-SRBC.


A) DIRECT ANTI-IG TECHNIQUE
B) INDIRECT ANTI-IG TECHNIQUE
% OF CONTROL RESPONSE
220
180
140
100
60
20
0
10^{-5}
10^{-6}
10^{-7}
DNP-CGG
TNP-KLH
KLH SIGNAL DOSE (µg/ml)

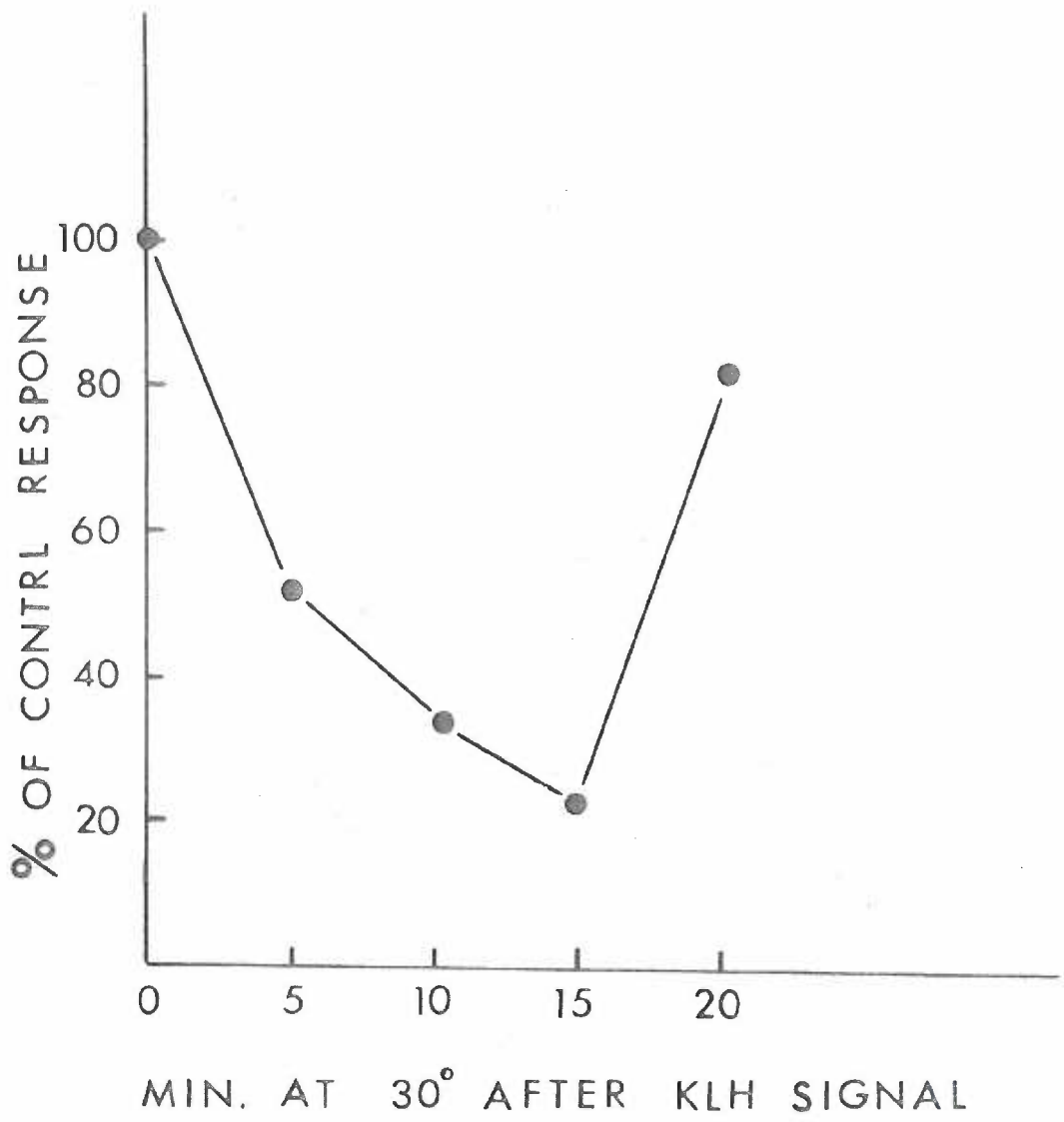
4. Is an Induction Step at Elevated Temperatures (37° or 30°) Required for Helper Cell Susceptibility to Anti-Ig and C?

To ask if elevated temperature is required for susceptibility to anti-Ig and C, KLH-primed cells were treated in the following manner: cells were not signaled (control cells) or signaled with low doses of KLH (10^{-6} µg or 10^{-7} µg) for 60 minutes at 4°. After washing, control and test cells were incubated at 37° or 30°, sampled at 5-minute intervals and then cooled at 4° for 5 minutes. Cells were then treated by the direct anti-Ig technique prior to C addition. Normal spleen cells were used to replenish B cells. Cells were then cultured for 5 days without antigen or with 0.02 µg TNP-KLH and assayed against TNP-SRBC. Two experiments were performed at each temperature; all four experiments gave similar results. The results from one experiment are presented in Figure 10. Data are expressed as % of control response with control cells signaled with 10^{-6} µg KLH, but not incubated at 30°. If signaled cells were incubated at 30° for 5, 10 or 15 minutes prior to anti-Ig and C treatment, the response is decreased 48%, 64% and 77% respectively compared to signaled cells not incubated at 30°. After 15 minutes incubation, there is a gradual return to control responses. These experiments indicate that cells signaled with low doses of KLH become susceptible to anti-Ig and C treatment only after induction at 37° or 30°. Elevated temperature is necessary to reveal induction, which suggests that active metabolism is required.

Figure 10

Induction of helper cell susceptibility to anti-Ig and C after low-dose KLH signal--dependence upon elevated temperature.

See text for detailed description of procedure. Data are presented as % of control response with control cells signaled with 10^{-6} µg KLH, but not incubated at 30°. Test cells were signaled with 10^{-6} µg KLH and incubated at 30°. All cells were treated by the direct anti-Ig and C technique. Normal spleen cells were used to replenish B cells.


% OF CONTRL RESPONSE
100
80
60
40
20
0
5
10
15
20
MIN. AT 30° AFTER KLH SIGNAL

5. Discussion

The important observation from these experiments is the modulation of responsiveness by low doses of antigen. After signaling with low doses of KLH, helper T cells become susceptible to inactivation by anti-Ig and C. Loss of helper T cell function is measured by the inability of such cells to cooperate with B cells in *in vitro* cell cooperation experiments. The inductive process can be initiated by antigen exposure at 4°, but requires elevated temperatures to be expressed, suggesting that metabolic activity is required. The susceptibility to anti-Ig and C is transient as it disappears by about 20 minutes. As stated previously, I can't exclude the possibility that the sera used possess some anti-membrane activity and that it is this specificity which is induced by signaling with low doses of carrier. If so, the experiments still suggest that low dose modulation of membrane components. If the anti-Ig antibodies were responsible for the observed effect on helper function, there are several possibilities to account for this:

1. The antigen receptor on thymus-derived helper cells is an Ig-like molecule. Low doses of specific carrier antigen serve as a signal for the appearance of these receptor molecules. This could occur through exposure of buried receptor molecules, rearrangement on the surface, or *de novo* synthesis of such molecules. The increased accessibility of Ig-like molecules would then render the cell susceptible to anti-Ig and C.

2. Cell contact with carrier antigen may signal the appearance of Ig-like molecules which serve a non-antigen receptor function.

3. Low doses of carrier may activate thymus-derived cells, rendering them able to bind Ig molecules. Activated T cells can bind aggregated Ig (VanBoxel and Rosenstreich, 1974) and I have no evidence that the anti-Ig sera used were aggregate-free. If anti-mouse Ig were bound to the cell via the Fc fragment, the enhancing serum could bind to the antibody and fix C. This mechanism seems unlikely since in some cases the direct anti-Ig technique resulted in loss of helper cell function.

4. Antigen-antibody complexes (KLH-anti-KLH) might be formed during signaling with KLH and be bound to the cell by either antigen binding to the antigen receptor or antibody binding via the Fc receptor (Yoshida and Andersson, 1972); in this case small amounts of anti-KLH antibody would be activating C. This scheme also seems unlikely as cells signaled with KLH and treated with C did not show loss of helper cell function (data not shown). This observation would also argue against the possibility that the low dose signal activates suppressor T cells since the latter would not require anti-Ig and C treatment for their effect.

These experiments cannot distinguish among the above alternatives 1 and 2 with respect to the mode of inactivation of helper T cells after signaling with KLH and subsequent anti-Ig and C treatment. Nor do they reveal the nature of the T cell antigen receptor. They do suggest, however, that picogram quantities of antigen can serve to modulate cell surface components and thereby regulate cell function.

HYPOTHESIS

Early signals in lymphocyte activation: the ability of a limited number of "hits" by a stimulating ligand to modulate membrane components and thereby alter immune competence.

The experiments suggest that picogram quantities of ligand (antigen or mitogen) can modulate surface membrane components of T and B lymphocytes. The doses of antigen required to induce these early signals are much lower than those reported to be mitogenic (Coutinho et al., 1974; Coutinho and Möller, 1975). Therefore, the question of mitogenicity is not pertinent because either Coutinho and Möller are correct and all antigens work through a second nonspecific mitogenic stimulus, or at the low doses used the mitogenic effects have been diluted out and I am dealing only with the antigen-specific properties of these materials. B lymphocyte unresponsiveness to an antigenic pulse after an initial encounter with a limited number of antigen or mitogen molecules might provide a regulatory means to discriminate between random "hits" by self-molecules and acute antigenic challenge by cross-reacting foreign molecules. Thus, random or limited "hits" by self-components would not break tolerance as the lymphocyte would be transiently unresponsive; however, this period of transient unresponsiveness is sufficiently short to permit the lymphocyte to respond to an acute antigenic challenge. Such unresponsiveness to the antigenic pulse could result from altered distribution or accessibility of membrane components, presumably antigen receptors.

Low levels of antigen also appear to modulate T lymphocyte membrane components, rendering the cells susceptible to inactivation by anti-Ig and C treatment. The experiments do not elucidate the mode of inactivation, but a reasonable interpretation is that low doses of antigen serve as a signal for the appearance of molecules which are Ig-like, thus rendering the T cell susceptible to anti-Ig and C. It isn't known if these molecules are antigen receptors or if they serve some non-antigen-related receptor function. Since the effect of carrier was antigen-specific, it is reasonable to assume that antigen receptors are involved. The transient appearance of such molecules on the cell surface would alter the cell's antigen responsiveness and could play a role in the cell interaction required for triggering of B cells. Regardless of the mechanism of inactivation, the data suggest that T cells like B cells can be modulated by small subimmunogenic numbers of antigen molecules. Both T and B cells appear to have a discrimination threshold which suggests a type of coincidence counting not unlike that in scintillation counting. The exact number of "hits" which registers a signal cannot be determined because the number of precursor cells cannot be measured.

The low dose signal to T cells would have to be viewed as an arming response. The first "hit" activates the cell and readies it for further "hits" by a true foreign antigenic insult; if it gets these additional "hits", it binds antigen and enters into the immune pathway. As a counterpoint the B lymphocyte responds in the reverse manner; it turns off at the first "hit" and then recovers, waiting for a multiple "hit"

event such as provided by an antigen focused on a macrophage or on a T-independent antigen. The two together provide T and B lymphocytes with the capacity to discriminate a true "signal" from "noise" and allow for a response to cross-reacting foreign antigenic molecules, but not to self-components, thus maintaining tolerance.

APPENDIX: CELL SEPARATION TECHNIQUES

For dissection of the immune response, it is desirable to obtain pure B and T cell populations and admix them in various combinations. Since different cell populations have different cell surface antigens, subpopulations can be depleted by specific antiserum and complement. For example, anti-Thy 1 serum and C can be used to deplete T cells and anti-Ig serum and C can be used for B cell depletion. Consequently, preparation and characterization of specific antiserum are important to define immunocompetent cell subpopulations and to study their functions and interactions. Methods have been described which separate T and B cells based on differences in the physical properties of the two cell types. Two reportedly simple and reproducible techniques devised for B cell depletion are use of nylon wool columns and antigen-antibody complexes.

The following section deals with production and characterization of cytotoxic antiserum and B cell depletion studies using nylon wool and antigen-antibody complexes. I will show that it is difficult to produce cytotoxic antiserum free of contaminating antibodies. I will also show that it is difficult to deplete cell suspensions of specific subpopulations using either cytotoxic antiserum and C or physical adherence methods. Depletion is not reproducible and is only relative, depending upon the method used to determine cell depletion. The problems associated with cell separation techniques and with the use of specific antiserum must be kept in mind since these techniques are used to assign specific functions to cells and their antigens in the generation of the immune response.

INTRODUCTION

Production of Anti-Thy 1 and Anti-Baθ Sera

Reif and Allen (1966) described a thymus cell alloantigen θ (new nomenclature, Thy-1) prepared by injecting C3H thymus cells into AKR mice. There are two known alleles of Thy-1, Thy-1.2 and Thy-1.1. The majority of mouse strains, including C3H and BALB/c, carry Thy 1.2; AKR strain carries Thy-1.1. Raff and Owen (1971) characterized the organ distribution of Thy-1 positive cells and found the approximate proportions: thymus (100%), spleen (30-35%), thoracic duct lymphocytes (80-85%), blood lymphocytes (70%), lymph nodes (65-70%), Peyer's patches (20-25%), and bone marrow (0%). There is a greater density of Thy-1 antigen on thymocytes within the thymus than on splenic derived thymocytes. As T cells leave the thymus and migrate to the spleen, there is a loss of Thy-1 antigen, rendering thymocytes more sensitive to anti-Thy-1 and complement inactivation than splenic T cells (Raff and Cantor, 1971). It is the Thy-1 bearing lymphocyte which is responsible for helper cell activity with thymus-dependent antigens (Raff, 1970; Chan et al., 1970).

A Thy-1 like antigen is also present on brain tissue (Baθ). Golub (1971) used mouse brain to immunize rabbits and goats for production of large quantities of anti-Baθ. The original report claimed anti-T cell specificity of the serum; cytotoxicity testing showed > 90% killing of thymocytes, 30% killing of spleen cells and < 1% killing of bone marrow

cells. Inactivation of T cell function was demonstrated by inhibition of a primary in vitro immune response to SRBC (thymus-dependent antigen) and of graft-versus-host reactions. The only evidence for absence of anti-B cell activity in the antiserum was its failure to act on antibody forming cells (AFC) when immune spleen cells were treated immediately prior to assaying for PFC; activity against B cells other than AFC was not determined. Since this report, Golub has shown that anti-Baθ serum possesses anti-stem cell activity directed against bone marrow and fetal liver stem cells. This was measured by in vivo functional tests, although by trypan blue dye exclusion testing there was no cytotoxicity for bone marrow cells (Golub, 1972). Anti-erythrocyte (Golub, 1973), anti-lymphoma (discussed in Golub, 1973; Toh and Cauchi, 1974) and anti-B cell activity (discussed in Golub and Day, 1975) of anti-Baθ serum have been described. Due to this cross-reactivity among brain and other tissues, difficulty in preparing specific anti-Baθ serum might be encountered. The best method for production of specific anti-mouse alloantiserum free of contaminating antibodies is to use congenic mice, that is mice which have been bred to differ only at a single genetic locus. While such antisera are useful in cytological studies, they can be produced only in relatively small quantities; it is technically difficult to prepare large quantities of antiserum in the mouse, so it is sometimes necessary to use heterologous species for production of the large volumes of antiserum needed for cell depletion studies.

