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Small B Cells as Antigen Presenting Cells in the Induction of Tolerance to Soluble Protein Antigens: A Dissertation

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SMALL B CELLS AS ANTIGEN PRESENTING CELLS IN THE INDUCTION OF TOLERANCE TO SOLUBLE PROTEIN ANTIGENS

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ABSTRACT

This thesis proposes a mechanism for the induction of peripheral tolerance to protein antigens. I have investigated the mechanism of tolerance induction to soluble protein antigens by targeting an antigen to small, resting B cells. For this purpose I have used a rabbit antibody directed at the IgD molecule found on the surface of most small, resting B cells but missing or lowered on activated B cells. Intravenous injection of normal mice with 100 μg of an ultracentrifuged Fab fragment of rabbit anti-mouse IgD (Fab anti-δ) makes these mice profoundly tolerant to challenge with nonimmune rabbit Fab (Fab NRG) fragments. This tolerance is antigen specific since treated mice make normal responses to an irrelevant antigen, chicken immunoglobulin (Ig). Fab fragments of rabbit Ig (rabbit Fab) not targeted to B cells do not induce tolerance as well as Fab anti-δ. Evidence suggests that the B cells must remain in a resting state for tolerance to be induced, since injection of $F(ab)'_2$ anti-δ does not induce tolerance. Investigation of the mechanisms of the tolerance, by adoptive transfer, have shown that rabbit Fab specific B cell function has been impaired. The major effect however is in helper T cell function, as shown by adoptive transfer and lack of help for a hapten response. In vitro proliferation experiments show that the T cell response has not been shifted toward activation of different T cell subsets which do not help Ig production, nor is there any change in the Ig isotypes produced. Suppression does not

appear to be the major cause of the helper T cell defect as shown by cell mixing experiments. This work shows that an antigen targeted to small B cells can induce tolerance to a soluble protein antigen, and suggests a role for small B cells in tolerance to self-proteins not presented in the thymus.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

abs	spleen cell absorbed
alum	aluminum potassium sulfate
anti-δ	Fab rabbit anti-mouse IgD
APC	antigen presenting cells
ars	azophenyl arsonate
B.pertussis	*Bordatella pertussis*
BGG	bovine γ globulin
BSA	bovine serum albumin
CD	cluster of differentiation
CD2F1	Balb/c x DBA/2 F1 mice
CFA	complete Freunds adjuvant
cpm	count per minute
δ	heavy chain of IgD
DTH	delayed type hypersensitivity
DNP	dinitrophenyl
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
g	unit gravity
H+L	heavy plus light chain Ig
HGG	human γ globulin
Ig	immunoglobulin
IFA	incomplete Freunds adjuvant
IL	interleukin

i.p.	ıtraperitoneally
i.v.	intravenously
IFN γ	interferon gamma
LN	lymph node
LPS	lipopolysaccharide
κ	Ig kappa light chain
μ	heavy chain of IgM
MHC	major histocompatibility complex
Mls	minor lymphocyte stimulating antigen
MLR	mixed lymphocyte reaction
NRG	non-immune rabbit immunoglobulin
O.D.	optical density
PBS	phosphate buffered saline
PCC	pigeon cytochrome c
SCID	severe combined immune deficiency
SRBC	sheep red blood cells
s.c.	sub-cutaneously
TCR	T cell receptor
TH	T helper cells
Tween-20	polyoxyethelene-sorbitan monolaurate
Vβ	variable region of the TCR β chain

CHAPTER I.

INTRODUCTION

This thesis will investigate the role of small B cells as antigen presenting cells in tolerance induction. I will introduce here the interactions of T cells, B cells and other antigen presenting cells (APCs), how tolerance is generated in the thymus, why peripheral tolerance is required, the role of APCs, the difference between effective and ineffective antigen presentation, and the consequences of ineffective antigen presentation. I will also give an overview of acquired tolerance to soluble protein antigens and the mechanisms proposed to mediated this tolerance. I will then describe why I think that small B cells are the APC in the induction of acquired and self tolerance to peripheral antigens.

Overview of the Immune Response.

The immune system contains two antigen specific cell types, T lymphocytes and B lymphocytes, which interact with a variety of other cell types including macrophages (MΦ) and dendritic cells. Each individual T or B cell is unique because it bears on its surface an individually rearranged antigen receptor which recognizes particular sites (epitopes) found on antigens (1). Upon antigen recognition

these cells interact to produce an immune response which may take one of several forms: antibody mediated, cell mediated, or cytotoxic. The type of the response is regulated by the location of the antigen in the body, the type of APC which picks up the antigen (B cell, MΦ, or dendritic cell), the type of help provided by specific cytokines, and by the antigen itself and its form (2, 3).

An antibody response is produced by B lymphocytes. B cells are derived from bone marrow precursor cells and express on their surface Ig molecules, which are identical in their variable regions to the Ig that the B cell will secrete upon activation. This Ig molecule serves as the antigen receptor on B lymphocytes. Some antigens (particularly carbohydrates and other molecules having repeating subunits) are able to extensively cross-link these Ig molecules on the B cell and activate the B cell to grow and differentiate into Ig secreting plasma cells without the help of antigen-specific T cells. However, most protein antigens are not able to extensively crosslink the antigen receptors on B cells and require T cell help to provide the signals necessary for growth and differentiation (4).

T lymphocytes are also derived from a bone marrow precursor and they mature in the thymus (5, 6). T cells have helper, cytotoxic and regulatory roles in the immune response. T cells recognize antigen in a different way than do B cells. B cells can 'see' antigen in solution or bound to the surface of a cell or organism by recognition of particular epitopes found in the primary sequence or tertiary structure of the antigen. T cells can only 'see' processed antigen (as

linear sequences of amino acids) bound in the cleft of a class I or class II major histocompatibility complex (MHC) protein on the surface of an APC (1). APCs pick up (through specific and nonspecific means) or synthesize proteins which are processed in some internal compartment and then become associated with MHC molecules which are expressed on the cell surface. Once activated by an APC, T lymphocytes become effector cells and perform the role prescribed by the circumstances of its activation (2).

B cells get help for antibody responses by acting as APCs to T cells specific for the same (or linked) antigen (7). However, small or unactivated B cells do not have the ability to activate a naive T cell which requires some additional signal or signals found on other types of APCs (8, 9).

This two (T cell/APC) or three (T cell/APC/B cell) cell interaction can generate a specific immune response to almost any protein antigen. The antigen receptors found on T and B lymphocytes are generated from random rearrangement of variable, joining, and/or diversity segments found in the gene complexes of the Ig and T cell receptor (TCR) genes. This random rearrangement has the potential to generate enormous diversity in both T and B cell receptors allowing recognition of almost any protein sequence which can be generated (10). However, the immune system must have a mechanism to inactivate or remove T and B cells which are reactive to self proteins and peptides in order to avoid generating immune responses toward self. During their maturation T and B cells reactive

with self antigens are deleted from the repertoire generating a mature population of cells without self-reactivity.

T Cell Maturation in the Thymus

Since this thesis will look at T cell tolerance I will confine my discussion of the selection process to the T cell arm of the immune system. T cells mature in the thymus (6, 11). T cell precursors migrate to the thymus from the bone marrow, where they arise from maturation of pluripotent stem cells (5, 6). Once in the thymus these cells begin to proliferate and rearrange their TCR genes. TCR alpha (α) and beta (β) chains are comprised of variable and joining, or variable, diversity and joining regions producing a very diverse population of T cells (10). Successful synthesis of the $\alpha\beta$ heterodimeric TCR leads to low levels of surface expression of that TCR with two accessory molecules, Cluster of Differentiation (CD)4 and CD8 (5, 12), which interact with either MHC class II or MHC class I molecules, respectively (13, 14).

MHC molecules are a set of polymorphic proteins with a large number of alleles (15). The molecules are structurally related to the Ig superfamily of proteins, with a constant membrane proximal region and a variable region which is very polymorphic between alleles (16). This variability changes the ability of these MHC molecules bind antigenic peptides as well as with TCRs. In the mouse

there are three expressed class I molecules K, L, and D; and potentially two class II molecules, I-A and I-E. Class I MHC molecules are expressed on virtually all cell types, while class II MHC molecules are expressed on a very limited variety of cells predominantly B cells, macrophages and dendritic cells, as well as some endothelial lineage cells which can be induced to express class II MHC by interferon γ (IFN γ) (15). CD4 and CD8 molecules interact with either class I or class II MHC, probably in some nonpolymorphic portion of those molecules. Those T cells bearing CD8 interact primarily with antigen in association with MHC class I and those T cells bearing CD4 interact with antigen in association with MHC class II (17, 18). At some time during T cell maturation these immature T cells lose expression of either CD4 or CD8, as a consequence of a selection process, and are then committed to interactions with either Class I or Class II MHC molecules respectively (7).

The T cell receptor also interacts with MHC molecules. It recognizes the antigen (in the form of a peptide) in association with one of the specific alleles of MHC (19).

Since the initial repertoire comes from a random rearrangement from hundreds of variable segments, TCRs do not always have affinity for self-MHC with or without antigen. The thymus must select those TCR with affinity for self-MHC. This positive selection appears to be mediated by an MHC class I and class II bearing epithelial cell population in the thymus (20-24).

Self proteins are processed and presented constantly by the MHC molecules present on all cells (25, 26). Since among the variations of TCR molecules are receptors reactive with protein sequences present in self proteins, the thymus has another critical job and that is to remove those T cells with receptors having self specificity.

Negative selection of the T cell repertoire appears to be mediated by a population of bone marrow derived cells (probably the dendritic cell located at the cortical/medullary junction) (27-30).

There is no evidence as yet which shows how positive or negative selection is mediated. For positive selection there are two prevailing theories to explain this finding. First, selection occurs by an affinity model. T cells bearing receptors with some affinity for self MHC are rescued while T cells bearing receptors with no affinity for self MHC go on to die via programmed cell death or apoptosis. This mechanism has no requirement for a particular peptide to be associated with the MHC molecules, but since MHC molecules are rarely found empty, peptides may be less important in this stage of selection. The second model is that there are a unique set of peptides associated with the thymic epithelium which are responsible for positively selecting the MHC linked repertoire of the T cell pool (31).

The mechanism of negative selection is also unknown but one theory for the mechanism of this selection is that signalling through the TCR/CD3 complex at this stage of maturation leads to cell death

(32, 33). Thymic dendritic cells present self peptides, and those T cells which have affinity for these self peptides and the associated MHC die.

After these selection events occur, the levels of the TCR increase and these more mature cells move into the medulla of the thymus, where they are exported into the peripheral circulation. This selected population of mature T cells bears receptors which have affinity for self-MHC (with or without peptide) but have been negatively selected to remove reactivity for self peptides presented on the self-MHC in the thymus (7).

Peripheral Tolerance

If thymic tolerance were complete, all proteins manufactured by the organism would be presented by the thymic APCs which would remove all the potential self reactive T cells. However, some proteins which are sequestered or developmentally regulated, may not be expressed in the thymus (34, 35). Some of these proteins, like myelin basic protein (found in the central nervous system), can then be immunogenic if the barrier to their recognition is broken and autoimmunity results. Some proteins, especially cytokines or hormones, are expressed locally and at concentrations too low for thymic presentation.

If all proteins are not presented in the thymus, then to avoid autoimmunity a peripheral tolerance mechanism must be employed to complete tolerance to self. Since specific immune recognition of protein antigens requires T cells, whether as effectors or as helpers, and T cells require an APC for antigen recognition, it is necessary to investigate the role of APCs in the induction of peripheral tolerance.

Antigen Presenting Cells

APCs are a specialized class of cells which all express, to varying degrees, the class II MHC molecules. The classical antigen presenting cells are MΦ, dendritic cells, and B cells.

MΦs are bone marrow derived cells, bearing Class II MHC molecules and having antigen presenting capabilities (36). There are substantial populations of MΦ in the gut and lung, lower numbers in the lymphoid organs: spleen, lymph nodes and thymus, and even lower numbers in the blood (as monocytes). These cells are characterized by two other features which separate them from the other types of APCs. They are highly phagocytic and are very adherent. Receptors for complement, Ig Fc regions, denatured proteins, mannose-fucose, and fibronectin mediate these functions (37). This makes the MΦ a scavenger cell picking up and degrading antigen/antibody complexes with and without complement attached, aggregated proteins, carbohydrate antigens as well as bacteria and

other aggregates. MΦ can also pick up antigens nonspecifically by pinocytosis. However, this nonspecific mechanism is not as efficient as that mediated by receptors. These phagocytosed materials are degraded, and some portions of them are processed and presented via MHC class II to T cells.

Dendritic cells are a loosely grouped class of cell found in many peripheral sites as well as in lymphoid organs. They are characterized by having an unusual veiled, interdigitating, or dendritic shape, high levels of MHC class II molecules, and the ability to capture antigens and present them to T cells (38). Dendritic cells are found in very low numbers in the epidermis, heart, liver, lung, gut, afferent lymph, blood, tonsil, peripheral LNs, spleen and thymus (38). These cells have been shown to have potent antigen presenting function (39), but it is not known how they capture antigen. These cells are not actively phagocytic; they do not express Ig Fc or complement receptors (with some exceptions) to capture antigen/antibody complexes; nor do they express the other types of scavenger receptors found on MΦ (38). Dendritic cells do, however, express molecules which are involved in adhesion and potentially signalling. These include : Cd11a (LFA-1), CD11c, CD54 (ICAM-1), CD58 (LFA-3), CD29 (β1 integrin), and B7/BB-1 (38). Very low numbers of these cells are required to mediated potent T cell responses both in vivo and in vitro (38). Langerhans cells in the skin, which have Fc receptors, are thought to convert into dendritic cells when they migrate (with antigen) into the LNs (38).