Production of Cytotoxic Anti-Immunoglobulin (Ig) Antiserum

B lymphocytes bear a dense layer of Ig molecules on their surface. 7S IgM and IgD-like molecules have been isolated from murine B cell surfaces (reviewed in Vitetta and Uhr, 1975). It is estimated that murine cells bear about 10^5 Ig molecules per B cell with the Fc region embedded in the membrane and the Fab (antigen-binding) portion oriented outward (Engers and Unanue, 1974). Miller et al. (1972) reported selective B cell killing using anti-kappa chain serum and complement (C) and numerous investigators have used anti-Ig and C to deplete B cells. However, not all anti-Ig antisera are cytotoxic for B cells (Lesley et al., 1971); the reason for this is not known. Nucleated cells show differences in lytic sensitivities to antibody and C (Ohanian and Borsos, 1975) and murine B cells in mitosis are resistant to lysis by cytotoxic anti-Ig and C even though they bear large amounts of surface Ig (Kerbel and Doenhoff, 1974). Such differential sensitivities could result from differences in antigen density, membrane resistance to lysis or C fixation and activation. Non-C fixing immunoglobulin classes have been identified in humans and guinea pigs and antibody of the IgM class is more efficient than IgG antibody in C fixation (Eisen, 1974).

Loss of cytotoxicity after absorption with Ig is one criterion used to determine the specificity of cytotoxic anti-Ig antiserum. However, contaminating molecules present in the Ig preparation could be responsible for this reduction. For example, β_2 microglobulin is a cell surface component which shares homology with Ig (Peterson et al., 1972);

B cells have surface receptors for C and C components can bind to Ig molecules. Antibodies to β_2 microglobulin or to C could be the cytotoxic moieties in some cytotoxic antisera and still appear to be absorbed out by Ig if the latter is impure.

Since cytotoxic anti-Ig is difficult to raise, one must ask if Ig molecules on cells can serve as target antigens for cell lysis or if cytotoxicity is due to contaminating antibodies.

Use of Nylon Wool Columns for B Cell Depletion

Julius et al. (1973) reported a rapid method for the isolation of thymus-derived (T) cells. They found that B cells stick preferentially to nylon fiber columns, whereas T cells do not. If spleen cells were passed through the columns, the effluent (non-bound) population was virtually devoid of B precursor and memory cell activity, but contained helper cell and cytotoxic precursor and effector cell activity. The effluent cells contained 85 to 90% T cells, less than 5% Ig-bearing cells and 10% null cells (Thy^-, Ig^-). Trizio and Cudkowicz (1974) used *in vivo* cell transfer experiments to show that, after column passage, effluent cells retained T helper activity and nylon-adherent cells retained antibody-forming capacity.

I used nylon wool column filtration to separate T cells from B cells; separation was monitored by the anti-Thy-1 reactivity of non-bound cells and by the ability of these cells to participate as specific helper cells in *in vitro* immune responses.

Cellular Immunoadsorbents for B Cell Depletion

Basten et al. (1972A) described a receptor for antibody-antigen complexes (Fc receptor) present on B cells and absent on T cells. The Fc receptor binds antigen-antibody complexes or aggregated Ig through the Fc portion of the Ig molecule. This finding was utilized to separate B cells from T cells by passing lymphocytes over antigen-coated (human gamma globulin) degalon beads after allowing the cells to bind antibody (anti-human gamma globulin). B cells were retained through formation of antigen-antibody bridges between the cells and the beads. Kedar et al. (1974) described a modification of Basten's method utilizing cellular immunoadsorbents for B cell depletion. Monolayers of sheep erythrocytes (antigen) sensitized with anti-sheep erythrocyte antibody were used to retain B cells. T cells would not be retained since they supposedly lack the Fc receptor.

Several laboratories have been unable to deplete B cells by using the above method of Basten et al. (1972A). Karpf et al. (1975) suggested that the apparent B cell depletion observed by Basten and his colleagues could have been due to blocking of B cell receptors by anti-Ig rather than by depletion. Wigzell et al. (1972) were unable to obtain B cell depletion using a bovine serum albumin (BSA), anti-BSA column. However, if they used a mouse IgG-2a, anti-IgG-2a column, only IgG-2a bearing cells but not other Ig-bearing (B) cells were depleted. Wigzell et al. (1972) suggest that the depletion achieved by Basten et al. (1972A) was due to anti-mouse Ig activity in the anti-HGG serum used, making the

columns behave like free anti-Ig columns where free anti-Ig binding sites are available to combine with Ig on the surface of the cells.

There are reports that T cells bear an Fc receptor; activated mouse T cells bind antigen-antibody complexes (Yoshida and Andersson, 1972), activated guinea pig T lymphocytes bind aggregated Ig (VanBoxel and Rosenstreich, 1974), and Basten et al. (1975), who initially reported that T cells lack Fc receptors, can detect Fc receptors on T cells using more sensitive techniques. Presently, the literature suggests that T cells bear Fc receptors and that B cell depletion by antigen-antibody complex binding is questionable.

EXPERIMENTAL RESULTS

A. Preparation and Testing of Anti-Thy 1 and Anti-Baθ Serum

1. Mouse Anti-Mouse Thy 1.2 Serum

AKR anti-C3Hθ was prepared according to the method of Reif and Allen (1966) with slight modifications. One hour prior to the removal of thymus glands, C3H mice were injected with Pelikan ink (colloidal carbon) (0.2 ml i.p., 1:2 dilution in normal saline) to stain and identify parathymic lymph nodes in order to avoid B cell contamination of thymocyte preparations (Blau and Gaugas, 1968). Mice were etherized and the thymus glands were removed. The parathymic lymph nodes were discarded, and excess bloody tissue around the thymus was removed. The thymus glands were stirred at 4° for 2 hours in Locke's buffer and forced through a wire mesh to obtain a single cell suspension. AKR mice received weekly injections of 0.5 ml cell suspension (2×10^7 cells/ml; i.p.).

Eight mice received nine weekly injections of thymus cells. At week 9, 0.2 ml CFA was injected i.p. weekly for three weeks to generate ascites fluid. Tissue injection was continued for three more weeks. Mice were eye bled at weeks 6, 7, 8, 9, 10 and 11 and the serum pooled. Final bleed (week 15) was not pooled with the sequential bleedings. Ascites developed in 3 out of 8 mice; ascitic fluid was collected by standard techniques at weeks 10, 11 and 12, keeping individual mouse pools. The fluid was heat inactivated at 56° for 30 minutes. Unless fluid was collected by shaking over glass beads, fibrin clots formed;

they were removed by 3X centrifugation at 20,000 x g for 20 minutes at 4°. The ascitic fluid was absorbed with an equal volume of packed BALB/c red blood cells.

Cytotoxicity was measured by the chromium release assay. At 1:5 dilution both sera were cytotoxic for thymus and spleen cells (Table 8). In general, ascitic fluid was of minimal cytotoxicity with maximal killing seen with fluid #1 (67% thymocyte, 17% spleen cell killing). These data are similar to those from two other preparations of mouse anti-mouse Thy-1 serum I prepared. These other two preparations used a larger number of mice (25 and 50 respectively). The serum and ascitic fluid were of low titer and the quantity insufficient for depletion studies. I discontinued preparation of mouse anti-mouse Thy-1 and used rabbits and sheep for production of anti-Baθ antiserum in order to generate sufficient quantities.

a. Testing Commercial AKR Anti-C3H θ Ascitic Fluid

AKR ascites anti-C3H θ serum was purchased from Bionetics (Litton Bionetics, Kensington, Maryland, lot #231-55-2). The Bionetics technical data for this lot number are summarized below:

Cytotoxic titer:	C3H thymus cells	50% or >	kill at 1:100
		> 90%	kill at 1:60
	C3H spleen cells	25% or >	kill at 1:40
Cytotoxic specificity:	C3H bone marrow cells	< 10%	kill at 1:10

The serum was prepared by injecting C3H thymocytes into AKR mice and generating ascites fluid. Further information regarding the anti-θ was

TABLE 8

CYTOTOXICITY OF AKR ANTI-C3H THY-1 SERUM

AND ASCITES FLUID

Serum	Dilution	Cytotoxic Index Target Cells	
		Thymus	Spleen
AKR anti-C3H Thy-1 serum:			
Pool	1:5	70	33
Final Bleed	1:5	100	33
AKR anti-C3H Thy-1 Ascitic Fluid			
#1	1:10	67	17
#2	1:10	11	0
#3	1:10	22	5

Cytotoxicity measured by chromium release assay and expressed as cytotoxic index (CI):

$$CI = \frac{\text{cpm (As + C)} - \text{cpm (NS + C)}}{\text{cpm (100\% lysis)} - \text{cpm (NS + C)}}$$

See text for serum and ascitic fluid preparation.

obtained from Dr. Kay of Bionetics by telephone. The cytotoxicity test used by Bionetics to generate these data was similar to my trypan blue dye exclusion test; however, Bionetics' test used 2 x 10^6 cells/ml, while I used 10^7 cells/ml. Dr. Kay stated the antiserum (1:10 dilution) inhibited the PHA response of 2.5 x 10^7 spleen cells/ml by 50% without affecting the LPS response. I tested the antiserum for spleen and thymus cytotoxicity. As seen in Table 9, there was incomplete thymus cell killing and minimal spleen cell killing. Furthermore, there was no reduction in the in vitro immune response to TNP-KLH or to TNP-T4 if cells were treated with anti-θ (1:20, 1:40, 1:80 dilution) and complement prior to culture (data not shown). Thus, although some thymus cells were killed, there was no effect on splenic helper T cells. Lack of cytotoxicity was corroborated by Dr. Arthur Malley, Oregon Regional Primate Center, using lot #231-55-1 (personal communication). Consequently this antiserum could not be used for depletion studies.

2. Preparation and Characterization of Sheep Anti-Baθ and Anti-T Cell Serum

a. Sheep 144 Anti-Baθ

Sheep anti-Baθ was prepared by injecting 5 pulverized BALB/c brains in complete Freund's adjuvant s.c. (4 ml total at 4 sites) on day 0. On day 26 the sheep was boosted with 2.2 x 10^8 BALB/c thymocytes i.v. (2.0 ml). Prior to injection, brain and thymus cells were treated with Tris-NH_4Cl to lyse red blood cells. Sera from day 35 and day 49 were pooled and will be referred to as 144 anti-θ. Prior to use, serum was heat

TABLE 9

COMPLEMENT DEPENDENT CYTOTOXICITY TESTING OF

BIONETICS ANTI-THY-1 SERUM

Serum	Dilution	% Dead Cells	
		Thymus	Spleen
Bionetics anti-θ	1:20	78	6
(lot #231-55-2)	1:40	63	5
	1:80	27	17
	1:160	39	22

Data are presented as % dead cells using thymus or spleen cells as target cells. Cell death determined by Cytograf analysis. At all dilutions tested, there was < 10% complement independent cytotoxicity.

inactivated (56°/30 min) to inactivate complement. Pre-immunization serum did not kill thymus or spleen cells (1:20 or greater dilutions).

Anti-θ serum 144 was tested for T or B cell inactivation by 1) cytotoxicity testing using trypan blue dye exclusion; and, 2) functional tests employing in vitro cell cooperation experiments. At 1:160 or 1:320 dilution, nonabsorbed serum inactivated B cell function; it obliterated the responses to both TNP-T4 and TNP-KLH (Table 10). However, by complement dependent cytotoxicity these dilutions killed only 30% of spleen cells (Figure 11). Liver absorption substantially reduced cytoxicity at these dilutions for both thymus and spleen cells, but there appeared to be a differential in the absorption of anti-B cell activity, since after absorption the TNP-T4 response was only reduced 11%, while the TNP-KLH response was still reduced 62%. Brain and liver absorbed out all cytotoxic activity by trypan blue criteria, but in in vitro immune responses the antiserum still showed marginal (30%) inhibition of the TNP-KLH response at 1:80 and 1:160 dilutions and considerable inhibition (71% and 19%) of the TNP-T4 response at 1:80 and 1:160 dilutions (Table 10). Figure 12 shows the degree of cell killing in the absence of added complement. Killing of thymocytes was more readily diluted out than was the killing of spleen cells. Liver absorption removed some complement independent cytotoxicity, but liver and brain absorption was more efficient.

Since there are reports that some anti-θ antiserum contain anti-Ig antibody (Baird et al., 1971), the serum was tested for reactivity with

TABLE 10

EFFECT OF ANTI-θ POOL 144 ON GENERATION OF IN VITRO IMMUNE RESPONSES

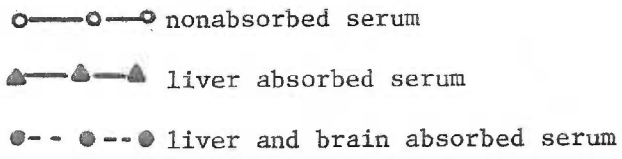
Absorption of Serum	Dilution	% Reduction of Anti-TNP Response Antigen In Vitro	
		TNP-KLH	TNP-T4
None	1:160	100	100
	1:320	100	93
Balb/c Liver	1:80	88	72
	1:160	62	11
Balb/c Liver and Brain	1:80	29	71
	1:160	27	19

KLH primed spleen cells were treated with antiserum and C prior to culture with TNP-KLH (0.02 g/ml) or TNP-T4 (3×10^6 PFU/ml). Direct anti-TNP PFC were measured 5 days later. % reduction obtained by comparing PFC/10 of cells treated with antiserum and C to PFC/10^6 of cells treated with C only.

Figure 11

Complement-dependent cytotoxicity of absorbed anti-θ 144.

Data are presented as % of dead cells using thymus or spleen cells as target cells. Cell death determined by automated Cytograf analysis.



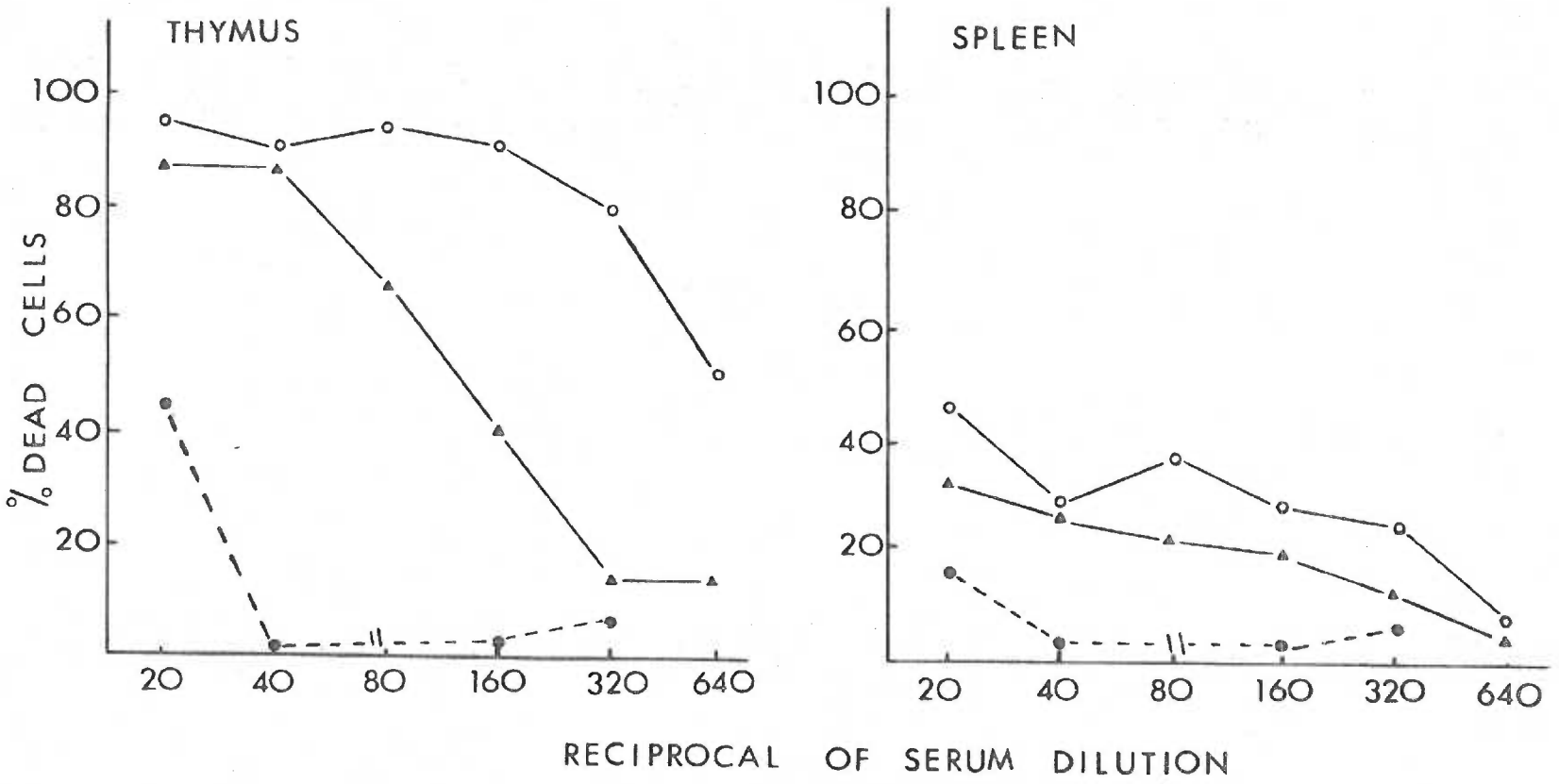
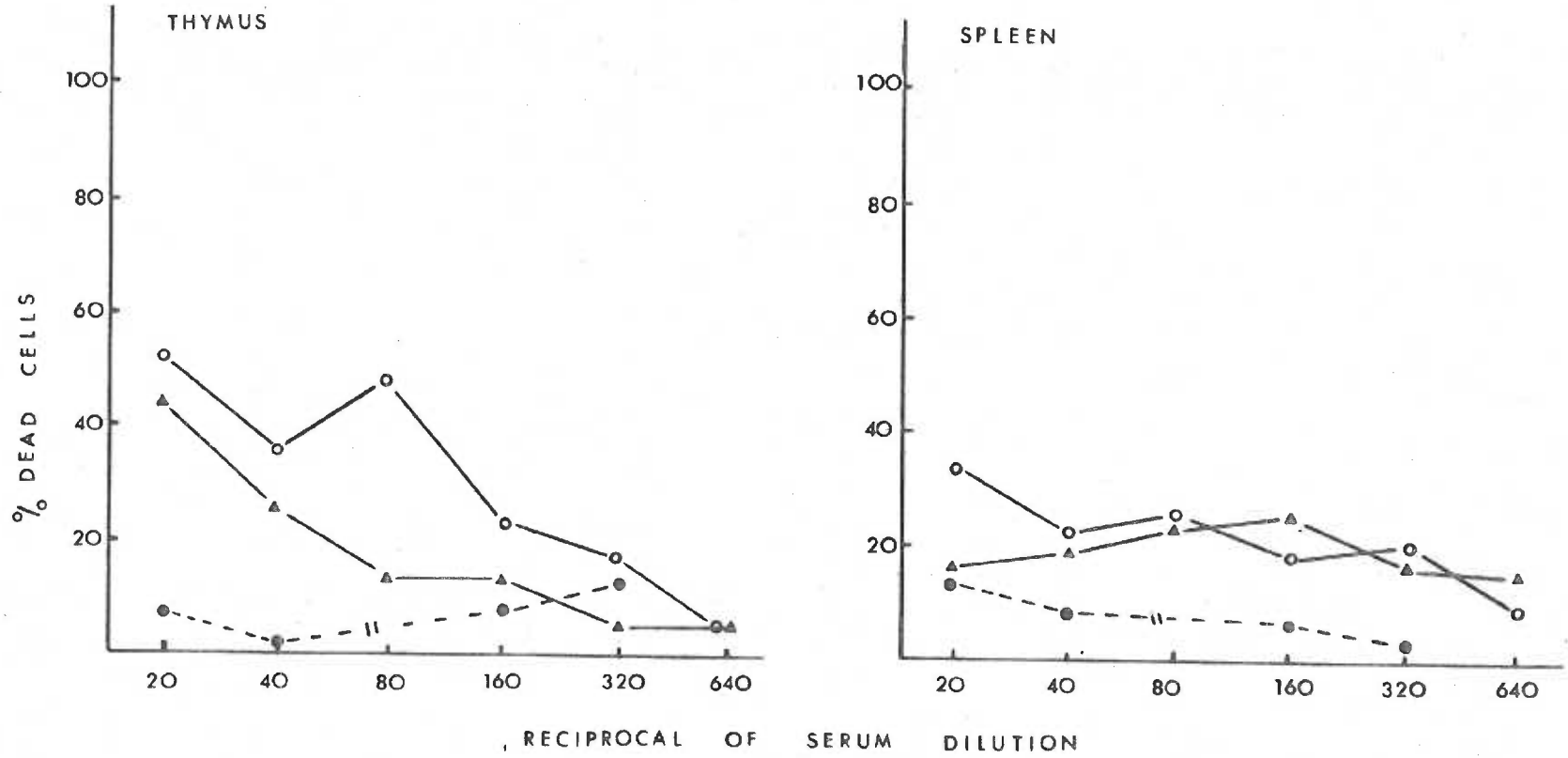

THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
20
40
80
160
320
640
RECIPROCAL OF SERUM DILUTION

Figure 12

Complement independent cytotoxicity of absorbed anti-θ 144.

Data are presented as % of dead cells using thymus or spleen cells as target cells. Cell death determined by automated Cytograf analysis.


THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
20
40
80
160
320
640
RECIPROCAL OF SERUM DILUTION

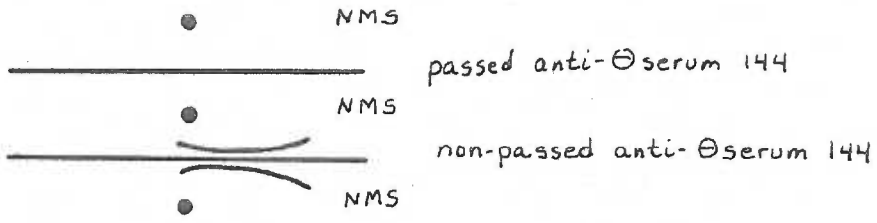
normal mouse serum (NMS) by immunoelectrophoresis. The antiserum reacted with both Swiss Webster NMS (undilute) and with a 50% saturated ammonium sulfate fraction of (BALB/c X C3H)F_1 serum (see photo #5-185). If this unidentified NMS component were lymphocyte associated and required for *in vitro* triggering, blocking of this component by the antiserum could result in lack of *in vitro* immune responsiveness and thus explain inconsistency between *in vitro* functional tests and cytotoxicity tests.

To remove the contaminating antibody the antiserum was passed over an NMS immunoabsorbent (NMS-IA) and concentrated to its original serum volume. When tested by IEP after column passage, the non-bound material did not react with the NMS (see photo #5-185); material bound to the immunoabsorbent and eluted with 3 M NaSCN reacted with NMS. Column passed serum was tested for cytotoxicity (Figures 13 and 14). There was a decrease in complement-dependent (Figure 13) and complement-independent (Figure 14) killing of thymus and spleen cells with thymocyte killing affected the most. Material eluted with 3 M NaSCN lacked cytotoxic activity (Figures 13 and 14). It is possible that 3 M NaSCN elution destroyed such antibody. Repeating the column passage did not alter the cytotoxicity of the non-bound fraction (data not shown).

The NMS-IA passaged serum was tested for its effect on *in vitro* immune responses to thymus-dependent (TNP-KLH) and thymus-independent (TNP-T4) antigens. In KLH immune spleen cells pretreated with antiserum (2X passaged over NMS-IA) and C prior to *in vitro* culture, the TNP-T4 response was decreased 99% at 1:160 serum dilution, although cytotoxicity

Photo 5-185

Anti-θ serum 144: removal of reactivity against normal mouse serum by passage over NMS-IA.



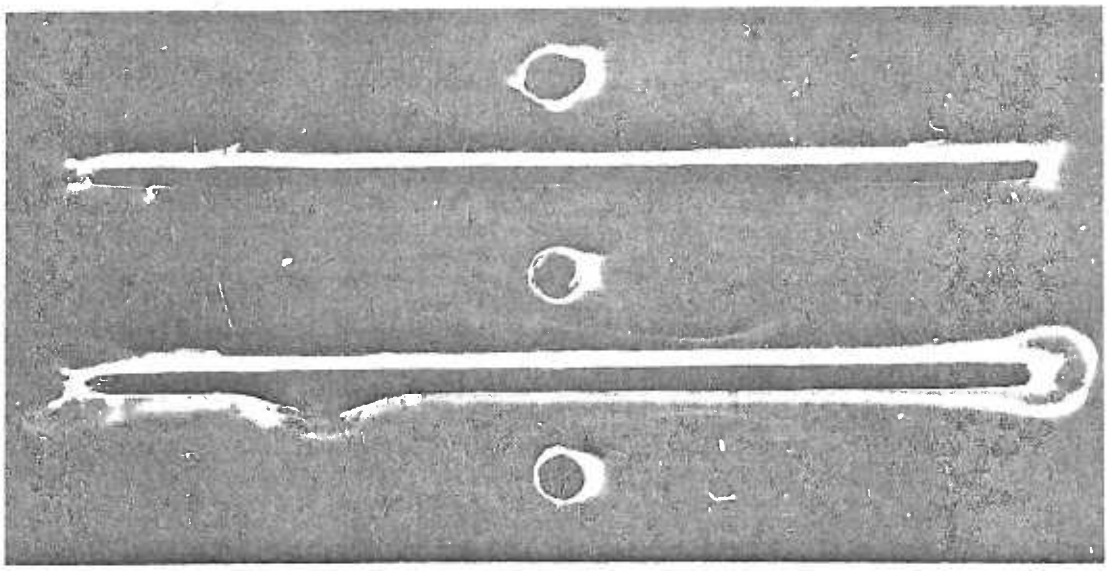


Figure 13

Complement independent cytotoxicity of NMS-IA passed pool of anti-θ 144 serum.

Data are presented as % of dead cells using thymus or spleen cells as target cells. Cell death determined by automated Cytograf analysis.

o—o—o nonpassed serum

▲—▲—▲ NMS-IA passed, nonbound

●--●--● NMS-IA passed, eluted

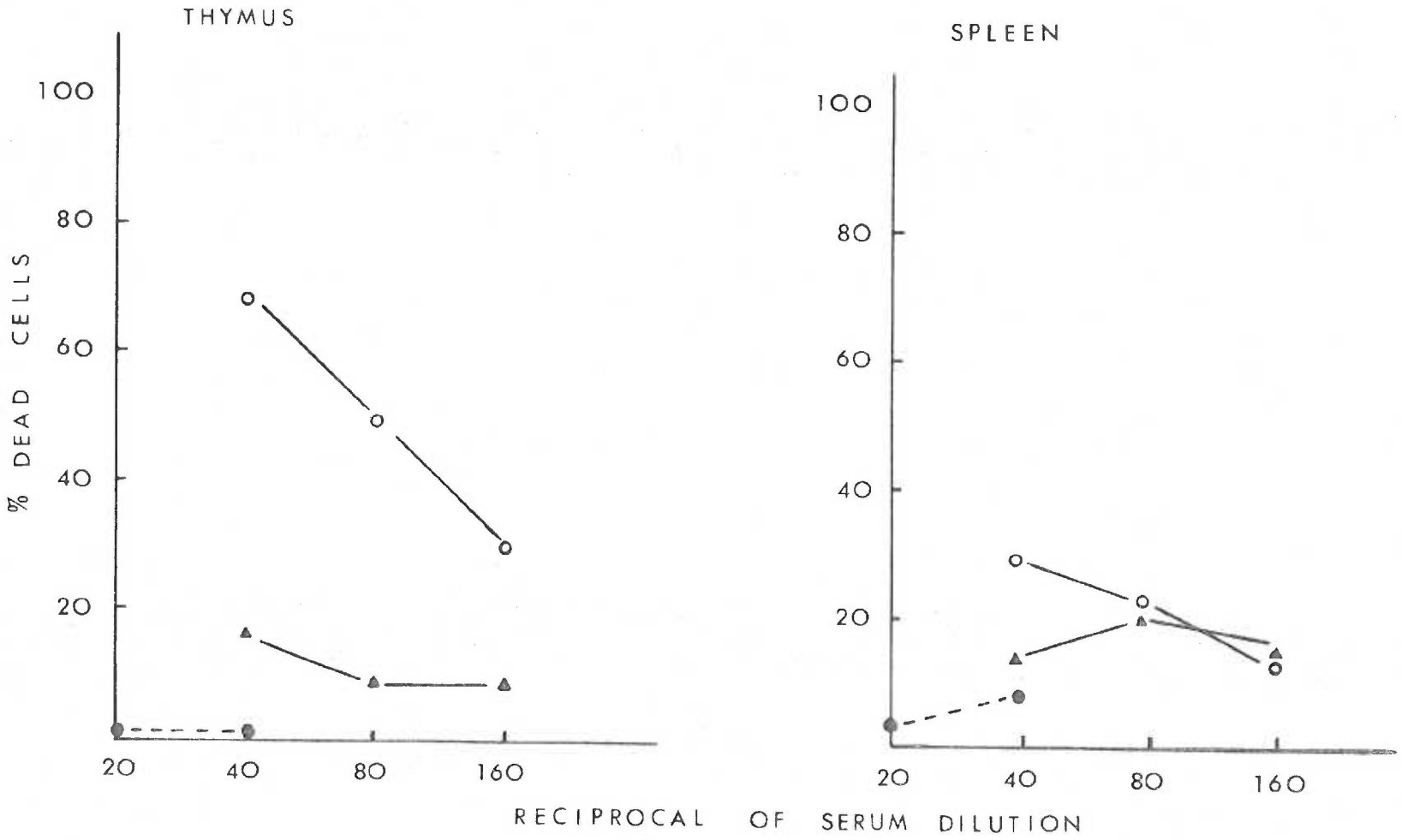

THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
20
40
80
160
RECIPROCAL OF SERUM DILUTION