B cells are also able to present antigen. As I have described, B cells must act as antigen presenting cells in order to receive T cell help for antibody responses to most protein antigens. B cells comprise approximately 40% of the cells in the spleen, and 20% of cells in the lymph node and peripheral blood (40). B cells have been shown to be very efficient APCs when the antigen is targeted to the B cell receptor (the surface Ig molecule) (41-43). In addition, B cells have receptors for lipopolysaccharide (LPS) and complement on their surface; these aid in antigen pick-up and also activation. These cells are also capable of processing and presenting antigen to T cell lines in vitro without being activated themselves (44).

All of these APCs process antigens in much the same way. Internalized antigens are routed to an endosomal compartment where they are degraded into peptides and become associated with class II MHC molecules which are then expressed on the cells surface.

Effective Antigen Presentation

Effective antigen presentation requires the interaction of a T cell receptor with self MHC plus a specific foreign peptide. This interaction is not sufficient, however, to activate a resting T cell (45). During T/B collaboration in the antibody response, there are additional signals required for this interaction to be productive from both the T cell and B cell perspectives. These additional signals

include adhesion and/or signalling by cell surface molecules and cytokines, some of which remain to be identified. A two signal hypothesis was first put forth by Bretscher and Cohn (46) for B cells and by Lafferty (47) for T cells.

The first signal to the T cell is from the interaction of the TCR with MHC plus peptide and is probably transmitted through the TCR associated CD3 complex (45). The second signal may not be unique. There are a variety of cell surface receptors (on T cells) and ligands (on APCs) which have adhesive and activating potential. These include CD2/LFA-3, CD28/BB-1, CD4/MHC class II, CD8/ MHC class I, and CD11a: CD18/ICAM-1 and 2 (45). In addition, there are those cell surface receptors with no known ligand which can activate T cells with antibody and some additional signal. These include Thy-1, Ly-6, CD-5, CD-6, CD-7, CD54, CD44, and CD45 (45). Much emphasis has been placed on the interaction of CD28 with its ligand B7/ BB-1 recently (48, 49), but it is unlikely that it is the only second signal which can help activate T cells. It is also possible that there are additional second signals which have not been identified yet.

These two or more signals combined lead to activation of the T cell. These T cells differentiate and expand as effector cells. A helper T cell can upon antigen recognition on a B cell provide the cytokine and cell surface mediated help in order to generate an antibody response.

Ineffective Antigen Presentation

Primed B cells and small B cells have been shown to present antigen to T cell clones, lines and primed T cells both in vivo and in vitro (42, 43, 50). However, there is evidence, both in vivo and in vitro, showing B cells to be poor APCs both in the allogeneic mixed lymphocyte reaction (MLR) and in primary antigen specific responses. Metlay and Steinman (39) have shown dendritic cells to be 50-100 fold better than large anti-Ig activated B cells as APC in MLR, and small B cells to be inactive. Lechler and Batchelor (51) showed that injected dendritic cells but not B cells induce rejection of renal allografts which have been transplanted into secondary hosts. In an adoptive transfer protocol, Sprent (8) looked at the cell type inducing primary helper T cell responses in vivo with sheep red blood cells and found that small thoracic duct B cells had no antigen presenting capacity. Lassila et al (9) showed that chicken B cells in chimeric animals were unable to make a thymus dependent antibody response without MHC compatible macrophages. Webb et al (52) and Cowing and Chapdelaine (53) showed APC activity by small B cell in MLR. However, since not all MLR reactivity measures a primary response (54), small B cells may have been activating mostly memory T cells in these reports. This evidence indicates that unmanipulated, small, resting B cells are poor antigen presenting cells for primary T cell responses, although they can provide the

necessary interactions to get help from an already activated T cell. What are the consequences of ineffective antigen presentation?

B Cells as Tolerogenic APCs

This thesis proposes that presentation of antigen by a small, resting B cell to a small resting T cell is tolerogenic and results in loss of T cell activity. This model is supported by evidence from Ryan et al (55) who showed that in vivo injection of small (accessory cell depleted) spleen cells from allogeneic donors into normal mice produced hyporesponsiveness (in MLR) which lasted at least 13 days post transfer. However, transfer of whole spleen containing MΦ and dendritic cells produced transient hyporesponsiveness followed by hyperresponsiveness. More recent evidence of B cell involvement in tolerance induction comes from Hori et al (56), who has shown that injection of F1 (bm12 x B6) accessory cell-depleted spleen cells into B6 mice results in prolonged survival of bm12 skin grafts and diminishes the MLR against bm12 cells. That the predominant MHC class II bearing cell in both these populations is a small B cell further supports our hypothesis.

Schwartz and colleagues (57-60) have shown that fixed APCs or signals which engage the TCR alone deliver to type 1 T helper cell lines (TH) an abortive signal which results in anergy. This abortive signal produces a rise in intracellular Ca^{++} and an increase in the

expression of IL-2 receptors, but no secretion of IL-2, and the cells remain unresponsive to complete activation signals for weeks (59). This model for T cell inactivation by ineffective antigen presentation is also appropriate for the signal provided by the small B cell to the naive T cell (61).

Acquired Soluble Protein Tolerance

Peripheral self tolerance is difficult to distinguish from tolerance acquired during maturation, and many investigators have used acquired tolerance to foreign antigens as a model for peripheral self tolerance. Acquired tolerance is a state of specific unresponsiveness brought on by previous exposure to a normally antigenic molecule in a form, dose or route which leads to this unresponsiveness (57).

A series of investigators, starting in the early sixties, showed that injection, intraperitoneally or intravenously, of soluble protein antigens (particularly when deaggregated or ultra-centrifuged) produced tolerance to a challenge with a more immunogenic form of the same antigen (usually complete or incomplete Freunds adjuvant). These antigens include bovine serum albumin (BSA)(Mitchison(62)), human gamma globulin (HGG)(Weigle et al (63)), bovine gamma globulin (BGG)(Dresser (64) and Clayman (65)), and flagellin (Shellam and Nossal(66) and Parish and Liew (67)). The cumulative evidence

from this type of tolerance induction is that low doses (less than one mg/mouse) of soluble (monomeric) antigen in either single or multiple doses induced tolerance in T cells (68-70). Higher doses of antigen were needed (greater than one mg/mouse) in order to induce B cell tolerance (70).

Tolerance in T cells is induced in a short time period. For human and bovine γ globulins 2-4 days were required before tolerance could be transferred (70, 71). Complete recovery from tolerance to these soluble protein antigens takes between 4-6 months (70, 71). Recovery does not appear to be related to the loss of circulating antigen, since passive immunization (which should absorb antigen into immune complexes) during tolerance did not affect recovery (71). However, by generating immune complexes which can accumulate in the follicular dendritic cells of the thymus (37) this may reinforce tolerance by aiding immunologic memory.

Mechanisms of Tolerance

T cell tolerance can occur by at least three known mechanisms: anergy, deletion, and suppression. These three mechanisms are not mutually exclusive and in some cases have been shown to overlap. Suppression has been shown to co-exist with nonfunctional T cells (whether by anergy or deletion is unknown) (72). Anergic cells have

been described which persist after the majority of antigen reactive cells have been deleted (73).

The easiest form of tolerance to understand is deletion. Once an antigen responsive population has been deleted it is impossible to activate that population. Deletion has been vividly demonstrated by Kappler et al utilizing the affinity of certain families of variable regions of TCR β chains (Vβ) T cell receptors with the class II MHC molecule I-E. They showed that in mice which express I-E in the thymus Vβ 17a$^+$ T cells were lost during maturation. There were immature Vβ17a$^+$ cells bearing CD4 and CD8 present in the thymus but no mature single positive cells present (CD4 or CD8 only) in these mice (29, 30). Apoptosis has been demonstrated in the thymus of mice transgenic for a TCR reactive with a peptide of ovalbumin (74). Upon addition of the specific ovalbumin peptide with which this TCR reacts, they could detect massive cell death and the ladderlike appearance of fragmented DNA in the thymus characteristic of apoptosis. Since the majority of T cells in the thymus never leave the thymus, this mechanism is thought to be the major mechanism of thymic tolerance (7).

Anergy is less simple. Anergic or unresponsive cells have been described in a number of systems of tolerance. Why the immune system would first turn a particular cell off and then leave in the circulation is unknown. Three theories cover this finding. First, that these cells are all in the process of dying and that at any one given time there are cells, not yet dead, which can be harvested but not

activated. This mechanism is implied in the anergic cells demonstrated by Webb et al and Rammensee et al (75, 76). Rammensee found anergy when they transferred cells bearing a foreign minor lymphocyte stimulating (Mls) antigen into mice. However, Webb repeated this experiment but first removed the thymus and found clonal deletion, after a period of cellular expansion. This implies that if there are no newly emerging cells from the thymus then the cells are deleted and possibly indicating that the anergic cells Rammensee found were short lived.

The second theory is that these cells perform some function which maintains the tolerant state of the individual. These cells are in fact obstructing, or reinforcing the tolerant state, or tolerizing other cells (34, 77-79).

One final theory involves the reactivation of these cells. Goodnow has a B cell tolerance system derived from mice transgenic for antibody and antigen (80). In these mice he has recently shown that tolerance can be broken by repeated stimulation (81). This would indicate a role for anergic B lymphocytes as antigen reactive cells held in an unresponsive state until highly stimulated.

Clonal anergy has been demonstrated in systems where there are an unusually large number of responding T cells (34, 76) or in mice transgenic for particular T cell receptors (73). Lo and Rammensee both took advantage of the families of Vβ TCRs responsive to MHC class II molecules or the minor stimulating antigen, Mls. In both of these cases with peripheral expression

through transgenic expression of a foreign MHC or transfer of Mls bearing cells, anergic T cells could be found in the circulation. Teh et al. have made mice transgenic for a TCR reactive with the male minor histocompatibility locus antigen, H-Y (73). When the transgenes were bred into male mice (which express H-Y), most of the T cells bearing the transgenic T cell receptor were anergic. When these anergic cells were transferred into secondary hosts, whether primed to H-Y or naive, the cells remained unresponsive (82).

The anergy or unresponsiveness induced has characteristics similar to the tolerance induced in vitro using cloned helper T cell lines and fixed APCs (58, 83), irradiated B cells (61), free antigen (84), plate bound MHC class II molecules (85), or inappropriate APCs (86). In all these papers, helper T cell clones were inactivated by exposure to antigen in an inappropriate manner or where there was an absence of secondary signals (see page 10, effective antigen presentation).

Suppression is the least clear system of all. Suppression or infectious tolerance was first described by Gershon and Kondo (87). They could demonstrate a population of T cells which could be adoptively transferred into secondary hosts and transfer the suppression. Suppressor cells fell into disrepute when the putative marker (I-J) for them was not found at its presumed location (by genetic mapping to a site in the MHC class II region). Also, efforts to clone suppressor cells have not been uniformly successful. In some cases cloned suppressor cells failed to have functional TCR (88).

However, even if there is no unique suppressor lineage, there are many functions of activated T cells which can, in an antigen specific manner, effectively stop an immune response. These include cytotoxic cells, and suppressive cytokine release such as IFN γ or IL-10. There are also systems of tolerance induction which do contain a population of cells capable of transferring unresponsiveness which is a hallmark of suppression (35, 89-91).

A response related to suppression is immune deviation or split tolerance. Parish and Liew (67) and Silver and Bennacerraf (92) have demonstrated under some conditions that antibody tolerance can co-exist with normal responses to delayed type hypersensitivity (DTH), a cell mediated immune response. Also, Otten et al (93) has shown split anergy in $CD8^+$ T cells, which no longer secrete IL-2 but do retain cytotoxic activity. We know that certain types of antigens and adjuvants (2, 94) influence the type of T cells activated and the types of cytokines that they produce. It has been postulated that B cell antigen presentation activates the type of helper T cell which proliferates, secretes IL-2, and is able to mediate DTH (96) (the TH1 type cell), but does not help antibody production (2, 95, 96). T helper subsets by virtue of the cytokines they secrete are mutually antagonistic toward one another. TH1 type cells secrete IFN γ, which inhibits the TH2 type cytokines, and TH2 cells secrete IL-10, which inhibits secretion of TH1 cytokines (2).

APC in Soluble Protein Tolerance

The APC which mediates acquired tolerance to soluble proteins has not been defined. Using low doses of deaggregated antigen, the most likely APCs are the relatively rare antigen specific B cells, which have antigen specific immunoglobulin molecules on their surface. These B cells could concentrate and process the antigen, but would not be activated by binding antigen since deaggregated antigen would not extensively cross-link B cell membrane Ig. As outlined above the interaction of an antigen specific resting T cell with an antigen specific resting B cell may be ineffective due to a lack of a costimulatory signal or signals, which are already present or unnecessary in previously activated cells.

The common thread which ties all of this together is that T cell tolerance may result from antigens presented by an ineffective APC which can pick up the antigen preferentially because of the concentration, location, and state of aggregation. Some of the features of soluble protein tolerance which favor small B cells as the APC are shown in Table 1.

The other APCs will not see these antigens because the macrophage receptor system is set up for aggregated proteins or antigen/antibody complexes, the antigen is in a location which does not favor pick up by dendritic cells (dendritic cells comprise less than 0.01% of the peripheral circulation (38)), and the concentration is too low for nonspecific antigen pick up via pinocytosis (41, 42).

TABLE 1

FACTORS WHICH INFLUENCE THE APC IN TOLERANCE TO SOLUBLE PROTEIN ANTIGENS

Route	I.V.	predominant MHC class II bearing cell is the small B cell
Dose	less than 1 mg.	most efficient receptor system is the antigen specific B cell
Form	deaggregated	unactivated, small antigen specific B cell, not picked up as particles by MΦs

Model for Soluble Protein Tolerance and Small B Cell Antigen Presentation

To test the hypothesis that small B cells are the APC involved in soluble protein tolerance in vivo, we needed a way to target an antigen directly to small, resting B cells. This target must be present on resting but not activated B cells, must not activate the B cells by binding, and must not have a high concentration in the circulation in a soluble form.

Our lab has previously shown that small B cells can process and present Fab fragments of rabbit anti-mouse anti-IgD (Fab anti-δ) very efficiently to our rabbit Ig specific helper T cell lines (97). Fab anti-δ alone does not activate B cells in vitro; they show no increases in MHC class II expression, size, or DNA content (97). Since we know that small, resting B cells can process this antigen while remaining in a resting state (44), we felt that this would be good system for looking at antigen presentation by small B cells to naive T cells.

The goal of this thesis is to show that an antigen targeted to small B cells in vivo can induce tolerance. I will also show that although the antigen specific B cells are affected the primary defect is in the T cell population. The response to the antigen has not been shifted toward different Ig isotypes nor toward a strictly cell-mediated response. In addition, the tolerance induced is not solely the result of suppression.

CHAPTER II.

MATERIALS AND METHODS

Mice. BALB/c x DBA/2 mice (CD2F1) and BALB/c mice were from the National Cancer Institute (Frederick, MD.). Mice with severe combined immune deficiency (CB.17 SCID mice)(98) and CB.17 mice were bred at the University of Massachusetts Medical Center animal facility and were from stock kindly provided by Donald Mosier (Medical Biology Institute, LaJolla, CA). Female, age matched mice were used in each experiment (except as noted), and the mice were 8-20 weeks old at the start of each experiment. The mice were injected and bled from their lateral tail veins.

Antigens and Antibodies. Rabbit anti-mouse IgD was prepared in rabbits as previously described (99). The antibody was purified from rabbit sera by affinity chromatography on IgD columns, removing anti-IgG and anti-IgM crossreactivity on appropriate columns first. Immunoprecipitation of δ (IgD) chains but not μ (IgM) chains from ^{125}I -labeled B cells was done to check specificity (97). Fab fragments were prepared by incubating intact antibody with papain coupled to agarose (Sigma Chemical, St. Louis, Mo) and then separated from Fc fragments by passage over Protein A sepharose columns. Separation of Fab fragments from whole rabbit Ig was confirmed by polyacrylamide gel electrophoresis. $F(ab)'_2$ fragments

were prepared by pepsin digestion. Rabbit anti-azophenyl arsonate (anti-ars) antibody was prepared in rabbits and purified by affinity chromatography. Nonimmune rabbit immunoglobulin (NRG), prepared from Cohn fraction II and III, was purchased from Sigma Chemical Co, and absorbed Fab NRG was prepared by incubating Fab NRG with whole murine spleen cells for one hour at 4° C. Chicken Ig was purchased from Calbiochem-Behring (La Jolla, CA). Bovine serum albumin (BSA) was purchased from United States Biochemical Corp. (Cleveland, OH). DNP (2,4 dinitrobenzenesulfonic acid sodium salt) from Eastman Kodak (Rochester, NY) was coupled to BSA, chicken Ig, and $F(ab)'_2$ NRG by the method of Mishell and Shigi (100) with approximately one DNP per molecule. Rabbit anti-mouse heavy plus light (H+L) chain or isotype specific horse radish peroxidase-coupled antibodies were purchased from Southern Biotechnology Associates (Birmingham, AL). Complete and incomplete Freunds adjuvant (CFA and IFA) were purchased from Gibco Laboratories (Grand Island, NY). Killed *Bordetella pertussis* (*B. pertussis*) was purchased from the Biological Laboratories at the State Laboratory Institute (Jamica Plain, MA). Endotoxin levels in antigens were measured by Pyrotell *Limulus* amebocyte lysate assay (Associates of Cape Cod Woods Hole, MA). All antigens used had less than 15 ng endotoxin/mouse at the maximum injected dose.

Other Reagents. For cell culture and washing, RPMI 1640 (Gibco Laboratories, Grand Island, NY) with fetal calf serum (FCS)

added (Hyclone Laboratories, Logan, UT) was used. Other reagents are listed as used.

Transfer of pulsed small B cells. Small B cells were prepared from pooled whole normal spleens from CD2F1 mice by centrifugal elutriation as previously described (42). In one experiment adherent cells were removed by allowing those cells to adhere to plastic for 2 hours (hrs) at 37° C. The cells were pulsed either overnight or for one hour at 37° C with Fab fragments of anti-δ and injected intravenously (i.v.) or intraperitoneally (i.p.) at various cell concentrations into normal CD2F1 mice. After seven days the mice were challenged with 100 μg Fab NRG precipitated in aluminum potassium sulfate (alum) and bled at weekly intervals for anti-rabbit Fab antibodies.

Tolerance Induction: Molecules used as tolerogens were centrifuged at 160,000 x gravity (g) in an airfuge (Beckman Instruments Palo Alto, CA) for 60 minutes and the top 60% of the solution was used. Tolerogens were injected in a volume of 0.2 ml in phosphate buffered saline (PBS) i.v into the lateral tail vein of the mice. After seven days the animals were challenged i.p. with 100 μg of Fab NRG in 0.2 ml precipitated in alum. Most animals also received 20 μg chicken Ig precipitated in alum at a separate site i.p. The animals were bled from the tail veins at weekly intervals and the serum tested for anti-rabbit Fab antibodies.

ELISA. Antibody titers were measured by a sandwich enzyme linked immunosorbent assay (ELISA) assay in which serial dilutions of mouse sera starting at 1:40 were incubated with antigen ($F(ab)'_2$ NRG, chicken Ig, rabbit anti-mouse Ig, or DNP-BSA) bound to flexible polyvinylchloride plates (Becton Dickinson Labware, Oxnard, CA) and blocked with 1% BSA in PBS. The plates were washed with PBS + 0.05% Tween 20 (polyoxyethelene-sorbitan monolaurate) (Sigma Chemical Co.) and incubated with rabbit anti-mouse IgG H+L chain or anti-isotype antibody coupled to horseradish peroxidase. The substrate and color developer were 3,3',5,5'-tetramethyl-benzidine (ICN Biologicals, Lisle, IL) with H_2O_2 added. The reactions were stopped by the addition of 2M H_2SO_4. Plates were read at 405 vs 530 nm on a Dynatech ELISA platereader (Dynatech Laboratories, Inc., Alexandria, VA) and antibody concentrations determined by comparing optical density (O.D.) with a mouse anti-rabbit Fab standard or purified mouse anti-ars antibodies of different isotypes which had been purified by affinity chromatography. Analysis of antibody titers was made by logistic analysis ("Immunosoft" copyright 1983 Dynatech Laboratories, Inc.) and those dilutions in the most accurate range of the plate reader were averaged to provide microgram/milliliter (μg/ml) equivalents.

Adoptive transfer. Balb/c mice were injected i.v. with 100 μg Fab anti-δ. On day five, spleen and lymph node cells were harvested. The spleen cells were depleted of T cells as previously described (97)

by treatment with an anti-T cell cocktail (anti-Thy-1, anti-CD4, and anti-CD8) followed by a mouse anti-rat kappa (κ) antibody and then agarose absorbed guinea pig complement (tolerant B cells). The lymph node cells were depleted of B cells by panning on rabbit anti-mouse Ig coated plates by the method of Wysocki and Sato (101) (tolerant T cells). These cells along with T and B cells collected and separated from untreated, control Balb/c mice (normal T and normal B) were transferred i.v. into SCID mice. Two days later the reconstituted SCID mice were challenged i.p. with 100 μg Fab NRG + 20 μg chicken Ig each precipitated in alum. In the cell titration adoptive transfer mice were challenged with 100 μg Fab NRG plus 20 μg chicken Ig precipitated in alum with 2 x 10^8 *B. pertussis* added. All SCID mice were shown to have less than 5 μg Ig/ml (by ELISA) prior to transfer. T cells were <1% sIg^+ and B cells were <1% Thy-1^+ when stained with biotinylated anti-mouse Ig or anti-Thy-1 plus FITC-avidin.

In vitro proliferation. CD2F1 mice were injected with 100 μg Fab anti-δ. Tolerant and control mice were challenged seven days later with 100 μg Fab NRG either precipitated in alum i.p. or emulsified in CFA subcutaneously (s.c.) at the base of the tail. After a further nine days spleens from the alum primed mice or draining LNs (inguinal and aortic) from the CFA mice were collected. Whole cell suspensions of LN or spleen were plated in RPMI 1640 with 10% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-

glutamine, and 5 x 10^{-5} 2-mercaptoethanol. 2,4, or 8 x 10^5 cells/well were plated with 50 or 100 μg/ml $F(ab)'_2$ NRG for 72, 96, or 120 hrs at 37°C in 5% CO_2 with ^3H thymidine added for the last six hours of the culture. Cells were harvested on a Skatron harvester (Skatron Inc Sterling, Va) and counted on an LKB betaplate scintillation counter (LKB Instruments Gathersburg, MD). Counts per minute over background (delta cpm) were calculated by subtracting proliferation without added antigen from proliferation with antigen.

Neonatal tolerance. Male and female CB.17 mice were made tolerant by i.p. injection of 500 μg Fab NRG emulsified in an equal volume of IFA in a total volume of 0.1 ml. within 24 hours of birth. All mice were bled at seven weeks of age and tested for anti-rabbit Fab antibodies by ELISA.

Mixing. Eight week old male and female CB.17 mice were made tolerant by i.v. injection of 100 μg Fab anti-δ as described. LNs and spleens from these mice (five days after anti-δ injection) and neonatal tolerant mice described above (at eight weeks of age) and normal unprimed mice were separated into T and B cells as described above. T and B cells from these mice were injected i.v. into eight week old SCID mice in various combinations, mixing normal with tolerant T cells plus normal B cells. After two days the mice were challenged with 100 μg Fab NRG and 20 μg CGG. The SCID mice were bled weekly and antibody titers measured by ELISA.

CHAPTER III.
RESULTS AND DISCUSSION

Transfer of Small Pulsed B cells

The first attempt to test whether B cells induce tolerance was to inject small and or large B cells, which had been pulsed with Fab anti-δ into mice and measure the subsequent response of these mice to a challenge with rabbit Fab precipitated in alum. This approach did not succeed.

The initial experiment involved normal spleen cells, depleted of T cells with antibody and complement, depleted of MΦ by plastic adherence, and elutriated for size. The cells were incubated overnight with Fab fragments of anti-δ. Normal mice were injected with these pulsed cells either i.v. or i.p. at either one or ten million cells per animal. The control groups were mice injected with 3×10^6 large pulsed cells or untreated mice. All mice were challenged after seven days with 100 μg Fab NRG precipitated in alum. The results are summarized in Table 2.

The second experiment involved different conditions for the preparation of the small cells. T cells were not removed and the cells were elutriated without any protein in the medium. The cells were incubated for one hour at 37° C with Fab anti-δ. The results are summarized in Table 3.

TABLE 2

TRANSFER OF PULSED CELLS #1

Number of cells	Route	Results
10 x 10^6	i.v.	primed
1 x 10^6	i.v.	primed
10 x 10^6	i.p.	unaffected
1 x 10^6	i.p.	unaffected
3 x 10^6 large cells	i.v.	primed

Table 2. Injection i.v. or i.p. of 1 or 10 x 10^6 small B cells or 3 x 10^6 large cells pulsed with Fab anti-δ overnight. Mice were challenged after seven days with 100 μg Fab NRG precipitated in alum i.p. Primed cells showed responses at day of challenge and/or higher overall response. Unaffected mice were the same as uninjected control mice.

TABLE 3

TRANSFER OF PULSED CELLS #2

NUMBER OF CELLS	ROUTE	RESULTS
4 x 10^6	i.p.	unaffected
4 x 10^5	i.p.	unaffected
4 x 10^4	i.p.	unaffected
3 x 10^6 large cells	i.p.	primed

TABLE 3. Injection of graded doses of small or large spleen cells pulsed with Fab anti-δ into mice. All mice were challenged after seven days with 100 μg Fab NRG precipitated in alum.

The last transfer experiment involved injection of pulsed cells twice a week for two or four weeks. In this experiment all the mice were primed (results not shown). This was probably due in part to the difficulty I had in actually injecting the mice intravenously.

Conclusions. Except for the mice receiving the highest doses of cells there was no effect of these small pulsed cells on the resultant immune response. The large cells did prime the mice, however.

This approach may not have induced tolerance for a number of reasons. It may have been that in this model system the transferred cells never reached the appropriate location for tolerance induction, or there was no 'space' in these normal mice for the injected cells. However, Rammensee (76) was able to transfer Mls disparate cells into normal mice and induce tolerance. Additionally, Victoria Yuschenkoff has been able, in our lab, to induce tolerance by transfer of whole spleen or LNs into normal mice, using mice transgenic for human Ig μ chains as donors for the transferred cells (102).

An alternate explanation is that the antigen did not remain presented for a long enough time to induce tolerance. Gosselin et al. (44) showed, with in vitro presentation of rabbit anti-mouse Ig by small B cells to T cell lines, a loss of antigen presentation (as measured by cytokine release) starting 12 hours after pulsing with antigen. This may indicate a gradual loss of antigen or antigen presenting capacity with time.