Figure 14

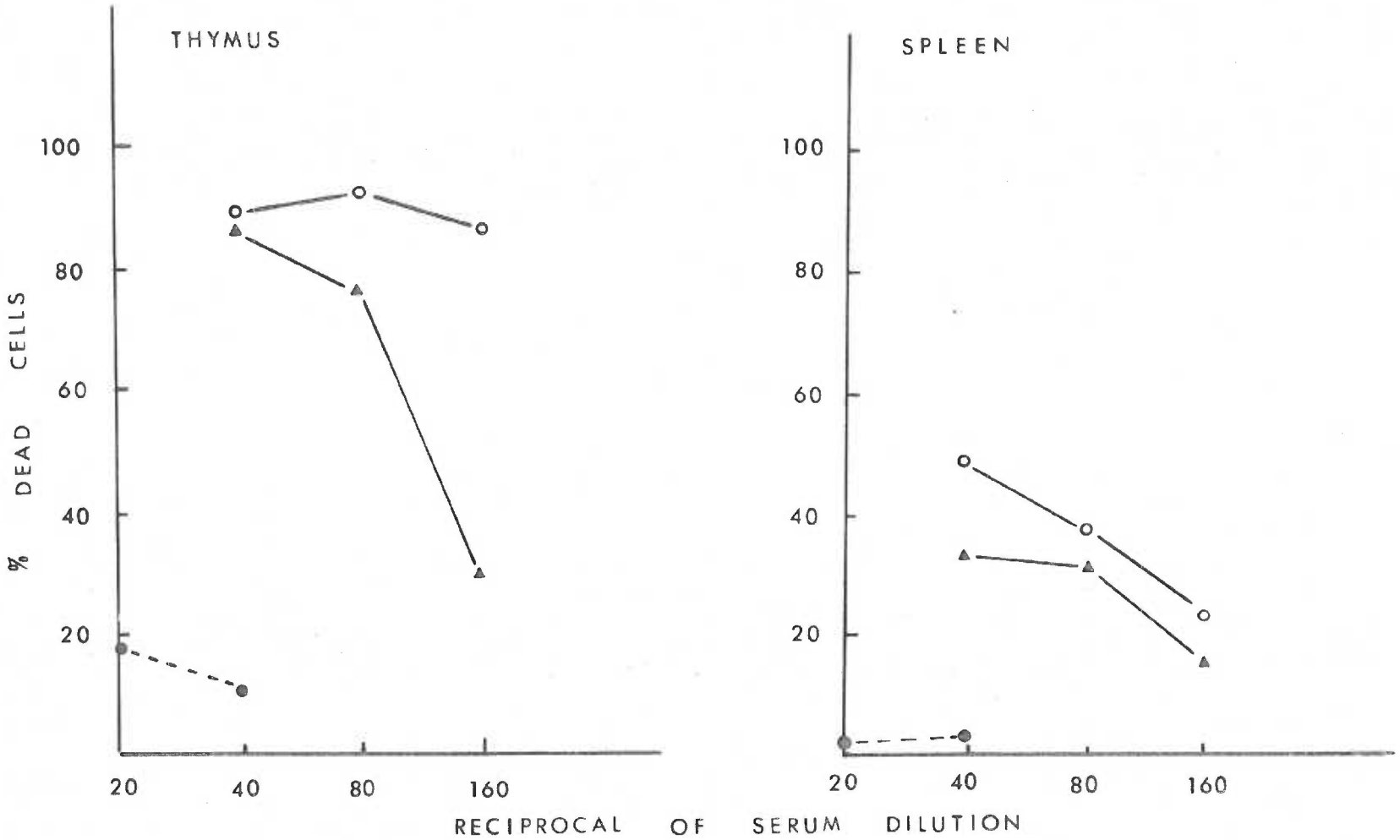
Complement dependent cytotoxicity of NMS-IA passed pool of anti-θ 144 serum.

Data are presented as % of dead cells using thymus or spleen cells as target cells. Cell death determined by automated Cytograf analysis.


nonpassed serum


NMS-IA passed, nonbound


NMS-IA passed, eluted


THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
20
40
80
160
RECIPROCAL OF SERUM DILUTION

tests showed only 13% killing of spleen cells and 30% kill of thymus cells (Fig. 14). The TNP-KLH response was reduced by 63%. At 1:40 or 1:80 dilution the response was reduced 100% to both TNP-KLH and TNP-T4. Thus, passage over NMS-IA did not remove the activity responsible for inactivation of B cell function; cell killing as measured by trypan blue dye exclusion did not correlate with functional inactivation.

The NMS-IA passed serum was absorbed with a variety of tissues to obtain a T cell specific antiserum. The serum was absorbed with C3H spleen, 6C3HED (C3H lymphoma, Thy 1.2 positive); BALB/c brain, thymus and spleen; and spleen and brain from nude mice (BALB/c genetic background). Mycobacterium tuberculosis was also used for absorption since CFA containing the bacilli was used for immunization; cross-reacting antigens on tumor cells and M. tuberculosis have been reported (Brunda and Minden, 1975), so it is possible normal cells also share such antigens. The results of such absorptions are seen in Table 11. Absorption with 6C3HED tumor cells or sequential absorption with BALB/c liver and brain removed cytotoxicity. Sequential absorption with liver and thymocytes did not absorb out activity and no other absorption was effective, although incomplete absorption cannot be ruled out in any instance.

The differentially absorbed samples were tested for their effect on in vitro generated immune responses (Table 12). The reduced TNP-T4 response indicates that BALB/c liver or nude spleen did not remove cytotoxicity for B cells. Sequential absorption with BALB/c liver and brain restored responses to control values or greater. Serum absorbed

TABLE 11

EFFECT OF DIFFERENTIAL ABSORPTION OF POOL 144 ANTI-θ ON COMPLEMENT DEPENDENT CYTOTOXICITY

	% Dead Cells							
	Thymus Antiserum Dilution				Spleen Antiserum Dilution			
Absorption	1:20	1:40	1:80	1:160	1:20	1:40	1:80	1:160
Liver[a]		81	69	40		31	16	27
Liver, brain[b]		3	4			7	4	
Liver, thymus[c]		74	59	38		20	17	17
Liver, thymus, 6C3HED[d]	11	4	0	15	0	0	0	0
Nude spleen[e]		67			17	14		
Nude brain[f]	75	49				3		
Balb/c spleen[e]		63				11		
C3H spleen[e]		67				14		
M. tuberculosis[g]		77				20		

% dead cells determined by analysis on Cytograf cell counter.

[a] 5 ml neat serum absorbed with 11 ml packed C3H liver.

[b] 2.4 ml neat serum absorbed with 3.5 ml packed C3H brain.

[c] 1.2 ml neat serum absorbed with 3.7×10^7 C3H thymocytes.

[d] 1.2 ml neat serum absorbed twice: initially 0.1 ml packed cells from 6C3HED ascitic fluid followed by a second absorption with 2.4×10^8 cells.

[e] 3 ml neat serum absorbed with 2.3×10^8 spleen cells from nude mice (Balb/c genetic background) or Balb/c or C3H.

[f] 2 ml neat serum absorbed with 2 ml packed nude brain.

[g] 8 ml neat serum absorbed with 16 mg H37RA Mycobacterium tuberculosis (Difco, lot #59586).

TABLE 12

DIFFERENTIAL ABSORPTION OF ANTI-Baθ POOL 144:

EFFECT ON GENERATION OF IN VITRO IMMUNE RESPONSES

AND CYTOTOXICITY TESTING

		% of Control Anti-TNP Response Antigen In Vitro				
		TNP-KLH		TNP-T4	% Dead Cells	
Absorptions	Exp. #	IgM	IgG	IgM	Thymus	Spleen
Balb/c liver	1	13	1	31	81	31
	2	0	1	38		
Balb/c liver and Balb/c brain	1	106	329	117	3	7
Balb/c liver, brain and 6C3HED	1	63	350	72	4	0
	2	71	131	47		
Nude spleen	2	0	0	0	67	14

KLH primed spleen cells were treated as described in Table 10. All antisera were used at 1:40 dilution. Serum absorbed as in Table 11. Cytotoxicity data taken from Table 11.

sequentially with liver and thymus or with liver, thymus and 6C3HED tumor cells reduced the IgM response to both antigens, but restored the IgG response to TNP-KLH to greater than control values. Since in vitro immune responses were reduced when spleen cell killing was not greater than 31%, there was poor correlation between inhibition of in vitro generated immune responses and cytotoxicity testing.

Studies on anti-θ 144 substantiate reports of anti-B and anti-T cell activity in anti-Baθ serum. I never obtained a B or T cell specific antiserum by absorption procedures; any effective absorption reduced both thymus and spleen cell cytotoxicity. Data in Table 10 suggested liver and brain could be used for differential absorption, but this did not hold up in later studies (Table 12). Sequential absorption with liver and brain or liver, thymus and 6C3HED tumor cells removed all thymus and spleen cell cytotoxicity as measured by trypan blue dye exclusion and restoration of in vitro immune responses (Tables 10, 11 and 12). Since brain and tumor cells have been reported to share antigens, absorption with 6C3HED would remove shared activity. 6C3HED absorption would also remove anti-θ activity since this tumor bears Thy 1.2 (C. Bianco, personal communication).

Serum passage over a NMS-IA did not render the serum T or B cell specific. However, this treatment did remove complement independent cytotoxic activity. Such cytotoxicity was also reduced by liver absorption and removed by sequential absorption with liver and brain.

b. Characterization of Sheep Anti-T Cell Serum (Feldmann)

The serum, a gift from Dr. Marc Feldmann (University College London, London, England) was prepared by injecting intravenously 2 x 10^8 BALB/c thymocytes into a sheep, and boosting several months later with 2 x 10^8 BALB/c thymocytes.

The serum was heat inactivated (56°/30 minutes) and absorbed twice with mouse erythrocytes prior to use. Using a one step cytotoxicity test and ^{51}Cr release assay, Dr. Feldmann determined that a 1:30 dilution of the serum with complement gave optimal thymus and spleen cell death. I used a two-step method assaying by dye exclusion, and found a 1:20 dilution of the serum with complement killed 90% thymocytes and 44% spleen cells, and 1:40 dilution killed 89% thymocytes and 33% spleen cells. When tested for its ability to reduce in vitro immune responses, the response to TNP-KLH was decreased > 95% (1:20 or 1:40 dilution), the response to TNP-T4 was not decreased at all at 1:40 dilution. Thus, the anti-T cell serum seemed to be T cell specific as judged by cytotoxicity testing with dye exclusion and by its effect on in vitro immune responses.

3. Production of Rabbit Anti-Mouse Brain Associated θ Serum (Anti-Baθ)

I prepared anti-Baθ serum in 7 rabbits following the method of Golub (1971). Pre-immunization bleedings of all rabbits showed minimal thymus or spleen cell cytotoxicity. The animals were bled frequently during the course of immunization and serum tested for thymus and spleen cell killing by trypan blue dye exclusion testing. Certain sera were

tested by functional assays by treating spleen cells with antiserum and complement prior to culturing with SRBC or TNP-T4 to determine the effect on the generation of an *in vitro* immune response.

a. Rabbit D57 Anti-Ba0 Serum

Rabbit D57 was injected on days 0, 8, 20, 48 and 103 with BALB/c brain in CFA. Early sera (days 13, 16, 22) showed 97-100% killing of thymocytes and 26-30% killing of spleen cells at 1:10 dilution (Figure 15). Complement independent killing was < 10% for both thymus and spleen cells. These sera were pooled (Pool A), absorbed and retested. Absorption with BALB/c liver reduced spleen cell killing from 30% to 15%; BALB/c brain completely absorbed out the activity. Liver-absorbed antiserum depleted the 1° SRBC response by 100% with no effect on the TNP-T4 response. By these criteria, pool A was T-cell specific and was used in cell depletion studies.

Individual testing of day 36 and 55 sera showed ∿ 100% thymus cells and ∿ 45% spleen cell killing at 1:10 dilution with 50% complement independent cytotoxicity for thymocytes (day 36 serum). A pool of these two bleeds (Pool B, 1:5 dilution) killed 97% thymocytes and 71% spleen cells after BALB/c liver absorption. Pool B also showed complement independent lysis, killing 61% thymocytes and 37% spleen cells. However, when tested by *in vitro* function, cells treated with Pool B showed no decrease in the TNP-T4 response and a 100% decrease in the SRBC response. Thus, functional tests indicated minimal B cell killing even though 71% spleen cells were killed as measured by cytotoxicity testing. This preparation was used in cell depletion studies.

Figure 15

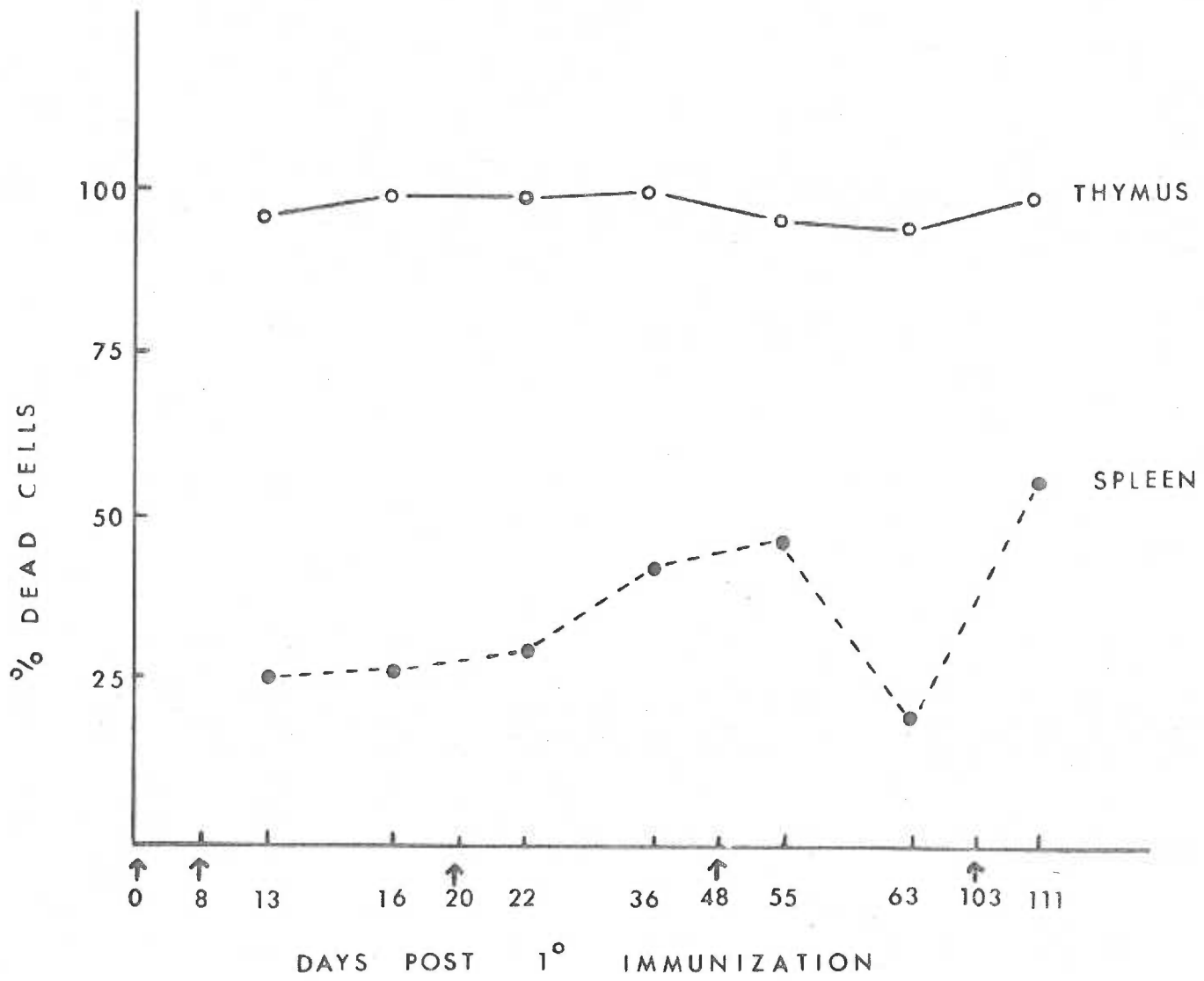
Complement-dependent cytotoxicity of rabbit D57 anti-Baθ serum during the course of immunization.