Since I was unable to induce tolerance by transfer of small B cells, I decided to abandon this protocol. I decided to try injection of soluble Fab anti-δ. Injection of soluble ultracentrifuged Ig molecules has been used very effectively in the past to generate antibody tolerance (63-65). Our laboratory had available rabbit anti-δ, which is specific for the majority of small B cells in the mouse. Also since there is only a low level of circulating IgD in the mouse there would be little immune complex (IgD anti-IgD complexes) formation. Since the Fab fragments do not activate small B cells (97), I can look at antigen presentation in vivo by small B cells.

Injection of Ultracentrifuged Fab Anti-δ

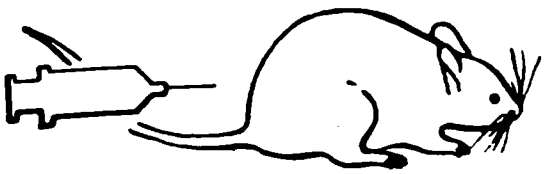
For this group of experiments I developed a technique from the protocols used in classical methods of tolerance induction for soluble protein antigens. Tolerance to ultracentrifuged Ig molecules has been induced using a single injection of as low as 100 μg (68). The tolerance requires 2-5 days to be induced (71, 103). Challenge was with heat aggregated (63), CFA emulsified (65, 71) or alum precipitated (62) antigen and was given 6 days to two weeks later in most cases. Figure 1 shows the general protocol followed in all the experiments to follow. All exceptions will be noted in each section.

Anti-δ injected into mice induces tolerance to rabbit globulin. Our model system involves injection of 100 μg of ultracentrifuged Fab rabbit anti-δ intravenously into normal CD2F1 mice followed by challenge with Fab NRG precipitated in alum seven days later. These animals become profoundly tolerant to rabbit Fab as measured by antibody production in an ELISA assay. Figure 2 and Table 4 show the results of one such experiment. These mice were followed weekly until day 64 post-challenge, when three of the six tolerant mice still made less than 5% of the mean control anti-rabbit Fab response. The difference between the control and tolerant mice is generally greatest at day 21 or 28 post challenge. At this point

Figure 1

PROTOCOL FOR TOLERANCE INDUCTION

TOLERIZE

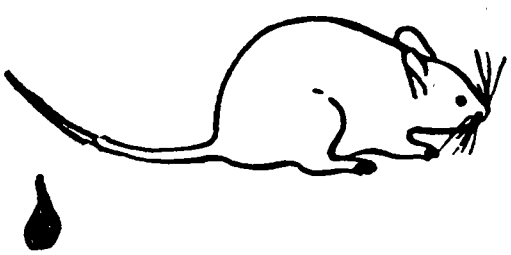


I.V. INJECTION OF SOLUBLE FAB <u>RABBIT</u> ANTI-MOUSE IgD (Fab anti-δ)

CHALLENGE

I.P. INJECTION OF ALUM PRECIPITATED FAB NON-IMMUNE <u>RABBIT</u> Ig (FAB NRG)

BLEED WEEKLY



MEASURE ANTI-<u>RABBIT</u> FAB BY ELISA

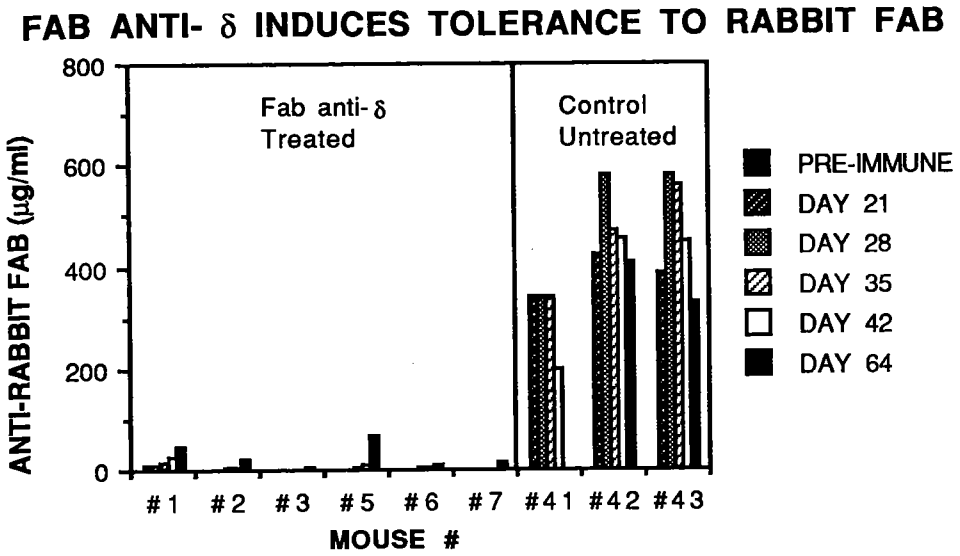


Figure 2. Serum anti-rabbit Fab antibody levels in µg/ml from mice injected i.v. with 100 µg ultracentrifuged Fab rabbit anti-δ or control untreated mice challenged with 100 µg Fab NRG precipitated in alum. Antibody levels were measured by ELISA at the indicated days post challenge.

TABLE 4

TREATMENT WITH DIFFERENT RABBIT Igs

	PRE	DAY14	DAY 21	DAY 28	DAY 35	DAY 42	DAY 64
	0.0	0.8	6.7	12.8	20.7	32.2	51
	0.1	0.7	0.04	2.4	3.6	4.0	35
	0.2	0.3	0.3	0.6	0.6	0.6	2.3
	0.3	0.9	0.8	1.8	2.5	5.1	126
	0.2	0.3	0.6	0.9	1.0	2.4	13
FAB	0.3	0.2	0.3	2.1	2.7	2.9	22
ANTI-δ	**0.18**	**0.53**	**1.46**	**3.43**	**5.18**	**7.87**	**41.62**
SD	**0.12**	**0.30**	**2.58**	**4.64**	**7.68**	**12.02**	**44.71**
	0.1	88	209	355	404		
	0.3	11	52.	125	106		
	0.1	25	100	146	160		
	0.1	32	257	358	307		
	0.4	29	188	191	216		
FAB	0.1	18	354	498	513		
ANTI-ARS	**0.18**	**33**	**193**	**279**	**284**		
SD	**0.13**	**28**	**109**	**148**	**154**		
	0.1	32	130	484	420		
	0.5	17	88	256	260		
	0.1	8	74	286	295		
	0.1	21	57	899	1023		
	0.3	36	52	243	292		
	0.02	59	98	1418	1697		
F2 ANTI-δ	**0.19**	**29**	**83**	**598**	**665**		
SD	**0.18**	**18**	**29**	**473**	**582**		
	0.0	9.9	41	96	76		
	0.1	17.5	109	319	441		
	0.1	1.7	13	46	71		
FAB NRG	**0.07**	**9.7**	**54**	**154**	**196**		
SD	**0.06**	**7.9**	**49**	**145**	**212**		
	0.2	49	120	1192	1244	974	834
	0.1	46	200	341	331	318	754
	0.1	50	482	1447	1221	1606	1698
CONT	**0.13**	**48**	**267**	**993**	**932**	**966**	**1095**
SD	**0.06**	**2**	**190**	**579**	**521**	**644**	**523**

TABLE 4. Shown are individual anti-rabbit Fab responses in μg/ml of the mice shown in Figure 2 and 6. Groups represented are from the top: Mice treated with 100 μg Fab anti-δ, Fab rabbit anti-ars, F(ab)'2 anti-δ, Fab NRG, or control untreated mice. All mice were challenged seven days after treatment with 100 μg Fab NRG precipitated in alum. Mice were bled weekly at the times shown at the top of the table and anti-rabbit Fab was measured by ELISA. Shown in bold are the averages and standard deviations for each group, which are plotted in Figures 2 and 6.

the control mice approach their peak response and the tolerant mice remain unresponsive.

Summarizing all of the data to date, 35 of 50 mice made less that 1% of the control response. Of the remaining mice only 5 made greater than 5% of the mean control response and only two mice made responses >10% (15 and 20%) as shown in Figure 3. Other mice, which were not tested at day 21 post-challenge, are shown in Figure 4 as percentage of the mean control response at day 14. These mice do not appear as tolerant as the mice shown in Figure 3. However, the control responses have not reached their peak at this time and do not reflect the full anti-rabbit Fab response and so the tolerance induced is underestimated. The average response of these 13 anti-δ treated mice is 3.5 μg/ml with only one mouse with a response greater than 10 μg/ml.

Antigen specificity and B cell function. Injection of mice with an anti-Ig may result in a global defect in B cell function. Down-regulation of membrane IgD via anti-δ has been implicated in tolerance induction in B cells previously (104). One way to look for a global defect in the B cell population is by an antibody response to an irrelevant antigen, which will show whether the tolerance induced is antigen specific. As shown in Figure 5, these animals all made normal responses to challenge with chicken Ig, also precipitated in alum, given i.p. at a different site. In all experiments to date the control and anti-δ treated mice have equivalent responses, except for

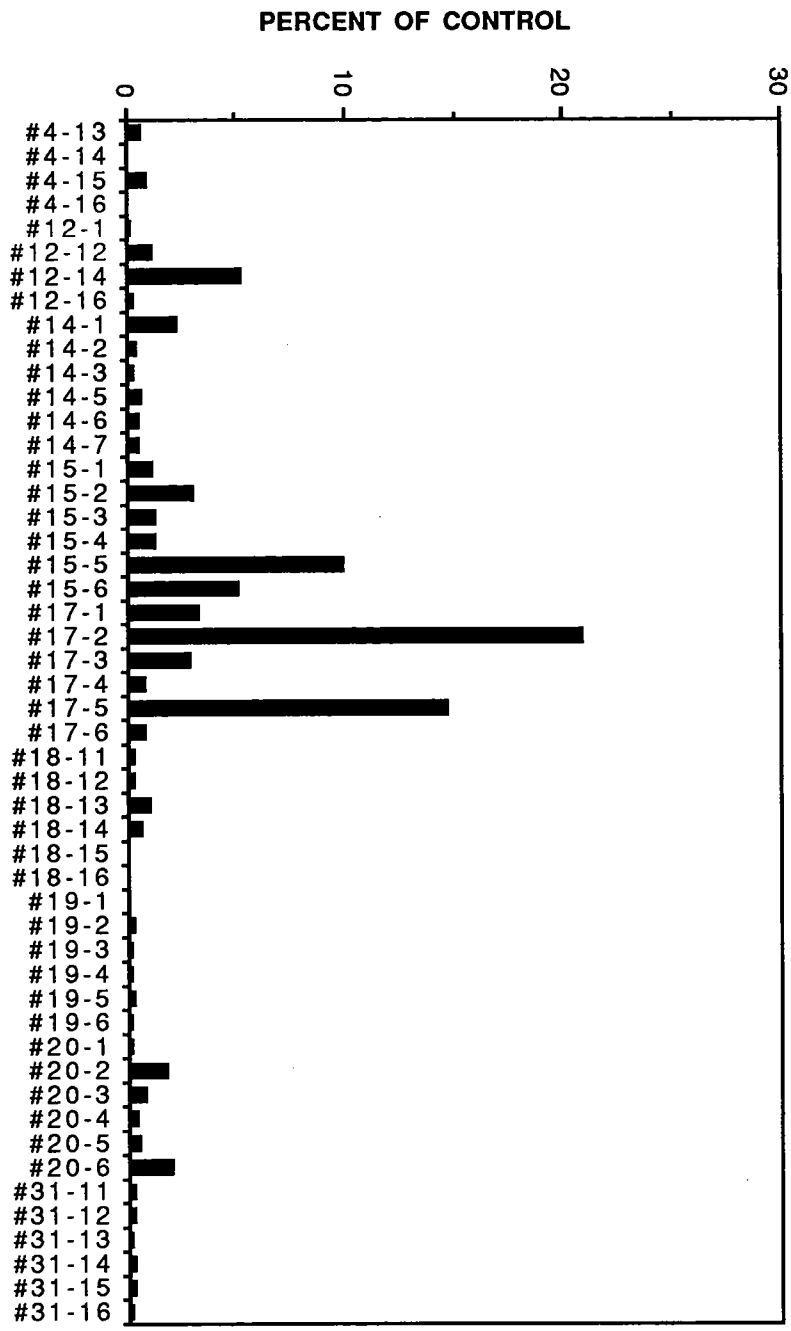


FIGURE 3. Individual anti-rabbit Fab responses from anti-δ treated mice expressed as a percentage of the mean control response at day 21 post challenge.