See text for details. D57 was injected on days indicated by arrows. All sera were tested at 1:10 dilution and were unabsorbed except days 55 and 111 which were absorbed with Balb/c liver. All sera showed complement independent lysis of < 10% for thymus and spleen cells except day 36, which killed 50% thymus cells in the absence of complement. The automated cytotoxicity test was used.

Target cells:

thymocytes ○—○—○

spleen cells ●- -●- -●


100
75
50
25
% DEAD CELLS
THYMUS
SPLEEN
0
8
13
16
20
22
36
48
55
63
103
111
DAYS POST 1° IMMUNIZATION

Day 111 bleed (liver absorbed) killed 90% thymocytes when titered from 1:10 to 1:160 dilutions. In vitro functional analysis showed 90% reduction in the SRBC and TNP-T4 response, indicating B cells were inactivated by the antiserum (1:5 dilution) and complement treatment. Sera which showed high spleen cell killing (day 55 and 111) were collected 7 to 8 days post-boost of brain in CFA.

b. Rabbit D56 Anti-Baθ Serum

Rabbit D56 was injected in the same manner as D57. Although all sera killed 100% thymocytes, they also showed high levels of spleen cell killing; early sera (day 13, 20, 36) killed at least 46% of spleen cells. A pool of these sera (Pool A) was cytotoxic for 85% and 68% spleen cells at 1:5 and 1:10 dilutions respectively with high levels of complement independent killing (95% thymus and 55% spleen cell killing at 1:10). Cells treated with Pool A (1:10 dilution) and complement were unable to generate an in vitro immune response to SRBC and the TNP-T4 response was also reduced by 57%. Day 55, 63 and 72 sera reduced the in vitro anti TNP-T4 response by > 90% (1:5 dilution). Day 111 likewise showed very high spleen cell killing (> 90%). Since this antiserum killed B cells as measured by cytotoxicity and by functional testing, it could not be used as an anti-T cell serum.

c. Rabbit 872 and 873 Anti-Baθ Sera

Rabbits 872 and 873 were injected on days 0 and 7 with brain in CFA. Because the animals were losing weight, brain was injected subcutaneously on days 14 and 31 without adjuvant. Cytotoxicity developed by

day 14; 873 serum (1:10 dilution) showed low cytotoxicity (82% thymus cell kill and 13% spleen cell kill), whereas 872 serum showed 30% spleen cell kill and 100% thymus cell killing. Rabbit 872 died before more bleedings were taken. 873 serum (day 44) was cytotoxic for 95% thymus cells and 27% spleen cells. Day 44 and 59 sera were pooled (Pool 873) and further characterized. Unabsorbed serum killed 30% spleen cells; liver absorption reduced this to 17% and sequential brain absorption to < 2%. Brain absorption completely removed cytotoxicity for thymus cells. The antiserum did not affect antibody secreting cells; SRBC or TKB-immune spleen cells treated with the antiserum (1:10 dilution) and complement immediately prior to assaying for PFC showed no reduction in the number of IgM or IgG PFC. Pool 873 was considered to be T cell specific.

All remaining sera from 872 and 873 were pooled and fractionated by 45% saturated ammonium sulfate. After reconstitution to 70% of the original volume and dialysis, the serum killed 100% thymocytes and 37% and 22% spleen cells at 1:5 and 1:10 dilutions respectively. However, after storage at -20° for several months, a 1:10 dilution killed 54% thymocytes and no spleen cells. It appears that prolonged storage at -20° adversely affected the cytotoxicity of the antiserum.

d. Rabbit H59, H62, H63 Anti-Baθ Sera

These rabbits were injected with brain in CFA on days 0 and 7 and bled on days 13, 16, 17, 23 and 24. All sera (1:5 or 1:10 dilution) killed 100% thymocytes before and after liver absorption. Unabsorbed sera killed 25% spleen cells; liver absorption reduced this to 15% kill.

However, functional tests showed that serum pooled from days 17, 20 and 23 (1:5 or 1:10 dilution) reduced both TNP-T4 and SRBC responses by 90%; thus, although the serum killed the appropriate percentage of cells in the spleen and thymus as measured by trypan blue dye exclusion testing, there was anti-B cell activity as seen by the reduction in the TNP-T4 response.

4. Preparation of Anti-Thy 1 Serum Using Thy 1.2 Positive Tumor Cells

The C3H lymphoma, 6C3HED, bears Thy 1.2 antigen (C. Bianco, personal communication). I confirmed this by indirect fluorescent staining of ascites tumor cells using AKR anti-C3H Thy-1 antiserum (a gift from Dr. P. J. Dawson, Department of Pathology) or AKR anti-C3H Thy-1 ascites fluid (Bionetics, lot #231-55-2) followed by fluorescein-conjugated rabbit anti-mouse IgG (MBA, lot #4454). Both test systems showed 65% Thy-1 positive cells and < 10% Ig positive cells. Since C3H and BALB/c share Thy 1.2 antigen but differ at many other genetic loci including the major histocompatibility locue (Staats, 1972), using 6C3HED as a cell source for heterologous anti-Thy 1 should reduce the level of contaminating (non anti-θ) antibodies.

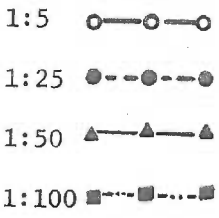
Two New Zealand white rabbits were injected subcutaneously and in the foot pads on day 0 with a mixture of CFA and 1.2×10^8 tumor cells. Tumor cells were pooled from 6 C3H male mice bearing an 11-day ascitic tumor generated by i.p. injection of cells from a solid tumor. Red blood cells were lysed with Tris-NH_4Cl prior to injection.

The rabbits were bled on days 10, 22, 29, 47 and bled out on day 54. Cytotoxicity testing from sequential bleedings for rabbit T24 are seen in Figure 16. Very early bleedings showed cytotoxicity against thymus cells; later bleedings were cytotoxic for spleen cells. This is compatible with the finding that thymocytes bear a greater density of Thy-1 antigen than do splenic T cells and are more susceptible to anti-Thy 1 and complement inactivation. Since mouse erythrocytes reportedly lack Thy-1 (Reif and Allen, 1964), day 54 antiserum was absorbed with BALB/c RBC. The antiserum was tested by cytotoxicity and functional tests. Cytotoxicity tests showed a high degree of complement independent killing which was reduced by antiserum dilution or by RBC absorption. A 1:25 dilution of unabsorbed serum and complement killed 97% thymus cells and 68% spleen cells. Unabsorbed antiserum at 1:50 dilution killed 59% thymocytes and 65% spleen cells; 1:100 dilution killed 40% thymocytes and 28% spleen cells. When tested for its effect on the in vitro immune response (Table 13), unabsorbed serum (1:25 dilution) reduced the response to TNP-KLH and TNP-T4 by 92% and 69% respectively. RBC absorption restored the response to TNP-T4, but the serum still reduced the response to TNP-KLH by 62%. At 1:50 serum dilution, the response to TNP-KLH was minimally decreased (20%) with no decrease in the TNP-T4 response; 1:100 serum dilution gave no decrease to either antigen. Thus even though cytotoxicity testing indicates that 48% of spleen cells were killed by anti-6C3HED serum (1:50 dilution) and complement, the immune response to TNP-KLH was decreased only 20% and there was no decrease in the in vitro

Figure 16

Complement dependent cytotoxicity of rabbit T24 anti-6C3HED serum. Data expressed as % dead cells using thymus or spleen cells as target cells. % dead cells determined by Cytograf analysis.

Dilution of serum tested:



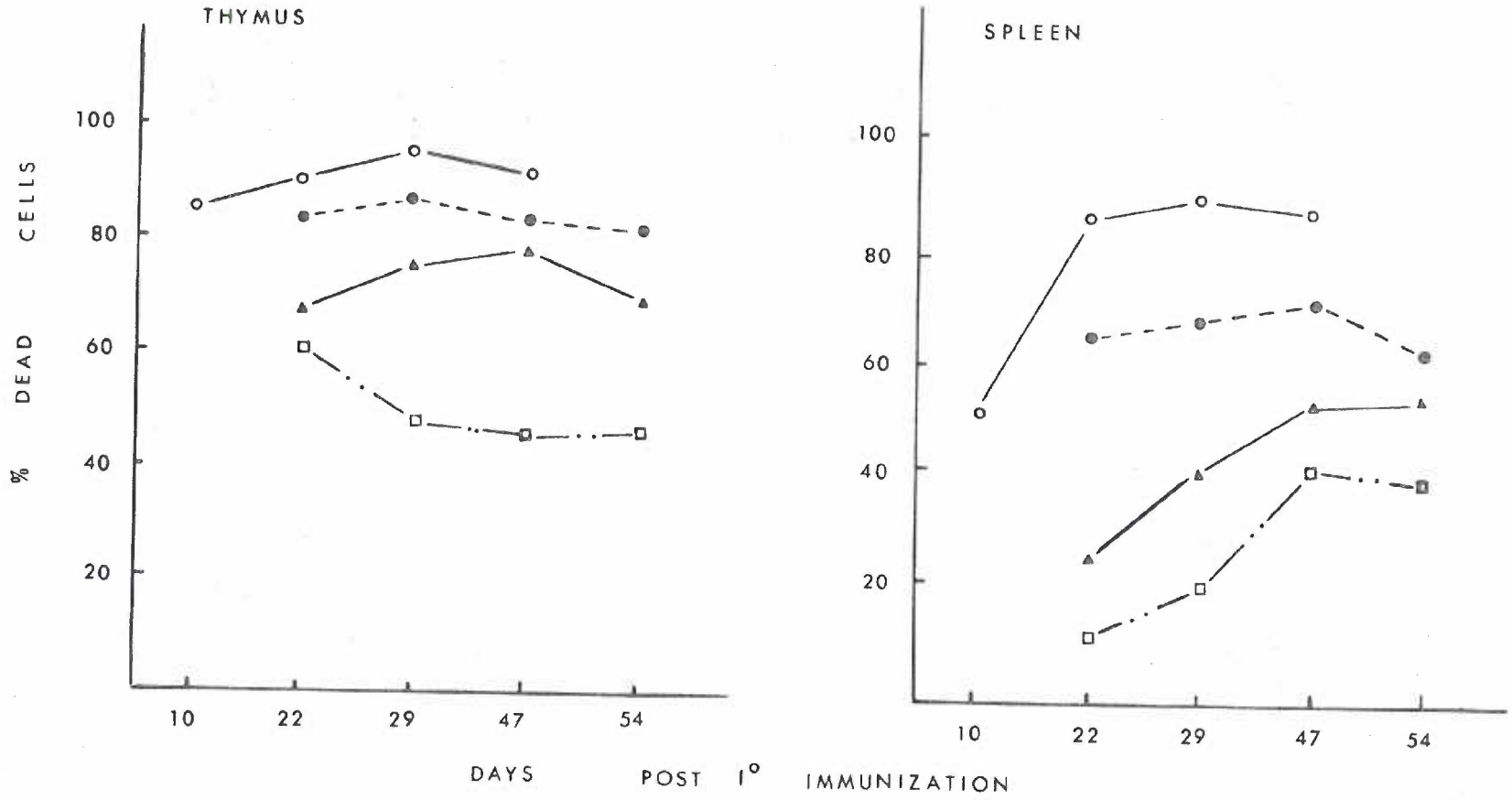

THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
10
22
29
47
54
DAYS POST 1° IMMUNIZATION

TABLE 13

EFFECT OF MRBC ABSORPTION ON ANTI-6C3HED SERUM

Serum	Dilution	In Vitro Immune Response % of Control Response Ag In Vitro[a]		Cytotoxicity Test % Dead Cells	
		TNP-KLH	TNP-T4	Thymus	Spleen
Unabsorbed	1:25	8	31	82(70)[b]	60(60)
MRBC Absorbed[c]		38	111	34(75)	58(48)
Unabsorbed	1:50	80	234	68(68)	52(53)
MRBC Absorbed		64	182	40(47)	28(45)
Unabsorbed	1:100	98	144	46(51)	38(38)

For in vitro functional test, spleen cells were treated with appropriate antiserum and C prior to culture with 0.02 µg TNP-KLH or 3 x 10^6 PFU TNP-T4. Values represent a pool of 8 replicate microcultures. Cells were assayed against TNP-SRBC.

[a]Control response is response of cells treated with C only.

[b]Number in parentheses indicates % of complement independent killing.

[c]4 ml of day 54 serum absorbed with 2 ml of packed Balb/c erythrocytes.

TNP-T4 response, indicating that the functional capacity of the system was not greatly affected.

Studies with this antiserum suggest that anti-Thy-1 -like antiserum can be prepared in rabbits by injecting Thy-1.2 positive tumor cells, 6C3HED. Absorption with mouse RBC removed anti-B cell activity and minimally affected anti-T cell activity. However, as seen in previous antiserum testing, there was a substantial amount of complement independent cytotoxicity, and cytotoxicity testing and *in vitro* functional analysis did not always correlate.

B. Testing Anti-Mouse Ig Sera for Cytotoxicity

I tested anti-mouse Ig antisera prepared in 9 rabbits, 5 goats and 1 sheep using various bleedings and dilutions. All 15 antisera contained anti-Ig as determined by Ouchterlony analysis. Four of the 15 antisera showed B cell cytotoxicity at some time during the course of immunization and cytotoxicity could be absorbed out by purified mouse Ig. Antisera showing cytotoxicity were an anti-kappa antiserum and two anti-IgG sera. Other anti-kappa chain, anti-IgG and anti-IgM were not cytotoxic.