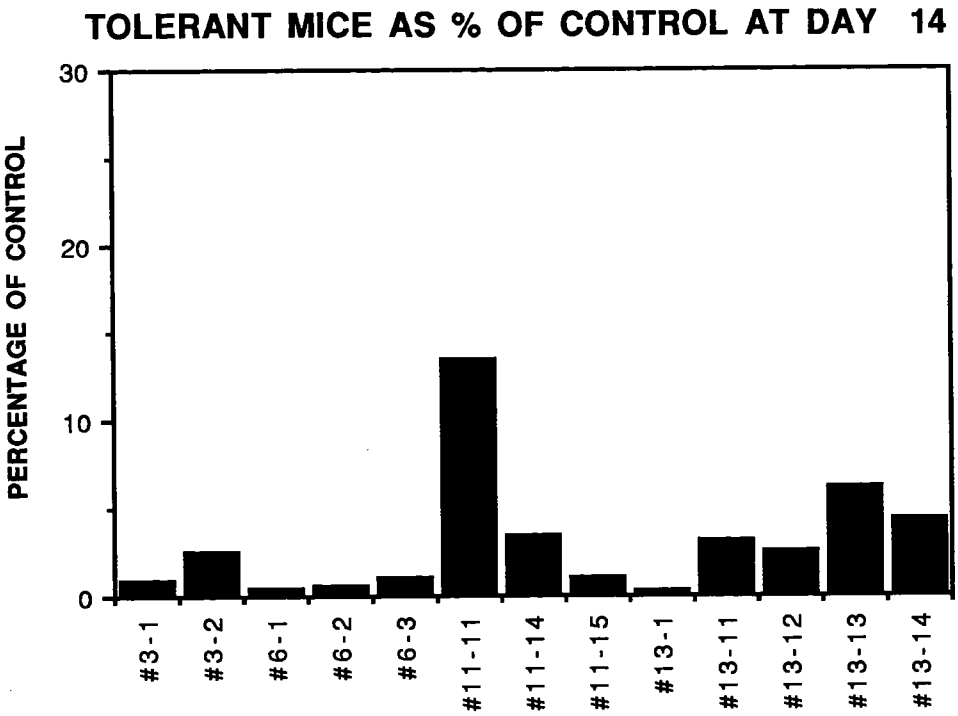


FIGURE 4. Individual anti-rabbit Fab responses from anti-δ treated mice as a percentage of the mean control response at day 14 post challenge.

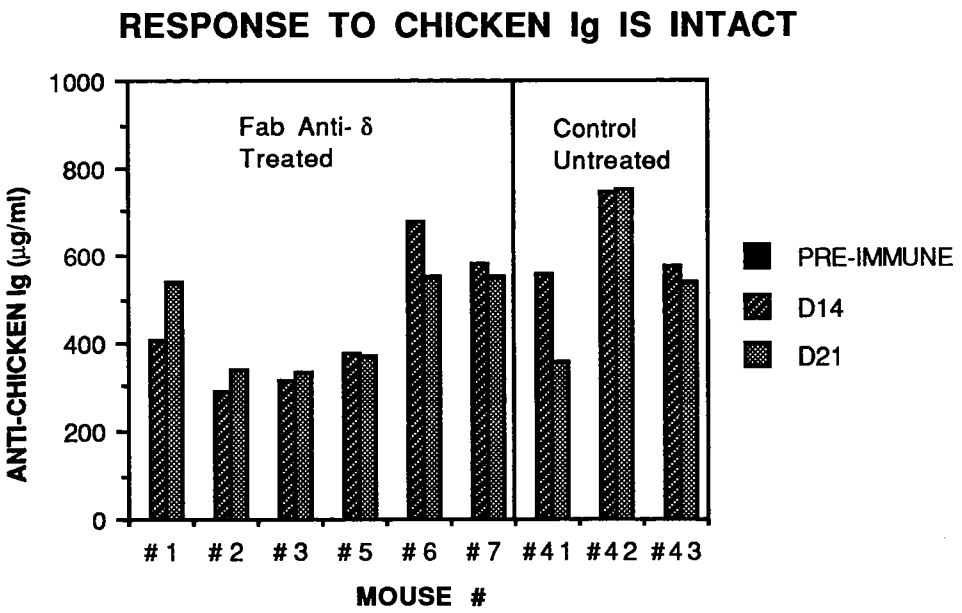


Figure 5. Serum anti-chicken Ig antibody levels in µg/ml from the same mice as shown in Figure 2. Mice were challenged at the same time as challenge with NRG with 20 µg chicken Ig precipitated in alum at a separate site. Antibody levels were measured by ELISA at the indicated days post challenge.

three mice which failed to respond to chicken Ig. The most probable explanation for these three failures is loss of the challenge antigen through leakage or missed injection. This indicates that the tolerance is antigen specific and that B cell function has not been generally affected by anti-δ.

B cells must be targeted for tolerance to occur. To show that the tolerance was due to targeting antigen to B cells rather than other types of APCs, we used a rabbit Ig molecule which was not targeted to B cells. For this purpose we used either Fab NRG or Fab rabbit anti-arsonate (anti-ars), a chemical compound not found on murine cells. Each of these antigens was ultracentrifuged and injected i.v., and the results are shown in Figure 6 and Table 4. Although both of these molecules, when injected at this dose (100 μg), reduce the anti-rabbit Fab response neither was as effective as Fab anti-δ. Whole nonspecific IgG molecules have been used in the past to induce low zone tolerance (63, 64), but, the half-life of whole rabbit IgG in the mouse is 5-6 days whereas the Fab fragments have a half-life of approximately 4 hours (105, 106). The Fab NRG may not be in the circulation long enough to efficiently induce tolerance in all cases.

In this and most other experiments with injection of Fab NRG, some diminished response was found (in all experiments 11 of 23 mice were <10% of controls at day 21 post challenge while 5 were > 50% of the controls) as shown in Figure 7 and Table 5. The

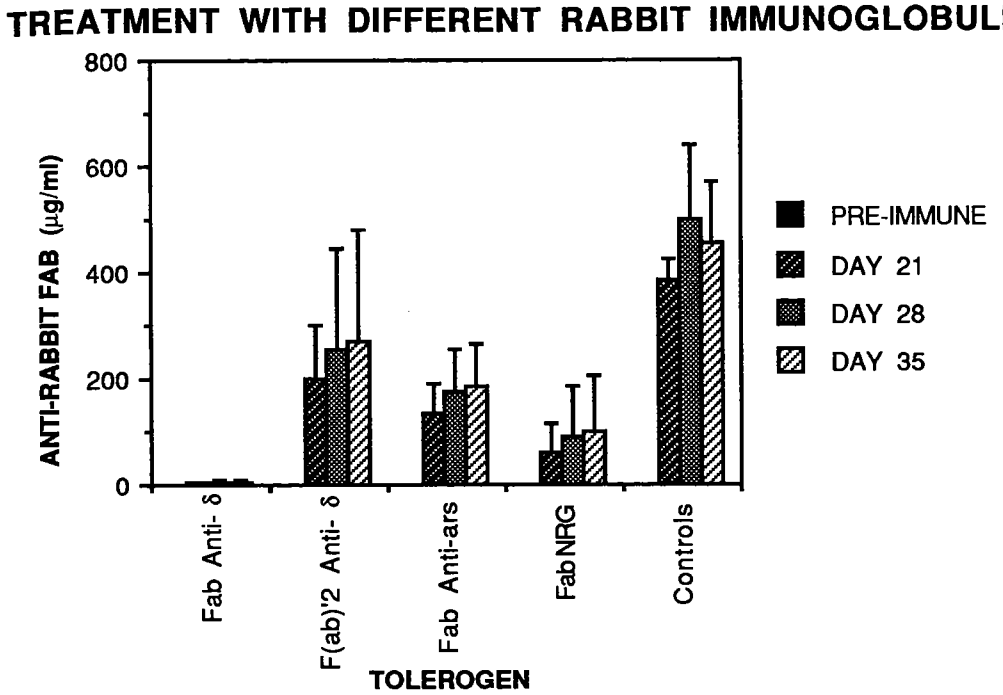


Figure 6. Mean serum anti-rabbit Fab antibody levels in µg/ml from five groups of mice treated with different forms and specificities of Fab rabbit Ig. On the left is the mean response of the 6 mice shown in Figure 1. Next are the average responses from 6 mice injected i.v. with 100 µg $F(ab)'_2$ anti-δ; 6 mice injected with 100 µg Fab rabbit anti-ars; 3 mice injected with 100 µg Fab NRG; and 3 control untreated mice. All mice were challenged as above and antibody levels measured by ELISA. Bars represent one standard deviation.

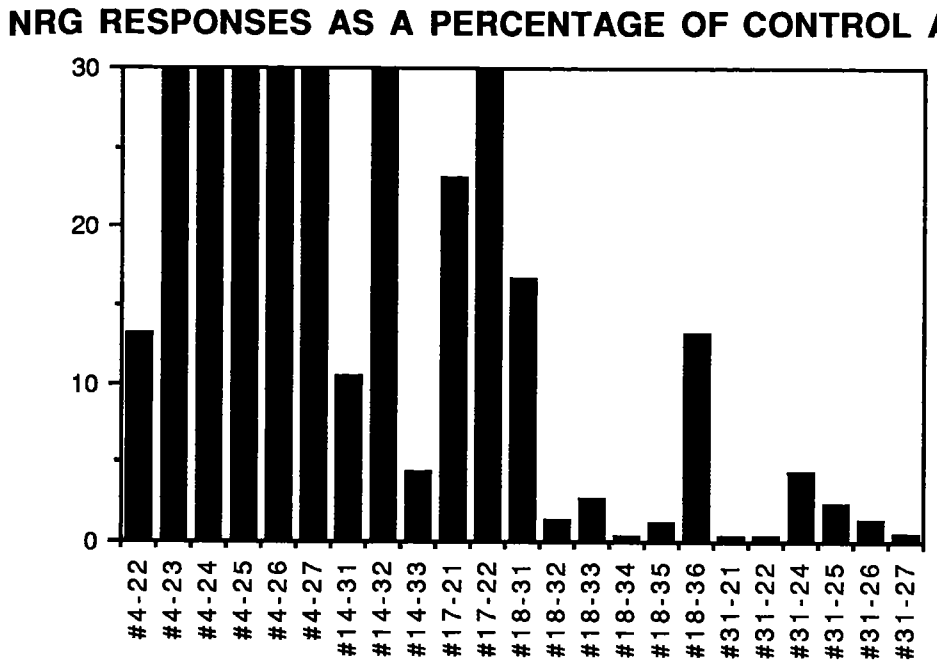


FIGURE 7. Individual anti-rabbit Fab responses from mice treated with 100 µg Fab NRG, as a percentage of the mean control response at day 21 post challenge.

TABLE 5

PERCENTAGE OF MEAN CONTROL RESPONSE FAB NRG TREATED MICE

NRG	% D21
#4-22	13.22
#4-23	140.76
#4-24	46.92
#4-25	257.35
#4-26	56.87
#4-27	88.15
#14-31	10.43
#14-32	32.09
#14-33	4.43
#17-21	23.03
#17-22	60.16
#18-31	16.62
#18-32	1.29
#18-33	2.66
#18-34	0.39
#18-35	1.13
#18-36	13.07
#31-21	0.36
#31-22	0.29
#31-24	4.36
#31-25	2.32
#31-26	1.31
#31-27	0.44

TABLE 5. Values shown are from mice treated with 100 μg ultracentrifuged Fab NRG i.v. followed by challenge with Fab NRG precipitated in alum. The mean uninjected control response from each individual experiment (shown are five independent experiments) was calculated and the percentage of the mean control response was calculated for the individual mice shown.

nonresponsiveness was not as profound as the tolerance induced by Fab anti-δ, and the results are not evenly distributed. There is a greater degree of tolerance induced in the later experiments. Since injection of small amounts of endotoxin can abrogate tolerance (107), it is possible that I had higher amounts of endotoxin in the earlier experiments. I did not begin to measure the levels of endotoxin until after the first set of experiments were done. I also became much more adept at i.v. injections with time. Since s.c. injection of antigen is likely to be picked up by a dendritic cell (38), better injection would avoid having competition between effective and ineffective APCs. Neither of these variables (endotoxin levels and injection success) appear to be as important for the anti-δ, which may indicate that the anti-δ is a much more effective tolerogen. There is a significant difference between the mice treated with Fab anti-δ and Fab NRG. The combined mean of the percent of control response for the 50 Fab anti-δ treated mice is 1.74% (standard deviation 3.78) while that for the 23 Fab NRG treated mice is 33.8% (standard deviation 59.9).

Dose response to NRG. The partial tolerance induced by i.v. injection of Fab NRG could have been due to anti-mouse activity found in the rabbit Ig preparation used, since the responses of mice treated with Fab rabbit anti-ars are closer to the control responses. With higher concentrations of NRG, lower affinity NRG binding B cells can be recruited to induce tolerance. However, anti-δ can induce

tolerance at doses as low as 1 μg (data not shown and see Figure 8 and Table 6A), and the difference between anti-δ (targeted to all B cells) and NRG (targeted to rabbit Ig specific B cells) should be accentuated by lower doses of tolerogen.

To investigate the differences in responsiveness of mouse spleen cell-absorbed Fab NRG (FAB abs NRG) versus Fab anti-δ, a dose response experiment was done. As shown in Figure 8 and Tables 6A and B, mice treated with lower doses of Fab abs NRG are less tolerant than mice treated with equivalent doses of Fab anti-δ. This difference is the most striking at the lowest dose, 0.5 μg/mouse. The mean of the 7 mice treated with 0.5 μg of Fab anti-δ was 18.6 μg/ml which is 13.4% of the mean control response. However, the mean of the 7 mice treated with 0.5 μg of Fab abs NRG was 205 μg/ml which is 148% of the mean control response. The mice treated with 5 μg of Fab abs NRG also made 2.5 times greater mean response (46.9 μg/ml) than mice treated with a 10 fold lower dose of Fab anti-δ.

These data indicate that although Fab NRG and Fab abs NRG can induce tolerance at higher doses (> 50 μg/mouse), antigens not targeted to B cells are ineffective at tolerance induction at lower doses (0.5 μg/mouse).

Divalent F(ab)'$_2$ anti-δ does not induce tolerance. Since activated B cells have been shown to initiate some T cell responses in vitro (39), activated B cells may no longer be tolerogenic as APCs.

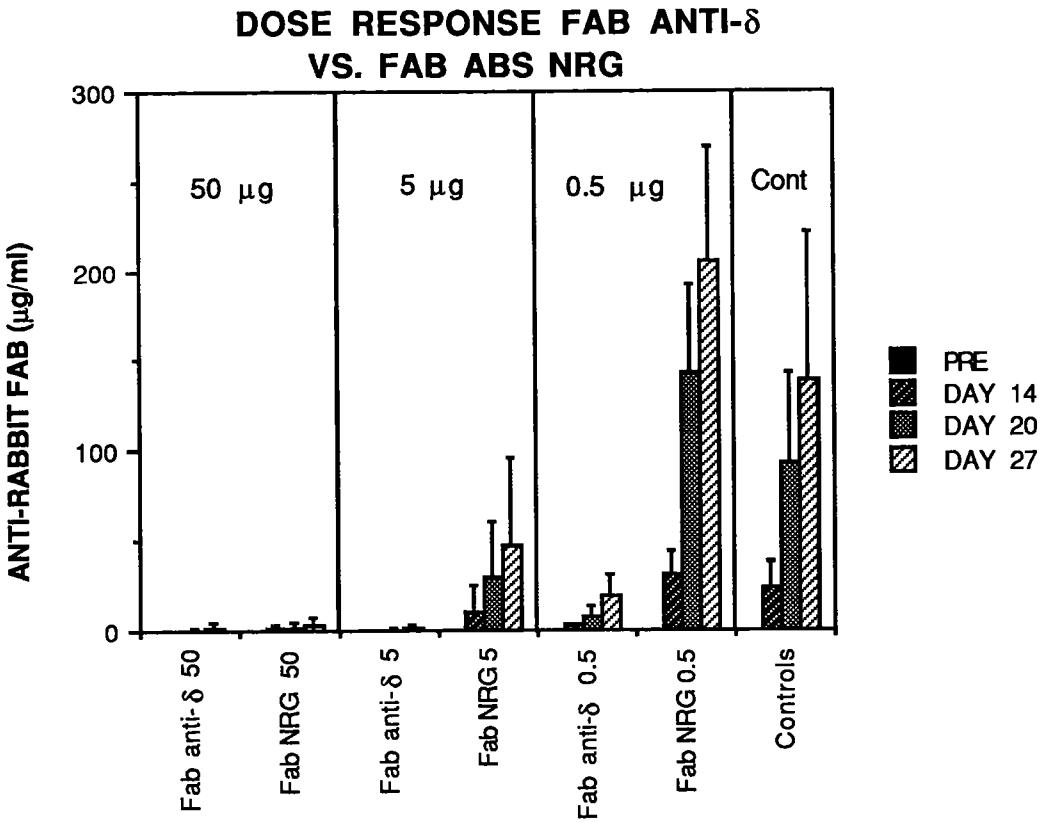


Figure 8 Mean serum anti-rabbit Fab levels in µg/ml from mice treated with 50, 5, or 0.5 µg/mouse of Fab anti-δ or Fab abs NRG. All mice were challenged as in Figure 2. There are 7 mice in each group. Bars represent one standard deviation.

TABLE 6A

DOSE RESPONSE

TREATMENT WITH FAB ABS NRG

	PRE	DAY 14	DAY 20	DAY 27
#31	0.03	28	90	136
#32	0.04	43	117	164
#33	0.03	36	112	182
#34	0.05	36	169	220
#35	0.06	46	193	329
#36	0.08	12	217	237
#37	0.05	15	106	169
AVG NRG/0.5	**0.05**	**30.86**	**143.43**	**205.29**
SD	**0.02**	**13.20**	**49.10**	**64.41**
#41	0.06	9.4	58	115
#42	0.06	4.8	16	26
#43	0.07	5.6	13	20
#44	0.08	42	89	118
#45	0.2	4.7	18	38
#46	0.1	0.1	0.5	1.5
#47	0.1	4.9	7.7	9.8
AVG NRG/5	**0.10**	**10.21**	**28.89**	**46.90**
SD	**0.05**	**14.27**	**32.28**	**48.94**
#51	0.2	0.3	0.5	1.2
#52	0.3	0.2	0.2	0.1
#53	0.1	0.1	0.2	0.1
#54	0.2	0.4	0.4	0.5
#55	0.1	3	7.7	12
#56	0.1	0.1	0.2	0.4
#57	0.1	2.8	4.7	7.9
AVG NRG/50	**0.16**	**0.99**	**1.99**	**3.17**
SD	**0.08**	**1.31**	**3.01**	**4.79**

CONTROLS

#61	0.2	18	58	78
#62	0.2	43	162	211
#63	0.1	16	92	129
#64	0.09	43	110	134
#65	0.2	20	68	103
#66	0.2	3.5	144	280
#72	0.2	23	13	36
AVG/CONT	**0.17**	**23.79**	**92.43**	**138.71**
SD	**0.05**	**14.49**	**51.48**	**82.48**

TABLE 6B

DOSE RESPONSE

TREATMENT WITH FAB ANTI-δ

	PRE	DAY 14	DAY 20	DAY 27
#1	0.04	1.4	9.2	25
#2	0.03	2.9	9.1	9.2
#3	0.03	0.9	7.1	12
#4	0.04	1.7	6.1	19
#5	0.03	4	17	43
#6	0.04	2.5	1	11
#7	0.04	3.1	6.5	11
AVG δ/0.5	**0.04**	**2.36**	**8.00**	**18.60**
SD	**0.01**	**1.08**	**4.82**	**12.14**
#11	0.03	0.03	0.02	0.02
#12	0.04	0.06	0.04	0.07
#13	0.08	0.1	0.7	4.3
#14	0.04	0.1	1.5	4.1
#15	0.04	0.1	0.1	0.1
#16	0.03	0.2	0.3	0.4
#17	0.03	0.3	1.2	2.6
AVG δ/5	**0.04**	**0.13**	**0.55**	**1.66**
SD	**0.02**	**0.09**	**0.60**	**1.96**
#21	0.03	0.1	0.1	0.1
#22	0.04	0.1	0.2	0.2
#23	0.03	0.06	0.06	0.03
#24	0.04	0.3	0.5	1.3
#25	0.09	0.4	0.4	1.9
#26	0.08	0.08	0.09	0.05
#27	0.06	0.2	1.7	8
AVG δ/50	**0.05**	**0.18**	**0.44**	**1.65**
SD	**0.02**	**0.13**	**0.58**	**2.89**

TABLE 6A and B. Shown are individual serum anti-rabbit Fab responses in μg/ml from mice shown in Figure 8. Groups include in A. mice treated with 50, 5, or 0.5 μg/mouse i.v. Fab abs NRG and control untreated mice. In B mice treated with 50, 5, or 0.5 μg/mouse i.v. Fab anti-δ. Shown in bold are the averages and standard deviation which are plotted in Figure 8.

Finkelman et al. have shown that i.v. injection of whole goat anti-mouse δ induces polyclonal B cell activation followed by the appearance of large numbers of surface IgG positive cells and T cell dependent IgG secretion (108). In addition, Golub and Weigle showed that injection of LPS around the time of the tolerogen (HGG) blocked the induction of tolerance (107). LPS can affect MΦ as well as B cells (94) and does not necessarily indicate only activation of B cells. We tested the effects of B cell activation in our system by injecting 100 μg of ultracentrifuged $F(ab)'_2$ fragments of rabbit anti-δ into mice followed by challenge as above. Unlike monovalent Fab fragments, this divalent antigen can crosslink the surface IgD molecules and could activate B cells in vivo. As shown in Figure 6 and Table 4, the $F(ab)'_2$ anti-δ did not induce tolerance. We were not able in this system to rule out effects caused by aggregation of the small amount of serum IgD (109) by $F(ab)'_2$ anti-δ , but the simplest interpretation of these results is that the B cells must remain in a resting state to induce tolerance.

Time of challenge. Tolerance generated in this system is not permanent. Over the times tested most mice made an antibody response to the alum depot of rabbit Fab. Since the original tolerogen is present for such a short time and mice are not challenged for an additional six days, it is possible that newly emerging immune cells can reconstitute the anti-rabbit Fab response. Alternatively, a very small number of peripheral pre-helper T cells

that escape tolerance induction during the anti-δ treatment may expand over the weeks following challenge.

To answer this question, I set up an experiment challenging mice at different times post treatment. Shown in Figure 9 and Table 7 are mice treated with 100 μg Fab anti-δ and then challenged at two or seven days post treatment. There is no striking difference in the anti-rabbit Fab produced by these two sets of mice. The 'day two' set were lower at all time points through day 56. All the 'day seven' mice were making some antibody by this time. The 'day two' mice were later and at day 63 three of four remaining mice were making antibody. The data above indicates that tolerance has been induced within two days after treatment shown earlier for other Ig tolerogens (71). Although there is a lower overall response in the 'day two' mice, the loss of tolerance is still occurs since three of four mice in the 'day two' group made antibody by day 64 post challenge. This may also indicate the the alum depot does not feed antigen into the thymus to continue tolerance induction in maturing T cells, as has been shown by Gahring and Weigle (69).

Conclusions. The data presented above show I can induce tolerance to rabbit Fab by i.v. injection of ultracentrifuged Fab fragments of rabbit Ig specific for IgD molecules found on the surface of the majority of small, resting B cells. Tolerance to soluble protein antigens is usually described as a loss of reactivity to a normally

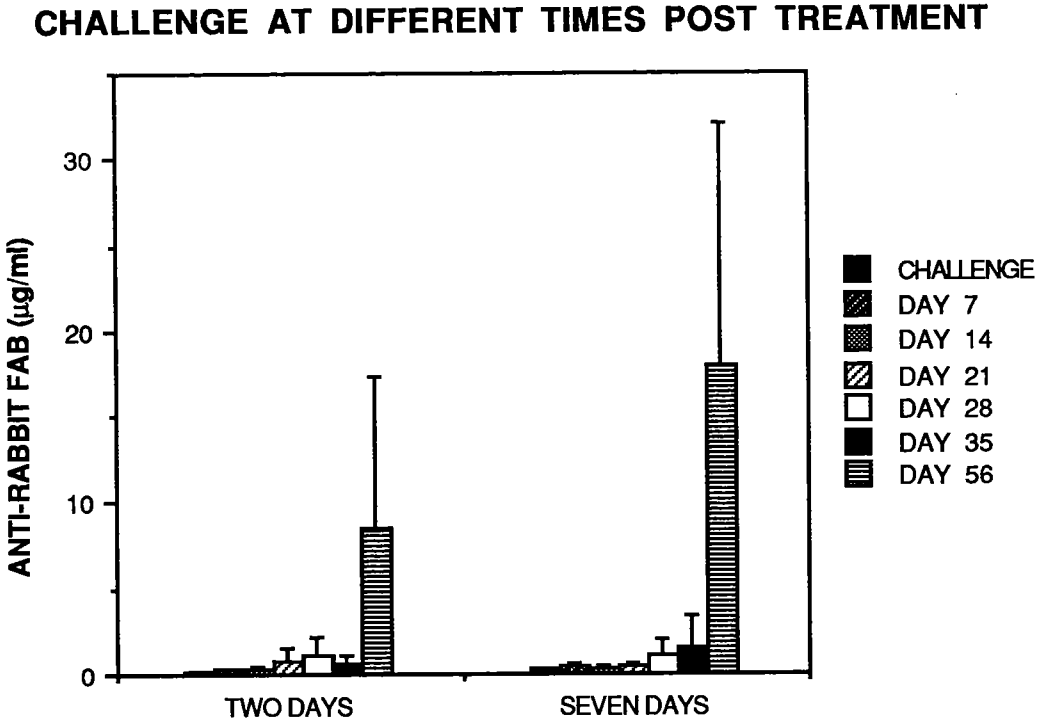


FIGURE 9. Mean serum anti-rabbit Fab response from mice treated with anti-δ and challenge on day two or day seven with 100 µg Fab NRG precipitated in alum. Mice were bled on the days post challenge indicated and anti-rabbit Fab levels measured by ELISA. Bars represent one standard deviation. Mean control responses for this experiment are shown in Table 7.

TABLE 7

CHALLENGE AT DIFFERENT TIMES POST TREATMENT

	DAY 28	DAY 35	DAY 49	DAY 56	DAY 63
#2	1.1	0.6		n.d.	n.d.
#3	0.6	1.1		21	29
#4	0.7	1.1		7.7	7.9
#5	0.1	0.1		0.1	0.1
#6	0.1	0.2		5.1	3.6
2 DAYS	**0.52**	**0.62**		**8.48**	**10.15**
SD	**0.43**	**0.48**		**8.93**	**12.97**
#11	0.3	0.2	2.1	1.8	
#12	1.9	2.9	9.2	21	
#13	0.1	0.1	0.3	2.8	
#14	1	0.9	14	26	
#15	1.4	4.7	32	39	
#16	0.3	0.2	4.1	17	
7 DAYS	**0.83**	**1.50**	**10.28**	**17.93**	
SD	**0.72**	**1.89**	**11.76**	**14.20**	
#41	80	108		190	300
#42	338	403		714	632
#43	0.4	0.6		0.5	0.6
#44	210	275		404	288
CONTROL	**157**	**197**		**327**	**305**
SD	**148**	**178**		**306**	**258**

TABLE 7 Shown are individual serum anti-rabbit Fab responses in μg/ml from mice shown in Figure 9. Groups include: Mice challenged with 100 μg Fab NRG two or seven days post anti-δ treatment or control untreated mice. Shown in bold are the averages and standard deviations for each group.
n.d.- not done.

immunogenic protein induced by administration of that protein by an alternate route, dose or form of administration (57).