1. Rabbit Anti-Mouse Kappa Chain Antiserum (497, 498, 499)

Kappa chain, purified from the urine of P10 plasmacytoma tumor-bearing mice, was a gift from Dr. C. Kimmel (University of Oregon). Rabbits 497, 498 and 499 were injected subcutaneously, intramuscularly and in the footpad with 250 μg kappa chain mixed with 250 μg methylated bovine serum albumin on day 0 and boosted with 250 μg on day 40, 50 μg at 9 months and 50 μg at 13 months. Sera from days 7 and 8 after the third injection were pooled and tested for cytotoxicity as shown in Table 14. Rabbit 497 serum showed no cytotoxicity at 1:5 or 1:10 dilution for spleen or for thymus cells. Serum of the same date from rabbit 498 was cytotoxic for spleen cells (47% at 1:10 dilution) and showed minimal thymus cell killing (15% at 1:10 dilution). The cytotoxicity of 498 serum was removed by absorption with purified mouse IgG (Table 15).

Sera obtained on days 53, 61, 74, 101, 111, 251, 255 and 258 from each of the three rabbits were combined and two identical pools were fractionated by three consecutive 33% saturated ammonium sulfate fractionations. Pool #1 was cytotoxic for spleen cells, but not for

TABLE 14

CYTOTOXICITY OF RABBIT ANTI-MOUSE KAPPA CHAIN ANTISERUM

Serum	Dilution	% Dead Cells[a]	
		Thymus	Spleen
Rabbit 497	1:5	< 10	12
	1:10	< 10	< 10
Rabbit 498	1:5	15	47
	1:10	15	47
	1:15	15	29
	1:40	< 10	12
Pool #1	1:5	< 10	40
	1:10	< 10	22
	1:20	< 10	< 10
Pool #2	1:5	< 10	10
	1:10	< 10	10
	1:20	< 10	< 10

[a] % dead cells determined by manual trypan blue dye exclusion testing. In all cases, complement independent killing was < 10%.

TABLE 15

ABSORPTION OF CYTOTOXIC ANTI-KAPPA CHAIN SERA WITH MOUSE IgG

µg/ml IgG Used for Absorption[b]	% Dead Cells[a]			
	Thymus Serum[c]		Spleen Serum[c]	
	498	Pool 1	498	Pool 1
0	14	< 10	43	32
0.1	13	NT[d]	19	NT
1.0	< 10	NT	23	NT
10	< 10	< 10	19	20
100	< 10	< 10	17	19
1000	< 10	< 10	< 10	< 10

[a] % dead cells determined by manual trypan blue dye exclusion test using thymus and spleen cells as target cells in complement dependent cytotoxicity.

[b] Mouse IgG (Pentex mouse IgG, Miles Laboratory) used to absorb a 1:5 serum dilution.

[c] The antisera were tested at 1:5 dilution.

[d] NT = not tested.

thymus cells (Table 14). This activity was removed by absorption with mouse IgG (Table 15). Pool #2 was not cytotoxic for spleen or for thymus cells. Since pools #1 and #2 were identically prepared from the same material, this result was unexpected, but indicated the fragility of cytotoxic antisera.

2. Rabbit Anti-Mouse Ig Antiserum

a. Rabbit 2148 and 2170

Rabbit anti-mouse IgG antiserum was prepared by injecting 2.0 mg mouse IgG (Pentex mouse IgG, Miles Laboratories) in CFA subcutaneously, intramuscularly and in the footpad. Rabbits were bled on days 0, 14, 18, 22, 23, 33, 43, and 72. They were boosted with human $F(ab')_2$ fragments from IgG on day 75 (1.0 mg i.v.) and day 92 (1.0 mg subcutaneously in CFA) and bled on days 79, 85, 92, 108, 120, 122 and 124. All bleedings reacted with mouse IgG (1 mg/ml, Pentex) and normal mouse serum (undilute) when tested by gel diffusion. 2148 day 22 bleed was cytotoxic for 33% spleen cells and < 10% thymus cells (Table 16). An aliquot of day 22 and 23 pool was absorbed with mouse IgG. Absorption with 500 µg/ml mouse IgG reduced spleen cell killing from 44% to 12% (Table 17). As of day 72, serum 2148 continued to be cytotoxic for spleen cells only. This is in contrast to serum 2170 which never showed appreciable cytotoxicity for spleen or thymus cells except day 33 serum which killed 10% spleen cells and 16% thymus cells.

Even though serum 2148 was cytotoxic, I wanted to increase the titer. Cytotoxic anti-Ig antibodies are probably directed to the

TABLE 16

SCHEDULE FOR PRODUCTION OF CYTOTOXIC ANTI-Ig ANTISERUM IN RABBIT #2148 AND #2170

Day	Injection	Serum Dilution	% Dead Cells			
			Spleen		Thymus	
			2148	2170	2148	2170
0	2 mg mouse IgG in CFA	-	-	-	-	-
14		1:5	18	< 10	12	< 10
		1:10	< 10	< 10	< 10	< 10
18		1:5	16	< 10	< 10	< 10
		1:10	16	< 10	11	12
22		1:5	33	< 10	10	10
		1:10	27	< 10	< 10	10
23		1:5	25	< 10	< 10	NT
		1:10	15	< 10	< 10	NT
33		1:5	30	19	61[a]	16
		1:10	19	NT	49	NT
		1:15	6	NT	24	NT
43		1:5	19	< 10	18	< 10
		1:10	16	< 10	10	< 10
72		1:5	33	< 10	< 10	< 10
75	1 mg HGG(Fab')$_2$	-	-	-	-	-
79		1:5	36	13	13	< 10
85		1:5	26	< 10	14	< 10
92	1 mg HGG (Fab')$_2$ in CFA	-	-	-	-	-
108		1:5	47	NT	19	NT
		1:10	35	NT	17	NT
120		1:5	45	39	< 10	10
		1:10	35	40	< 10	< 10
		1:15	11	35	< 10	NT
122		1:5	50	44	23	12
		1:10	25	34	21	< 10
		1:15	21	36	12	< 10
124		1:5	39	29	15	18
		1:10	36	25	< 10	10
		1:15	24	18	< 10	NT

% dead cells determined by manual trypan blue dye exclusion using thymus or spleen cells as target cells. All sera showed < 10% complement independent cytotoxicity except where indicated.

NT = not tested.

[a]Complement independent thymus cell killing was 24% at this dilution.

TABLE 17

ABSORPTION OF 2148 SERUM WITH MOUSE IgG

µg/ml Mouse IgG Used for Absorption	% Dead Cells	
	Thymus	Spleen
0	14[a]	44[a]
0.1	15	46
1.0	20	47
10	13	30
100	10	12
1000	13	12

% dead cells determined by manual trypan blue dye exclusion testing. Complement independent killing was < 10% at all dilutions tested.

Pentex mouse IgG used to absorb 1:5 dilution of 2148 serum (day 22 and 23 pool).

[a]Antiserum used at 1:5 dilution.

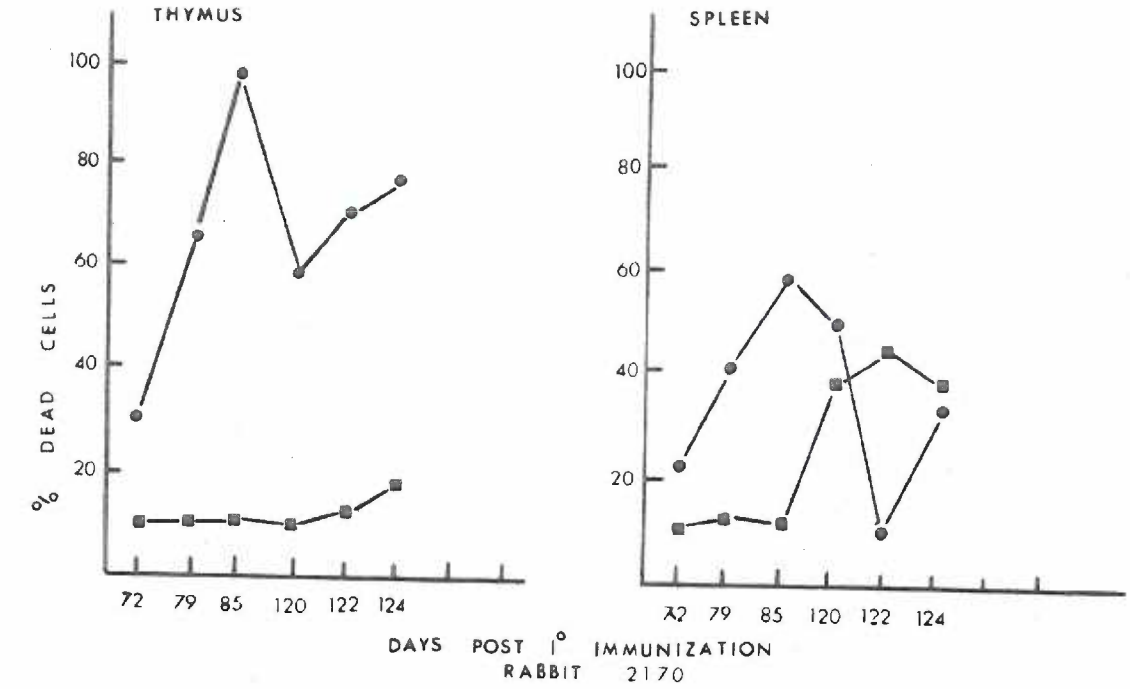
antigen-combining portion of the Ig molecule, so I reasoned that a boost with $F(ab')_2$ fragment of Ig might serve to raise the cytotoxic titer. Since I had a large amount of human IgG (HGG) I prepared $F(ab')_2$ fragments from this material. Gel diffusion analysis showed no cross-reactivity between human and mouse IgG using anti-mouse IgG or anti-human IgG antiserum; nevertheless I thought it possible that similarities in tertiary structure between the two species of $F(ab')_2$ might serve to boost the response. After two injections of $F(ab')_2$ (day 108) serum 2148 showed increased spleen cell killing to 47% with a slight increase in thymus cell killing (19%). By day 120, both 2148 and 2170 showed spleen cell killing (45% and 39% respectively) with < 10% kill of thymocytes. Injection of human IgG $F(ab')_2$ fragments after priming with mouse IgG seemed to provide a boost for cytotoxic antibody production. The boost sera cross-reacted with HGG in gel diffusion. The antisera were stored frozen at -20°; after storage, bleedings were retested when other experimental data from functional tests suggested the antiserum was affecting T cells. C-dependent spleen and thymus cell cytotoxicity is shown in Figure 17. Storage at -20° ranged from 4 weeks (day 124 serum) to about 5 months (day 14 serum). Prior to storage 2148 sera, with the exception of day 33, showed minimal thymus cell killing; after storage, at least 60% of thymus cells were killed by all sera. Spleen cell killing before and after storage also did not correlate, although the discrepancy was not as pronounced as seen with thymus cells. Serum 2170 also showed increased thymus cell killing after storage (Figure 17).

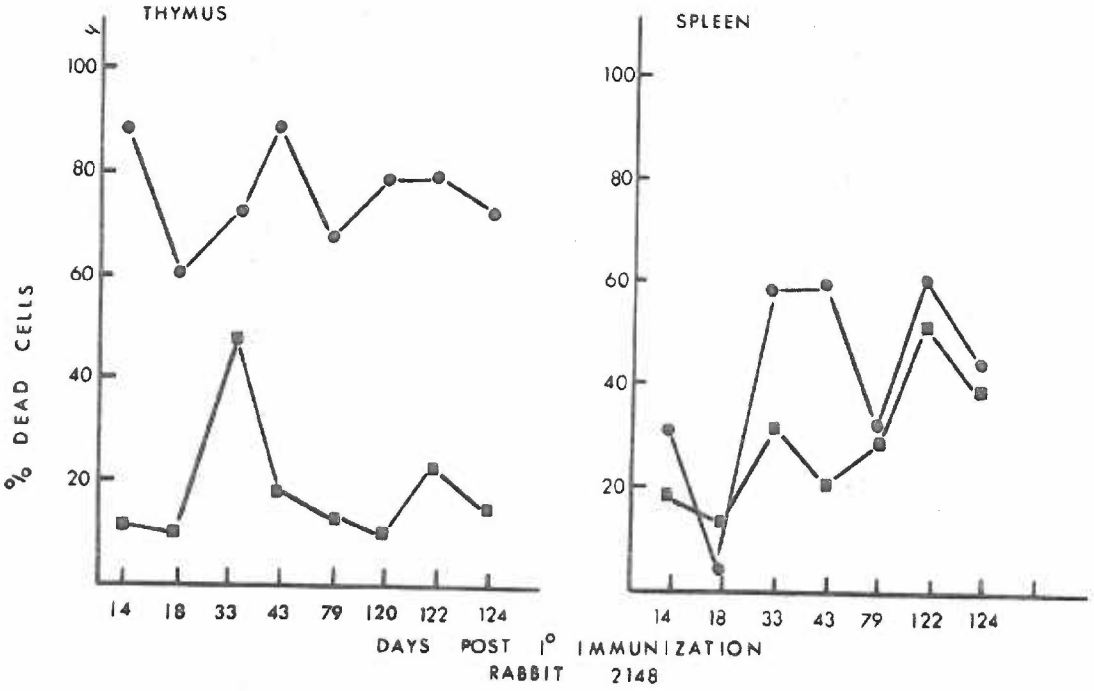
Figure 17

Effect of -20° storage on cytotoxicity of serum 2170 and 2148. See text for details. Serum was tested by complement dependent trypan blue dye exclusion test prior to or after storage at -20° for 4 weeks to 5 months.

●—●—● after storage

■—■—■ before storage


THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
72 79 85 120 122 124
DAYS POST 1° IMMUNIZATION
RABBIT 2170


THYMUS
SPLEEN
% DEAD CELLS
100
80
60
40
20
14 18 33 43 79 120 122 124
14 18 33 43 79 122 124
DAYS POST 1° IMMUNIZATION
RABBIT 2148

Sera were pooled and fractionated by Sephadex G-200 chromatography to remove thymocyte cytotoxicity. After fractionation, neither the IgG rich nor the IgM rich fractions showed C dependent cytotoxicity for thymus or spleen cells at 1:8, 1:16, 1:32 or 1:64 dilutions. However, by indirect fluorescent staining, the IgG and the IgM fractions stained approximately 60% of spleen cells and no thymus cells. Thus, Sephadex G-200 fractionation removed the nonspecific cytotoxicity, but did not restore the level of specific B cell cytotoxicity even though the anti-Ig could be shown to bind to spleen cells.