One consequence of these methods of administration is that the antigen may be processed by an alternate or inappropriate APC. APCs, like MΦ, gather antigen by pinocytosis and via receptors for Fc and complement on their cell surfaces. Dendritic cells gather antigen by an unknown mechanism, but they are present in their highest numbers in tissue and not blood (38). In the case of low doses of soluble protein antigens, the small antigen specific B cells may be the only cells in the right location with the ability to pick up enough antigen to affect the T cell response. Although these cells are present at low frequency (between one in 10^4 or 10^5 for protein antigens) (110), they can pick up and present very low concentrations of antigen in vitro through antigen specific receptors (41-43). If no other APC can gather enough antigen, these few small B cells could present it over time (with a persistent or endogenous antigen) to the antigen specific T cells. In our model the concentration of anti-δ antigen is 100 μg/mouse but can be as low as 1 μg/mouse (data not shown and Figure 8 and Table 6B). The anti-δ is immediately diluted into the blood volume of the mouse, which is approximately 2.5 ml, for a final concentration of 4-40 μg/ml of anti-δ. Tony et al. (42) have shown, using in vitro antigen presentation by whole spleen cells or small B cells, that doses of less than 100 μg/ml of $F(ab)'_2$ NRG are ineffective at inducing T cell proliferation. However, as little as 0.1 μg/ml of F(ab)'2 anti-δ, presented by small B cells, could maximally

stimulate the same T cell hybridomas. Without too much extrapolation of in vitro data to in vivo results it is possible to conclude that the concentration of the Fab anti-δ injected is too low to be effectively picked up by nonspecific means.

As shown in the dose response data, in Figure 8 and Tables 6A and B, the lowest dose of Fab abs NRG was completely ineffective at inducing tolerance. At higher doses (50 and 100 μg/mouse) Fab NRG and Fab abs NRG may be able to find enough rabbit Fab specific B cells within the short lifespan of Fab fragments (105, 106) and induce tolerance.

$F(ab)'_2$ anti-δ does not induce tolerance. Activated B cells have been shown in vitro to be better APCs than small B cells by Metlay and Steinman (39). One explanation for a different functional state with activation are the cell surface molecules which are expressed after activation. One such molecule B7/BB-1 has recently been described as the ligand for the T cell activation antigen CD28 by Linsley et al (49) and Koulova et al (48). Small resting human B cells do not express the B7/BB-1 molecule, but it can be induced upon activation (48). If this is the costimulatory signal which differentiates a tolerogenic signal from an activating signal, then our evidence for prior activation of B cells would fit in with this finding.

Mechanisms of Tolerance

Tolerance in the T cell compartment has been shown to occur by at least three different mechanisms (57). Clonal deletion and anergy have both been shown to account for tolerance in the thymus (111, 112) and in the periphery (76). In addition, suppression has been described in peripheral tolerance to BSA (89) and to high doses of fowl gamma globulin (90).

B cell tolerance is usually associated with high antigen doses (greater than one mg/mouse), which results in both T and B cell tolerance (62, 64, 80, 113). With the dose of antigen I used I expected to find tolerance in the T cell compartment only.

T cell help is compromised in Fab anti-δ treated mice. To investigate the T cell compartment in anti-δ treated mice, we used a hapten carrier model. DNP is a hapten which requires carrier specific help in order to elicit an antibody response. We challenged mice treated with Fab anti-δ and control untreated mice with $F(ab)'_2$ NRG modified with DNP. As shown in Figure 10 and Table 8, these mice make less than 15% of the control anti-DNP response at day 21 post challenge. Mice treated with soluble Fab NRG (data not shown) make an intermediate anti-DNP response which is commensurate with their anti-rabbit Fab response. Anti-rabbit Fab responses for anti-δ treated and control mice are shown in Figure 11 and Table 8. This

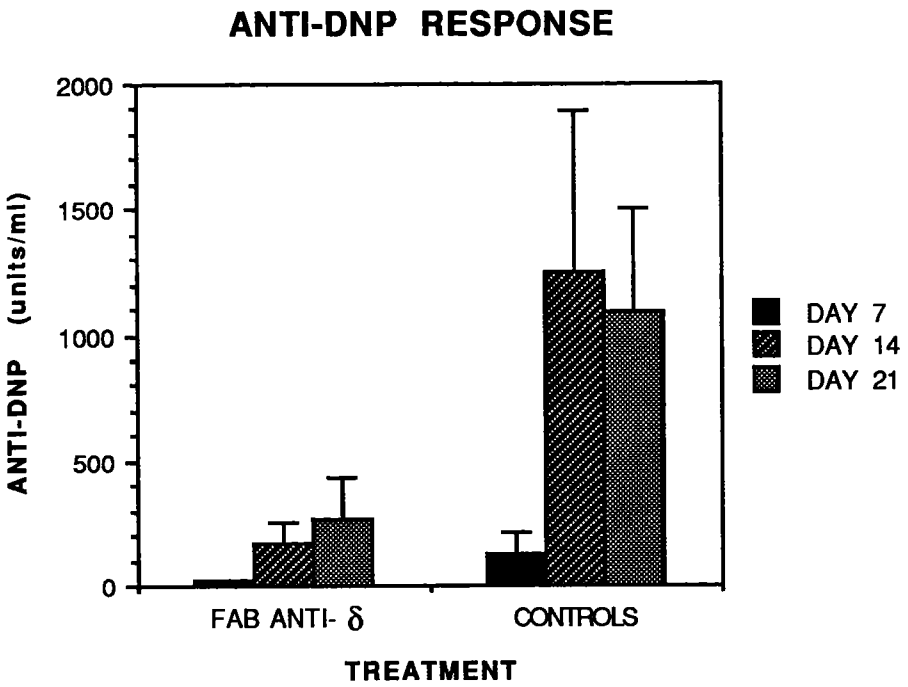


Figure 10. Top: mean anti-DNP levels in units/ml using as a standard antiserum a control (day 28 post challenge with DNP-BSA in alum) serum arbitrarily assigned 1000 units/ml. Shown are mice injected i.v. as in Figure 1 with Fab anti-δ or control untreated mice, challenged after 7 days with DNP modified $F(ab)'_2$ NRG precipitated in alum. Mice were bled weekly post challenge and antibody levels measured by ELISA. Bars represent one standard deviation. Fab anti-δ group contains 6 mice and the control group contains 3 mice.

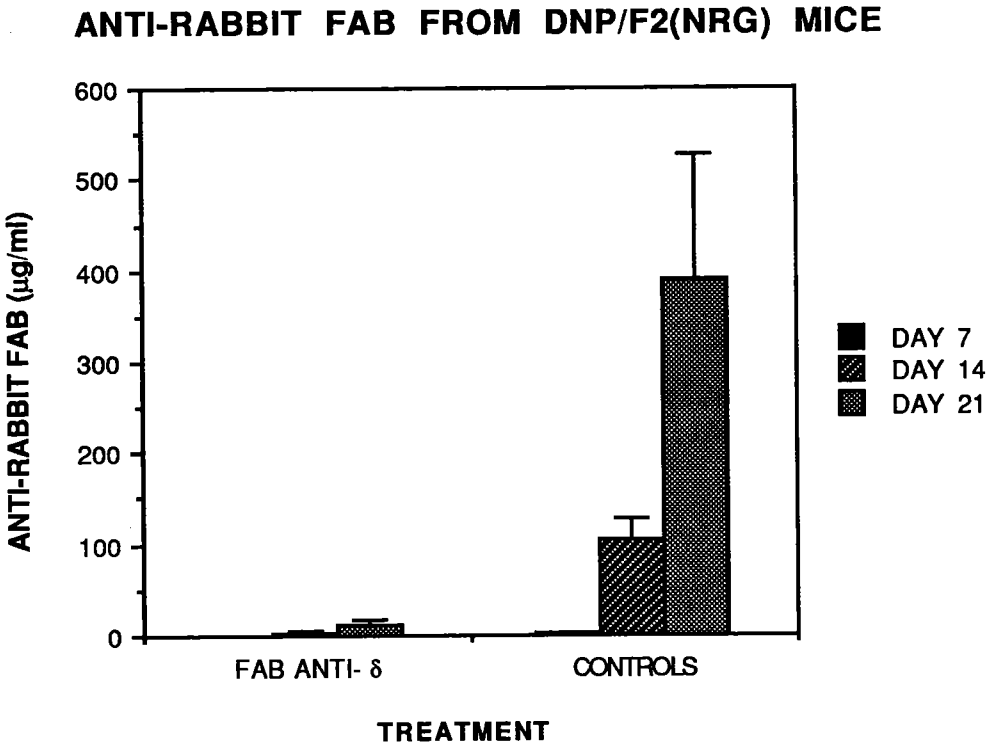


Figure 11. Mean anti-rabbit Fab responses in µg/ml for the mice shown in Figure 10.

TABLE 8

ANTI-DNP RESPONSE TO DNP-F_2(NRG)

	PRE	CH	DAY 7	DAY 14	DAY 21
#1	20	32	27	171	255
#2	13	18	21	193	274
#3	7.4	13	14	89	170
#4	8.9	11	13	310	590
#5	12	14	14	150	187
#6	7.2	15	17	94	137
ANTI-δ	**11.42**	**17.17**	**17.67**	**167.83**	**268.83**
SD	**4.83**	**7.63**	**5.43**	**81.04**	**165.66**
#41	6.4	8.3	220	1727	1220
#42	9.3	7.5	65	525	618
#43	8.9	9.7	92	1508	1416
CONT	**8.20**	**8.50**	**125.67**	**1253.33**	**1084.67**
SD	**1.57**	**1.11**	**82.80**	**640.19**	**415.86**

ANTI-RABBIT FAB RESPONSE TO DNP-F_2(NRG)

	PRE	CH	DAY 7	DAY 14	DAY 21
#1	0.2	1.1	0.9	6.5	10.8
#2	0.2	0.4	0.3	1.6	9.8
#3	0.2	0.6	0.5	1.2	4.4
#4	0.2	0.3	0.3	3.0	22.0
#5	0.2	0.3	0.3	3.2	17.0
#6	0.1	0.3	0.3	0.6	4.1
ANTI-δ	**0.2**	**0.5**	**0.4**	**2.7**	**11.4**
SD	**0.04**	**0.3**	**0.2**	**2.1**	**7.1**
#41	.03	0.1	3.5	116.0	621.0
#42	.1	0.1	3.5	77.0	248.0
#43	.1	0.1	2.1	120.0	514.0
CONT	**0.1**	**0.1**	**3.0**	**104.0**	**461.0**
SD	**0.04**	**0.0**	**0.8**	**23.8**	**192.1**

TABLE 8. Shown are individual anti-DNP responses in units/ml for the mice shown in Figure 10. Groups represented are : mice made tolerant with anti-δ or untreated control mice challenged with 100 μg DNP coupled to F(ab)'$_2$ NRG precipitated in alum.
On the bottom are shown are individual anti-rabbit Fab responses in mg/ml from mice shown in Figure 10 and in the top panel.
Shown in bold are the averages and standard deviations which are plotted in Figure 10 and 11.

data is consistent with a loss of the rabbit Fab specific helper T cell function.

To look separately at T and B cell function in these anti-δ treated mice, T or B cells from anti-δ treated BALB/c mice were transferred, before challenge, with normal B or T cells into SCID mice. The SCID recipient mice were challenged and bled weekly, and the data from this adoptive primary antibody response is presented in Figure 12 and Table 9. SCID mice which received tolerant T plus tolerant B cells, as expected, made less than one μg/ml of anti-rabbit Fab. Tolerant T plus normal B gave slightly higher anti-rabbit Fab titers which were 2% of the control response at day 21. Tolerant B plus normal T gave low anti-rabbit Fab responses which reached 34% of control responses by day 35. These data indicate that there is a loss of rabbit Fab specific helper T cell activity in the anti-δ treated mice.

The responses of individual mice in the adoptive transfer model are variable. Standard deviations are probably not the best representation of the spread of the data among these mice. The two groups of mice, in this transfer, with significant antibody responses are the normal T plus tolerant B and normal T plus normal B groups. For these sets of mice I have shown the individual data points for day 35 post-challenge directly on the figure in symbols. There is a greater degree of spread among the normal T plus normal B group with the range of antibody responses from 147 μg/ml to 1488 μg/ml.