All subsequent sera were stored at -70° under nitrogen gas in an effort to avoid adverse effects on cytotoxicity.

b. Rabbit H60

Injection of HGG $F(ab')_2$ fragments boosted cytotoxic antibody titers in rabbits 2148 and 2170. I tried to repeat this by injecting rabbit H60 with 1.0 mg of human $F(ab')_2$ subcutaneously in CFA on day 0; H60 was bled on days 7, 10, 13, 17, 21 and 37, boosted s.c. with 2.0 mg mouse IgG (Pentex) in CFA on day 37 and bled on days 44 and 50. No significant cytotoxicity (< 10% killing) was present against spleen or thymus cells at any bleed. By Ouchterlony analysis day 21 serum (only serum tested) reacted with $F(ab')_2$ fragment of HGG but not with mouse IgG. Day 44 serum reacted with mouse IgG. The injection schedule for H60 differs from 2148 and 2170 in that H60 was not initially immunized with mouse IgG. It is not clear if this alteration of the immunization schedule accounts for the lack of cytotoxic anti-Ig antibodies.

c. Rabbits H10, G42

Rabbits were injected with 2 mg mouse IgG in CFA s.c., i.m. and f.p. on days 0 and 21 and bled on days 28, 38 and 42. G42 prebleed serum was not cytotoxic for thymus or spleen cells; H10 prebleed serum killed 58% thymus cells (1:5 dilution) and no spleen cells. Liver absorption reduced pre-immunization cytotoxicity for thymus cells to 35%. After immunization, all H10 sera killed thymus cell and spleen cell and G42 sera killed spleen cells only. However, liver absorption reduced thymus cell killing and completely removed spleen cell killing from both antisera. Even after liver absorption, both antisera reacted with mouse IgG when tested by gel diffusion. These sera were not used as cytotoxic anti-Ig sera.

d. Rabbits 66, 67, 69

This serum was a gift of K. Pratt and was prepared by injecting goat anti-mouse Ig, mouse Ig complexes formed at equivalence (5.2 mg of mouse IgG) in CFA into rabbits f.p. and s.c. Approximately one year later rabbits were boosted i.v. with 100 µg mouse IgG and bled out 7 days later. Serum from the three rabbits was pooled (pool 66-69).

Table 18 shows cytotoxicity testing for the pooled antiserum. At 1:75 dilution the serum killed 15% thymus cells and 32% spleen cells. After absorption with mouse IgG, cytotoxicity was reduced to 3% for thymus cells and 10% for spleen cells. This antiserum was used as a cytotoxic anti-Ig antiserum.

TABLE 18

CYTOTOXICITY OF 66-69 ANTI-IgG

Dilution	% Dead Cells[b]	
	Thymus	Spleen
1:20	47	35
1:50	< 10	34
1:75	12	30
1:100	15	32
1:150	11	< 10
1:500	< 10	< 10
1:75[a]	3	10

[a] 1 ml of a 1:75 serum dilution absorbed with 250 µg mouse IgG (Pentex).

[b] % dead cells determined by trypan blue dye exclusion in complement dependent cytotoxicity test. % dead cells measured by Cytograf analysis.

e. Rabbit Anti-MIgG2bκ

The serum was a gift from Dr. E. Rabellino, National Jewish Hospital, Denver. It was raised in rabbits against MOPC-195 protein ($IgG_{2_b}\kappa$). After heat inactivation for 30 minutes at 56° the serum was absorbed with BALB/c thymocytes (5 x 10^7 cells per ml serum) for 60 minutes at 4°. At 1:5 dilution the serum killed 11% thymocytes and 21% spleen cells in a C dependent cytotoxicity test assayed by trypan blue dye exclusion. By indirect fluorescent staining the serum at a 1:10 dilution stained 53% of spleen cells and 2% thymus cells. This serum was used in studies where a cytotoxic anti-Ig serum was required.

3. Goat Anti-Mouse IgG Antiserum

In addition to the rabbit anti-mouse Ig sera tested, five goat anti-mouse IgG sera were tested for complement dependent cytotoxicity. These sera were obtained from several different sources and prepared in different manners, but all contained anti-mouse IgG when tested by IEP and Ouchterlony analysis; they all fixed C and could be used as facilitating antisera in the plaque assay to detect IgG PFC. All of the sera were tested for cytotoxicity at 1:5 and 1:10 dilutions; 5 of the 6 sera were not cytotoxic for spleen cells or thymus cells. One serum tested killed 29% spleen cells, but also killed 21% thymus cells. The sera were not further characterized.

4. Sheep Anti-Mouse IgM Antiserum

A sheep anti-IgM serum prepared against mouse tumor 104E (μ,λ) was a gift from Dr. A. Malley of the Primate Center. Serum diluted 1:5, 1:10 or 1:20 killed neither spleen nor thymus cells in complement dependent cytotoxicity testing.

5. Other Modifications

I attempted to render anti-Ig serum cytotoxic for spleen cells by modifying the procedures as follows:

Addition of Ca^{++}. 10^{-3} M Ca^{++} ($CaCl_2 \cdot 2H_2O$) was added to MEM in case Ca ions, necessary for C activation, were limiting. There was no difference in the % kill in the absence or presence of Ca .

C titer. C was titered at 1:3, 1:4, 1:6 and 1:9 dilutions. Optimal % killing was observed at 1:3 dilution.

Absence of FCS. FCS was omitted to eliminate potential anti-complementary effects of the serum. No increase in cytotoxicity was observed when FCS was omitted.

Use of a facilitating serum. The rabbit anti-mouse Ig serum was facilitated by adding an anti-rabbit Ig serum. Presumably, as in the enhancement of IgG plaques, this step would provide additional binding sites for C and allow for more effective killing. Cells were allowed to bind varying dilutions of rabbit anti-mouse Ig at 4° for 60 minutes, washed and incubated with varying dilutions of anti-rabbit Ig for 60 minutes at 4° prior to washing and addition of C. Two different anti-rabbit Ig antisera were used; goat anti-rabbit Ig (a gift from Dr. R. M.

Parkhouse) and sheep 93 anti-rabbit Ig were used. Neither facilitating serum increased spleen cell killing.

Use of rabbit serum as C source. Rabbit serum is a potent source of C for lysis of antibody-sensitized mouse cells (discussed in Boyse et al., 1970). Whole rabbit serum as a source of C was used at 1:1, 1:3 or 1:6 dilutions, but it did not increase spleen cell killing with any of the anti-Ig sera tested.

C. Use of Nylon Wool Columns for B Cell Depletion

Initial experiments were designed to test the efficacy of the columns in removing B cells. In Experiment 1 (Table 19) spleen cells were passed over glass wool columns only or over glass and nylon wool columns. Nonadherent cells were used as target cells in a cytotoxicity test using anti-Baθ and C to determine the number of T cells. Mouse erythrocytes in the spleen cell suspension did not affect column passage. Approximately 70% of the cells were recovered after passage over glass wool columns; nylon wool passage reduced recovery to ∿ 30%. Anti-Thy-1.2 and C treatment killed 25 to 30% of the cells; since this is the approximate % killing of unpassed spleen cells, there was no enrichment for T cells in Experiment 1.

In Experiment 2 (Table 19), spleen cell recovery after passage ranged from 22 to 46% and the effluent cells showed T cell enrichment (26 to 56%).

Although passage over glass wool columns gave ∿ 30% loss of cells in Experiment 1, I continued to use these columns because Dr. Julius stressed that this step was essential for removal of dead cells and macrophages which interfere with the nylon wool separation (personal communication).

The increased percentage of cells killed by anti-Baθ and C after column passage could be explained by increased cell fragility from column passage; thus, functional tests were important to substantiate B cell depletion. I tested the effluent cells for their ability to generate

TABLE 19

B CELL DEPLETION BY NYLON WOOL COLUMNS

Exp. #	Passage	No. of Cells Filtered (x 10^7)[a]	% Cell Recovery	% Kill By Anti-Baθ and C[b] After Passage
1	Glass wool	4.5	76	26
	Glass wool and nylon wool	3.4	31	30
		5.0	35	31
2	Glass wool and nylon wool	1.2	22	41
		1.8	46	56

[a] 1.8-2.5 ml spleen cells loaded in all experiments.

[b] Rabbit 873 anti-Baθ used at 1:5. % dead cells determined by manual trypan blue dye exclusion test.

an in vitro immune response; B cell depletion should be reflected in a decreased response. TKLH primed spleen cells were passed over both glass and nylon wool columns. The effluent cells were recovered, washed, brought to 10^7 cells/ml and cultured with TNP-KLH or TNP-T4 for 5 days; anti-TNP PFC were measured. As seen in Table 20, column passage depleted all IgG PFC. The IgM response to TNP-T4 was reduced by 75% and the IgM response to TNP-KLH was reduced 72%. The partial decrease in the in vitro immune response to TNP-T4 suggested B cells were in the effluent populations and that separation was incomplete.

I tested the effect of changing the nylon wool packing on cell separation. The same lot number of nylon wool was used and identical procedures were followed for all columns. Four experiments are shown in Table 21. In all experiments, cell recovery from glass wool columns ranged from 31 to 53% (data not shown). Glass wool passage alone removed a substantial number of PFC (32% [Experiment 5] to 76% [Experiment 7]), but did not enrich for T cells as measured by anti-Baθ and C killing.

The experiments showed variation in PFC depletion regardless of column packing. The greatest PFC depletion was observed when glass wool column passage alone substantially reduced the response. In experiments 6 and 7 passage over glass wool decreased the responses 56% and 76% respectively; after nylon wool passage, the PFC response was reduced $\sim$ 90%. In experiments 4 and 5, there was a 42% and 32% reduction of PFC by glass wool passage; the nylon wool pass further reduced the responses in some but not all cases.

TABLE 20

GENERATION OF IN VITRO IMMUNE RESPONSES

AFTER PASSAGE OVER NYLON WOOL COLUMNS

	PFC/10^6[a]			
	Glass and Nylon Wool Passaged		Nonpassaged	
Antigen In Vitro	D	I	D	I
0.2 µg TNP-KLH	148	0	481	586
3 x 10^6 PFU TNP-T4	636	0	2542	0

[a] D = direct IgM PFC; I = indirect IgG PFC.

Cells assayed on day 5 of culture against TNP-SRBC.

TABLE 21

EFFECT OF NYLON WOOL PASSAGE ON B CELL DEPLETION OF IMMUNE SPLEEN CELLS IMMEDIATELY PRIOR TO PLAQUE ASSAY

Exp. #	No. of Cells[b] Passed (x 10^7)	% Reduction in DPFC[e] After Passage: Degree of Nylon Wool Packing[a] 0.9/6	1.2/6	0.9/9	0.6/4	0.6/6	0.6/7	0.6/9	Glass Wool Only
4	5.4	73	68		52	66			42
5	6.4	66		62	53	53	40	39	32
6	1.2	95		90	100	100	98	92	56
7	4.1[c]					100			
						100			
						100			
						100			
		% Passed Cells Killed by Anti-Baθ and C[d]							
6		91		90	95	92	90	92	25
7						86			
						89			
						77			
						77			

[a] 0.9/6 = $\frac{\text{gm nylon wool}}{\text{ml packed volume}}$

[b] 1.5 to 2.0 ml cell volume loaded in all experiments. TNP-KLH immune spleen cells used in exp. 4; all other experiments used SRBC immune animals.

Control # PFC/10^6 : Exp. 4 = 683
Exp. 5 = 154
Exp. 6 = 433
Exp. 7 = 683

[c] 4 replicate columns run in experiment 7.

[d] Rabbit D57 pool anti-Baθ used at 1:5 dilution. % dead cells determined by C dependent cytotoxicity test using manual trypan blue dye exclusion.

[e] After column passage, effluent cells were assayed for direct PFC against TNP-SRBC (Exp. 4) or SRBC (Exp. 5, 6, 7).

Since 0.6 gm nylon wool packed to 6 cc was reported to be optimal (Julius et al., 1973), four such columns were run on the same cell suspension (Experiment 7). The PFC response was decreased 100%, but again glass wool passage alone substantially reduced the response (76%). In experiments 6 and 7 where PFC depletion was achieved, there was an enrichment for T cells. In four other experiments using this packing ratio, two experiments gave 100% PFC depletion and two other experiments gave 55 and 53% B cell depletion.

D. B Cell Depletion by Use of Cellular Immunoadsorbants

I used the technique of Kedar et al. (1974) to deplete B cells. Three criteria were used to measure the ability of SRBC-aSRBC monolayers to deplete B cells: 1) the number of antibody secreting cells (PFC) remaining after monolayer absorption; 2) the number of PFC precursors remaining after monolayer absorption as measured by culturing non-adherent cells (NA) in vitro with antigen; and, 3) the percentage of non-adherent cells bearing surface Ig. Serum 95-97-aSRBC was titered to determine the amount needed to sensitize the SRBC monolayers. Table 22 shows the result of an experiment in which 5.5×10^7 spleen cells were adsorbed to monolayers sensitized with different dilutions of aSRBC and the NA fraction was monitored for PFC. At dilutions up to 1:20 there was $\sim$ 95% PFC depletion in the NA population. About 30% of the cells adsorbed to the aSRBC sensitized monolayer or to the NRS control monolayers (sensitized with NRS absorbed 3X with SRBC). Adsorption to NRS monolayers might be attributed to residual aSRBC antibody in NRS. Alternatively, cell adsorption may be nonspecific and not mediated via the lymphocyte Fc receptor. In the original procedure, 50 to 60% of cells bound to SRBC-aSRBC monolayers and only 10 to 15% of cells bound to SRBC-NRS monolayers. However, this experiment (Table 22) suggested PFC could be depleted by adsorption to sensitized monolayers; in subsequent experiments I used a 1:15 dilution of Ra 95-97 antiserum to sensitize the monolayers.

TABLE 22

EFFECT OF DILUTIONS OF ANTI-SRBC ON THE ABSORBING CAPACITY OF SRBC-ANTI-SRBC MONOLAYER

Antiserum	Dilution	% Cells Remaining Unattached to Monolayer	PFC/10^6[c] Unattached Cells
Anti-SRBC[a]	1:5	72	3
	1:10	68	4
	1:20	65	3
	1:30	63	26
Normal Serum[b]	1:5	80	80
	1:10	76	90

[a]Antiserum from rabbit 95-97 used to sensitize monolayers.

[b]SRBC monolayer sensitized with normal rabbit serum absorbed 3X with SRBC.

[c]Spleen cells primed 4 days earlier with SRBC and assayed against SRBC by the Cunningham technique.

I tested for the ability of the NA cells to generate in vitro antibody responses (Table 23). Spleen cells primed to KLH or to TNP-KLH were adsorbed to the monolayers, NA cells collected, washed and cultured with antigen for 5 days. If B cells were adsorbed to the sensitized monolayer, the NA cells should show a diminished in vitro immune response. In Experiment 5 the in vitro response to both TNP-KLH and TNP-T4 was reduced by at least 90%. Cells passed over NRS sensitized monolayers responded at control levels. In subsequent experiments (7, 14, 15) there was only partial B cell depletion. Occasionally the in vitro responses of the NA cells were reduced, but most responses were at or above control values. To avoid overloading monolayers, 2.5×10^7 cells rather than 5.5×10^7 cells were allowed to adsorb to each monolayer in Experiment 15. Reducing the cell load did not deplete B cells. In Experiment 15 the number of PFC in the NA fraction was monitored prior to putting the cells in culture. Direct PFC were reduced by 96% and indirect PFC reduced by 99% in the NA fraction. Even though depleted of PFC, the NA fraction generated an in vitro immune response. In these experiments there was no apparent correlation between the magnitude of the PFC response and the efficiency of B cell depletion.

Another aSRBC antiserum (H61) was tested for its ability to sensitize SRBC monolayers (Table 24). When used at dilutions of 1:20 to 1:2560, there was no significant depletion of the in vitro immune response to thymus-dependent (SRBC or TNP-KLH) or thymus-independent (DNP-dextran or TNP-T4) antigens.

TABLE 23

GENERATION OF IN VITRO IMMUNE RESPONSES USING UNATTACHED CELLS AFTER MONOLAYER ADSORPTION

	% of Control Response[a]				
	Exp. 5		Exp. 7	Exp. 14	Exp. 15
Antigen In Vitro	Anti-SRBC[b]	NRS[c]	Anti-SRBC	Anti-SRBC	Anti-SRBC
TNP-KLH, 0.02 µg					
D[d]	10	107	6		126
I					137
TNP-KLH, 0.2 µg					
D				11	287
I				65	134
TNP-T4[e]					
D	6	93	35	81	121
I				126	200
% of Cells Remaining Unattached to Monolayer	44	26		50	59

[a]Control response is PFC/10^6 of unfractionated cells. 2.5 x 10^7 cells were loaded per monolayer. Spleen cells in experiments 5 and 7 were primed to KLH-B; cells used in experiments 14 and 15 were from TKB-primed mice boosted with 100 g TKLH 4 days prior to use. Each value represents a pool of 8 replicate cultures. Cells assayed against TNP-SRBC by Cunningham technique.

[b]Rabbit 95-97 anti-SRBC (1:15 dilution) used to sensitize monolayers.

[c]Normal rabbit serum (1:15 dilution) absorbed 3X with SRBC used to sensitize monolayers.

[d]D = direct IgM PFC.
I = indirect IgG PFC.

[e]TNP-T4 used at a final concentration of 3 x 10^6 PFU per culture.

TABLE 24

GENERATION OF IN VITRO IMMUNE RESPONSES BY REMAINING UNADHERENT CELLS[a] AFTER MONOLAYER ADSORPTION

Exp. #	Antiserum Dilution[c]	DPFC/10^6 Cells Antigen In Vitro[b] SRBC	TNP-T4	TNP-KLH	DNP-D
8	1:20	860	822		
	1:40	740	788		
	1:80	1131	530		
	1:160	539	712		
	Unfractionated	499	169		
9	1:160		184	105	211
	1:320		165	119	186
	1:640		183	150	279
	1:1280		117	75	251
	1:2560		104	65	139
	Unfractionated		75	63	219

[a] Spleen cells were primed to SRBC 4 days prior in Experiment 8; spleen cells used in Experiment 9 were primed to TNP-KLH. Cells were assayed against TNP-SRBC except cultures stimulated with SRBC in vitro which were assayed against SRBC. Each value represents a pool of 8 replicate microcultures.

[b] Antigens used in vitro: TNP-KLH 0.02 μg/ml; TNP-T4 3 x 10^6 PFU/ml; SRBC 3 x 10^6 erythrocytes/ml; DNP-D 10^{-2} μg/ml.

[c] Rabbit H61 antiserum used to sensitize monolayers.

Using H61 aSRBC, I repeated the monolayer adsorption on NA cells to remove residual B cells. The original technique states that additional depletion could be obtained by repeating the adsorption on fresh monolayers (Kedar et al., 1974). In this experiment cells were adsorbed to sensitized monolayers, NA cells removed and adsorbed to fresh sensitized monolayers. The NA cells were cultured with antigen for 5 days and anti-TNP PFC were determined. Repeated absorption did not deplete in vitro generated PFC (Table 25). B cells in the NA fraction were monitored for surface Ig by indirect fluorescent staining (Table 26). In Experiment 11, NA cells were monitored for Ig positive cells and PFC depletion. NA cells showed a 91% decrease in the PFC response compared to non-fractionated cells (1:80 dilution aSRBC). However, 40% of the NA cells were positive for Ig, while the non-fractionated population contained 41% Ig positive cells. Cells incubated with only fluoresceinated goat anti-rabbit Ig were negative, suggesting staining was not due to Fc binding of fluoresceinated reagent or to fluorescein uptake by dead or damaged cells.

The indirect staining method could give false positive staining cells if rabbit aSRBC serum used for sensitization passively adsorbed to NA cells, since fluoresceinated goat anti-rabbit Ig was used in the test. In Experiment 12, 27 to 30% of NA cells stained Ig positive by the direct staining technique. Thus both direct and indirect staining procedures indicate a substantial number of NA cells bear surface Ig.

TABLE 25

GENERATION OF IN VITRO IMMUNE RESPONSES BY UNATTACHED CELLS AFTER REPEATED MONOLAYER ADSORPTION

Antiserum Dilution[a]	# of Monolayer Adsorptions	Anti-TNP Direct PFC/10^6 Cells Antigen In Vitro[b]		
		TNP-KLH	TNP-T4	DNP-D
1:10	1	1137	2917	160
	2	1294	2022	1835
1:20	1	2071	1913	2173
	2	2246	1973	459
1:40	1	1699	2264	441
	2	844	1713	786
Unfractionated		1272	1189	612

[a]Rabbit H61 anti-SRBC serum used to sensitize monolayers.

[b]Antigen concentrations in vitro:

TNP-KLH . . . 0.02 µg/ml
TNP-T4. . . . 6×10^6 PFU/ml
DNP-D . . . . 10^{-2} µg/ml

Each value represents a pool of 8 replicate microcultures. Cells were assayed for direct PFC against TNP-SRBC by the Cunningham technique.

TABLE 26

CHARACTERIZATION OF UNATTACHED CELLS AFTER MONOLAYER ADSORPTION:

EFFECT ON PFC[a] AND ON CELLS BEARING SURFACE Ig

Antiserum	Dilution[b]	% Cell Recovery	% of Control PFC	% Ig Staining Cell		
				Direct Stain		Indirect Stain
				Exp. 11	Exp. 12	Exp. 12
Anti-SRBC	1:80	56	9	40	NT	NT
	1:160	46	20	65	38	38
	1:320	46	50	29	47	NT
	1:640	52	57	39	45	29
	1:1280	50	58	60	61	27
	1:2560	61	62	52	51	30
NRS	1:80	72	37	60	NT	NT
Unfractionated		100	100	41	57	48

[a]Spleen cells were primed to SRBC 4 days prior control PFC/10^6 cells assayed = 1032.

[b]Rabbit H61 anti-SRBC serum used to sensitize monolayers. Normal rabbit serum (NRS) was absorbed 3X with SRBC prior to use for monolayer sensitization.

Why was B cell depletion observed only occasionally? The hemolysin used was low titered, but contained IgG antibody, the antibody class which, when complexed with antigen, binds to B cells (Kerbel and Davies, 1974). Anti-SRBC serum (agglutination titer 1:256, antibody class not determined) used by Kedar et al. (1974) depleted B cells by 86% when used at 1:300 dilution and by 72% when used at 1:1000 dilution; 1:3000 dilution resulted in only 30% decrease. However, 1:50 dilution of antiserum was used to prepare monolayers for all specificity studies.

The methods I used to assay B cell depletion indicate cellular immunoabsorbents did not give consistent B cell depletion. After adsorption, NA cells often showed depletion of antibody-secreting cells (PFC) although the NA cells stained for surface Ig and generated in vitro immune responses. This seems to indicate that PFC differ from resting B cells in adherent properties, consistent with the view that there is heterogeneity in B cell populations.

DISCUSSION AND SUMMARY

Since it is desirable to obtain pure B and T cell populations for dissection of the immune response, depletion techniques are important tools. However, it is critical to assess efficacy of depletion methods which are based on cytotoxic antiserum and C treatment or physical adherence methods if these techniques are to be used to assign specific functions to cells and their antigens in the generation of the immune response.

Due to an inability to raise or to obtain commercially high-titered cytotoxic mouse anti-Thy-1.2 serum, I used heterologous species to prepare anti-Baθ serum. Of the 7 rabbits used, only 3 (873, 872 and D57) produced a T cell specific serum as measured by trypan blue dye exclusion testing or functional assays. Certain bleedings from these animals showed anti-B cell activity as did sera from the other four rabbits. In general, cytotoxic antibodies developed about 14 days post initial immunization. T cell specific antiserum was obtained both early (14-21 days) and late (44-59 days) in immunization. High spleen cell killing was often seen one week after a boost with brain in CFA; killing would decrease with time after the boost. Repeatedly there was no correlation of B cell killing as measured by trypan blue dye exclusion testing and functional analysis. Serum stored at -20° for several months lost cytotoxicity for both thymus and spleen cells.

Initial studies suggest anti-Thy 1.2 serum produced in rabbit T24 against Thy-1.2 bearing tumor cells is T cell specific. However, more studies must be done in order to rule out contaminating antibodies raised against cross-reacting tumor and B cell antigens.

Sheep 144 anti-Baθ serum was not T cell specific. The serum was passed over a NMS-immunoabsorbant, and absorbed with a wide variety of tissues without rendering it anti-T cell specific. Absorption with brain or tumor cells removed activity against both T and B cells. In retrospect it is apparent why using brain as the immunizing tissue produces a serum grossly contaminated with other specificities. Toh and Cauchi (1974) have reported anti-lymphoma activity present in anti-Baθ serum. However, this report appeared three years after the initial report by Golub (1971) which claimed anti-T cell specificity. Anti-erythrocyte activity (Golub, 1973) and anti-stem cell activity (Golub, 1972) were also reported to be present in such antisera. However, no data regarding anti-lymphoma or anti-B cell activity have been presented, although noted in unpublished observations (Golub, 1973; Golub and Day, 1975).

Only 4 out of 15 anti-mouse Ig antisera were cytotoxic for spleen cells but not thymus cells in a complement dependent cytotoxicity test (498, 2148, 66-69, anti-M$1g_2G_2b\kappa$). Cytotoxicity could be absorbed out by mouse IgG (3 of 4 sera tested). Five rabbits were injected with mouse IgG and 1 (2148) produced cytotoxic antibodies. 2148 serum was cytotoxic 3 weeks after immunization; the other 4 anti-IgG antisera were not cytotoxic. Rabbit 2170 was cytotoxic after boosting with human $F(ab')_2$ fragments. Both 2148 and 2170 produced cytotoxic "anti-Ig" antibodies after such a boost; however, the sera were never absorbed with mouse IgG to strengthen the assumption that cytotoxic antibodies were anti-Ig. This is important since G42 and H10 sera were cytotoxic,

but activity could be removed by liver absorption. Since the activity of 2148 and 2170 sera were lost, presumably due to improper storage, I cannot assess the efficiency of such an immunization procedure; however, H60 did not produce cytotoxic anti-Ig after priming with human $F(ab')_2$ and boosting with mouse IgG.

Cytotoxic anti-Ig appears to be extremely labile in that the sera lost activity by storing at -20° and acquired nonspecific thymus cell killing. Although all sera were subsequently stored at -70° under N_2, I have not systematically tested sera to determine if -70° is suitable for long-term storage. All attempts to render a non-cytotoxic antiserum cytotoxic failed. These included changing test reagents and their concentrations, isolation of IgG and IgM from the anti-Ig serum, and use of a facilitating serum to enhance killing. My data suggest it is difficult to obtain anti-Ig serum that is cytotoxic for B cells. All sera contained anti-Ig activity and selected sera stained B cells but not T cells (2148) and fixed C (all goat anti-mouse IgG), but only 4 out of 15 were cytotoxic for B cells.

Since different cell populations bear specific antigenic markers, use of specific cytotoxic antiserum and C is an ideal approach for cell depletion. However, my experiments using anti-Baθ and anti-Ig sera suggest that such antisera must be well characterized prior to use. More than one procedure for characterization should be performed, as I observed a lack of correlation in antiserum specificity using cytotoxicity tests and *in vitro* functional analysis.

It isn't known why these two methods do not correlate. Dr. G. M. Iverson also has noted inconsistencies between cytotoxicity testing and in vitro functional analysis when using anti-Ig serum and C to deplete B lymphocytes (personal communication). He finds that, after anti-Ig and C treatment, B cells appear dead as judged by their ability to take up trypan blue, but that these "dead" cells are functional by in vitro testing. He attributes this discrepancy to the B cell's ability to repair its damaged membrane in vitro, as such "repair" can be blocked by NaN_3. Such a cell repair mechanism could explain my finding that cells appear dead as judged by dye uptake, but are not depleted by in vitro functional analysis. However, the reverse situation also occurs. Cells may be viable as judged by trypan blue dye exclusion, but unable to function in vitro. This finding could be explained if cell death were sometimes caused by a time-dependent lytic process which only develops after the time course of a cytotoxicity assay. Such a discrepancy could also be explained by non-cytotoxic blocking of cell surface components.

Frequently a high degree of complement-independent killing was observed in trypan blue dye exclusion testing.

Killing in the absence of exogenous complement could occur via complement-dependent lysis or complement-independent lysis. Both fetal calf serum and complement were heated to 56° to inactivate, but not remove, complement components. Since C1, C2 and C5 are extremely heat labile and are required for the classical or the alternate pathway, conventional complement pathways should not be operating (Eisen, 1974). Recently, a new pathway of complement action has been described in a

complement-mediated bactericidal system (Moreau and Skarnes, 1975). In this system only early components of human complement (C1, C4 and C2) plus certain serum euglobulins were required for killing Shigella sonnei. It is possible that other as yet unidentified complement pathways exist which do not utilize the heat labile components and that these may somehow be activated here.

An alternative is that complement components are not involved in some forms of cell killing. "Cytotoxicity" as measured by trypan blue dye exclusion would result from membrane perturbations rather than cell death. Serum components (antibody?) binding to the cell would induce sufficient membrane damage or perturbations to allow for dye uptake and apparent cell death. There is some evidence for this in the work of Rubens and Dulbecco (1974) who showed that antibodies directed at the cell surface may augment the uptake of cytotoxic drugs even if the drugs are not bound to the antibody.

The two procedures I used for B cell depletion based on physical adherence did not give consistent results. As discussed in the introduction (Appendix), other workers have used nylon wool columns with apparent success. The variation seen among my experiments could be attributed to technical errors in column packing or cell passage even though the identical procedure was followed each time. In addition to my finding, Dr. S. Kontainen and Dr. L. Herzenberg have independently found (personal communication) that batches of Leuko-Pak vary in ability to deplete B cells and that these columns may remove a subpopulation of suppressor T cells (Dr. Kontiainen, personal communication). Nylon wool

passage is used for B cell depletion by many investigators. Since the technique may not always be reliable, careful analysis of cells recovered from the column is important; it cannot be assumed that 85 to 90% of effluent cells are T cells as stated originally (Julius et al., 1973).

Use of cellular immunoadsorbants for B cell depletion was not a useful technique either. However, this depletion technique depends upon B cells binding antigen-antibody complexes and, as discussed in the introduction to the Appendix, there are controversies regarding the ability to deplete B cells by such a method. My data agree with those investigators who are unable to achieve B cell depletion by this means (Karpf et al., 1975; Wigzell et al., 1972).

The difficulties that I have discussed associated with cell depletion studies should be considered when such techniques are used, not only in general T or B cell depletion studies, but also in staining and blocking studies where cells or their antigens are assigned specific functions in the generation of the immune response.

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