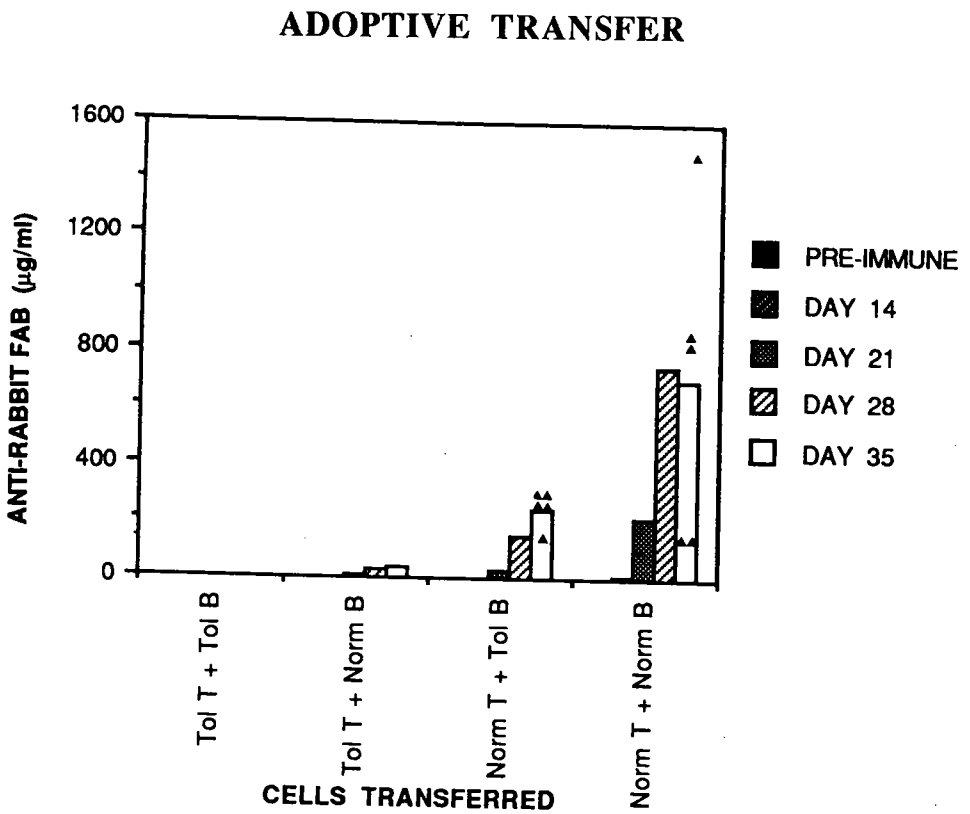


Figure 12. Mean anti-rabbit Fab response in µg/ml from SCID mice reconstituted with T and/or B cells from normal, untreated BALB/c mice or BALB/c mice injected with 100 µg Fab anti-δ as in Figure 2. Each group shown contains 5 SCID mice which received 80 x 10^6 pooled spleen B cells and 20 x 10^6 pooled lymph node T cells from either tolerant or normal mice. Mice were bled weekly and anti-rabbit Fab levels measured by ELISA. The symbols represent individual mice at d35 post challenge for the last two groups (Norm T + Tol B and Norm T + Norm B). Also included in this experiment but not shown were 3 mice which received 20 x10^6 normal T cells alone or 80 x 10^6 normal B cells alone. Neither of these groups made an anti-rabbit Fab response.

TABLE 9
ADOPTIVE TRANSFER

	PRE	DAY 14	DAY 21	DAY 28	DAY 35
#11	0.1	0.9	1.4	1.7	2.1
#12	0.07	0.5	0.5	0.7	0.6
#13	0.1	0.9	1	2.3	1.9
#14	0.05	1.3	1.7	5.7	17
#15	0.1	0.9	1.4	1.3	1.1
TOL T+ TOL B	**0.08**	**0.90**	**1.20**	**2.34**	**4.54**
SD	**0.02**	**0.28**	**0.46**	**1.97**	**6.99**
#21	0.1	11	131	720	819
#22	0.1	9.3	232	952	852
#23	0.2	1.6	7.4	83	147
#24	0.2	18	606	1779	1488
#25	0.1	9.4	70	159	162
NORM T + B	**0.14**	**9.86**	**209.28**	**738.60**	**693.60**
SD	**0.05**	**5.84**	**236.74**	**688.13**	**559.74**
#31	0.05	0.08	0.1	0.3	0.3
#32	0.1	0.2	1.9	16	31
#33	0.06	7.4	17	36	93
#34	0.1	0.2	0.4	1.9	3.1
T ONLY	**0.08**	**1.97**	**4.85**	**13.55**	**31.85**
SD	**0.03**	**3.62**	**8.14**	**16.55**	**43.06**
#41	0.05	0.2	0.2	0.2	0.2
#42	0.1	0.3	0.4	0.5	0.5
#43	0.04	0.2	0.2	0.5	0.9
#44	0.1	0.5	0.4	0.5	0.6
B ONLY	**0.07**	**0.30**	**0.30**	**0.43**	**0.55**
SD	**0.03**	**0.14**	**0.12**	**0.15**	**0.29**
#51	0.05	0.6	0.4	0.5	0.4
#52	0.2	1.1	5.8	34	59
#53	0.05	1.5	5.7	11	19
#2	0.1	0.7	0.9	13	34
#4	0.06	3.3	14	77	95
TOL T/NOR B	**0.09**	**1.44**	**5.36**	**27.10**	**41.48**
SD	**0.06**	**1.10**	**5.46**	**30.43**	**36.81**
#61	0.1	3	8.8	77	160
#62	0.06	2.3	31	229	287
#64	0.1	3.1	34	134	233
#5	0.1	3.9	21	106	234
#6	0.1	3	33	204	264
TOL B/NOR T	**0.09**	**3.06**	**25.56**	**150.00**	**235.60**
SD	**0.02**	**0.57**	**10.70**	**64.57**	**47.89**

TABLE 9. Individual serum anti-rabbit Fab response in μg/ml from SCID mice reconstituted with T and/or B cells from normal and anti-δ tolerant Balb/c mice as shown in Figure 12. Also shown are mice which received 20 x 10^6 normal T cells only or 80 x 10^6 normal B cells only. Mice were bled weekly at the times indicated post challenge and anti-rabbit Fab levels measured by ELISA. Shown in bold are the averages and standard deviations plotted in Figure 12.

Even though the responses of the normal T plus tolerant B group fall within the range of the normal T plus normal B group, there is an apparent decrease in the responses generated using B cells from tolerant mice with normal T cells, indicating that there may also be some effect in the B cell compartment. B cells from tolerant mice plus normal T cells transferred into SCID mice made approximately one-third of the mean response of mice which received normal B plus normal T cells. This response was delayed and did not reach the control response by the termination of the experiment (day 35). This delayed response may be due to recovery of B cell function, or the clonal expansion of a smaller number of responsive B cells.

As a control for reconstitution these mice were also challenged with chicken Ig and then tested for anti-chicken Ig antibodies at day seven and day fourteen post challenge. All four groups shown made similar levels of anti-chicken Ig, shown in Figure 13 and Table 10. This indicates again that there is no effect of Fab anti-δ on the chicken Ig specific B cell population. SCID mice which received normal T cells only or normal B cells only followed by challenge as above did not make anti-rabbit Fab or anti-chicken Ig antibodies indicating that the mice did not have sufficient T or B cells of their own to mount an immune response and that the level of T and B cell contamination in the transferred cell preparations was not a factor in the responses.

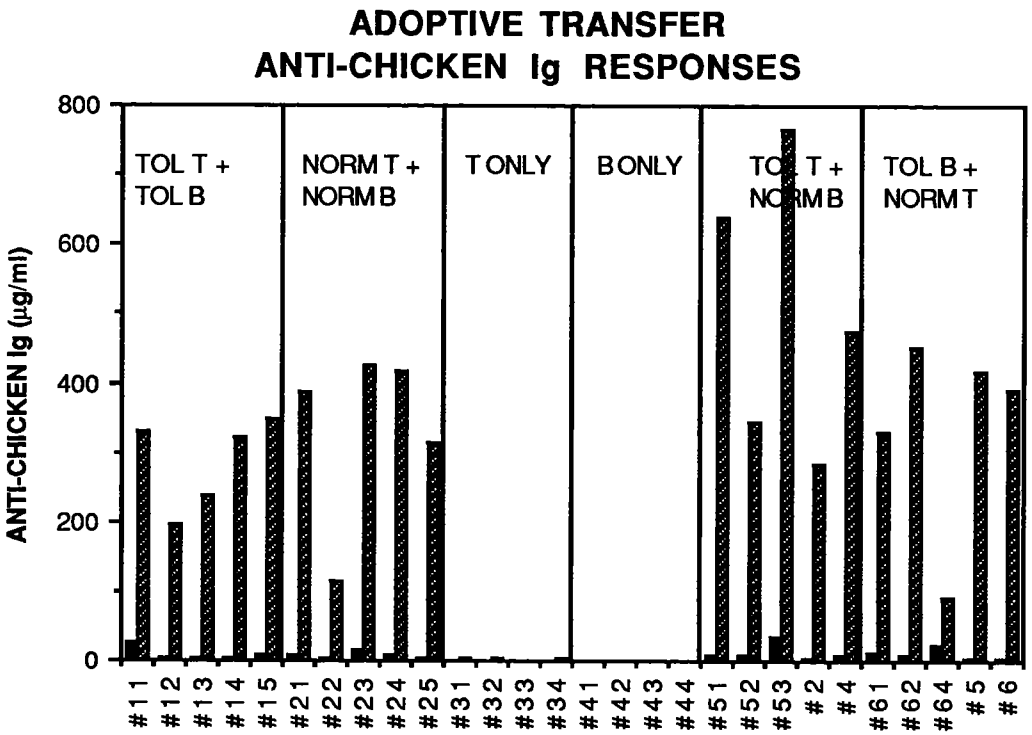


FIGURE 13. Individual serum anti-chicken Ig responses in µg/ml from the mice shown in Figure 12.

TABLE 10
ADOPTIVE TRANSFER- CHICKEN Ig RESPONSE

	DAY 7	DAY 14
TOL T + B		
#11	25	328
#12	3.8	194
#13	4.4	236
#14	3.5	323
#15	9.3	349
NORM T + B		
#21	5.9	387
#22	3.3	114
#23	16	425
#24	8.7	417
#25	5.5	314
T ONLY		
#31	0.004	2.8
#32	0.004	2.1
#33	0.02	0.1
#34	0.1	5.2
B ONLY		
#41	0.4	0.6
#42	0.5	0.5
#43	1.1	1
#44	0.8	1.1
TOLT/NOR B		
#51	5.9	641
#52	7.6	346
#53	35	767
#2	4.6	282
#4	6.6	474
TOLB/NOR T		
#61	11	330
#62	9.4	452
#64	23	91
#5	4.2	417
#6	4.7	390

TABLE 10. Individual serum anti chicken Ig responses in μg/ml from the mice shown in Figure 12 and Figure 13.

For the rabbit Fab specific B cells the interaction with Fab anti-δ would be a cross-linking interaction, since both Ig molecules (rabbit and mouse) recognize each other, and both the IgM and IgD molecules may be involved with the same Fab fragment. Therefore, the antigen specific B cells could get a signal through their antigen receptors in the absence of T cell help. Goodnow et al (80) have shown using antibody transgenic mice that transfer of mature antigen specific B cells into antigen bearing, T cell tolerant mice results in B cell anergy. Also, B cell tolerance may require the involvement of sIgM which can occur only in the rabbit Fab specific B cell population. This rabbit Fab B cell defect accounts for only a small part of the very profound loss of the anti-rabbit Fab response in mice treated with anti-δ.

In vitro proliferative responses of anti-δ versus control mice.

Treatment of mice with Fab anti-δ results in an inability of these mice to generate an antibody response to rabbit Fab. This lack of an antibody response is only partly due to lowered rabbit Fab specific B cells function. I have shown in the previous section that there is a lack of Fab specific T cell help in Fab anti-δ treated mice. Antigen presentation by B cells has been proposed to alter the type of T cell that is activated (95, 96, 114). We may have skewed the response of the T cells toward a completely cellular response. There is evidence that some tolerance protocols spare DTH like responses and ablate antibody production (92, 115). Whiteley et al. found, in tolerant

animals, a proliferating population of T cells which was unable to help antibody production (116). It is possible that the tolerance induced was merely the change from activation of T cells able to help antibody production to a proliferating population of non-helper T cells. These could be cells which mediated DTH like responses or a suppressor population.

To look at the T cell response in anti-δ tolerant mice, I set up in vitro proliferation experiments. Although none of these experiments is conclusive, the cumulative data show occasional, but much reduced responses of anti-δ tolerant mice to rabbit Fab. I had a great deal of difficulty in getting normal alum or CFA primed mice to make consistent responses to rabbit Fab in vitro. With most of the conditions I used, which gave responses, there was a very high background which was between 40-60% of the total response. I used as media RPMI with FCS or normal mouse serum as well as a hybridoma media HL-1 (Ventrex Laboratories, Portland,ME) with no added serum. I varied the cell number from 10^4-$3x10^6$ cells/well, and I used a variety of incubation times from three to six days. I also tried to reduce the background responses by isolating T cells from the spleen or lymph nodes and plating them with mitomycin c treated unprimed spleen cells as APCs. The most consistent results were obtained with RPMI with 10% FCS added using whole spleen or lymph node and incubating for 72-96 hours.

Shown in Figures 14 and 15 and Table 11 are data from two experiments using whole spleen from alum challenged animals and

draining LN from CFA primed animals. These two types of lymphoid organs gave the most consistent responses for the adjuvants used. The opposite combinations (LN from alum primed mice and spleen from CFA primed mice) gave similar results to those shown, when there was a proliferative response in the normal mice. Other proliferation experiments were done using B cell depleted (on anti-Ig coated plates) spleen or LNs with mitomycin c treated unprimed spleen as a source of APC. In these experiments the levels of proliferation are lower but the overall pattern of the response is the same. There is a consistent low level of proliferation in the anti-δ tolerant cells. This proliferation is very low or absent in the alum primed mice and greater in the CFA primed mice.

There is no evidence that I have activated a proliferating population of cells which do not help antibody production. There is very little antigen specific proliferation in the tolerant mice.

Adjuvants. Although most of our tolerance induction has been tested with alum precipitated challenge, we wanted to know if mice were still tolerant after challenge with other adjuvants since different adjuvants may stimulate different subsets of T cells (117, 118). We treated animals with Fab anti-δ and then after seven days challenged them with 100 μg Fab NRG either precipitated in alum, or precipitated in alum plus 2 x 10^8 *B. pertussis* (both injected i.p.), or emulsified in CFA and injected s.c. at the base of the tail. As shown in Figure 16 and Table 12, the alum challenged animals with or

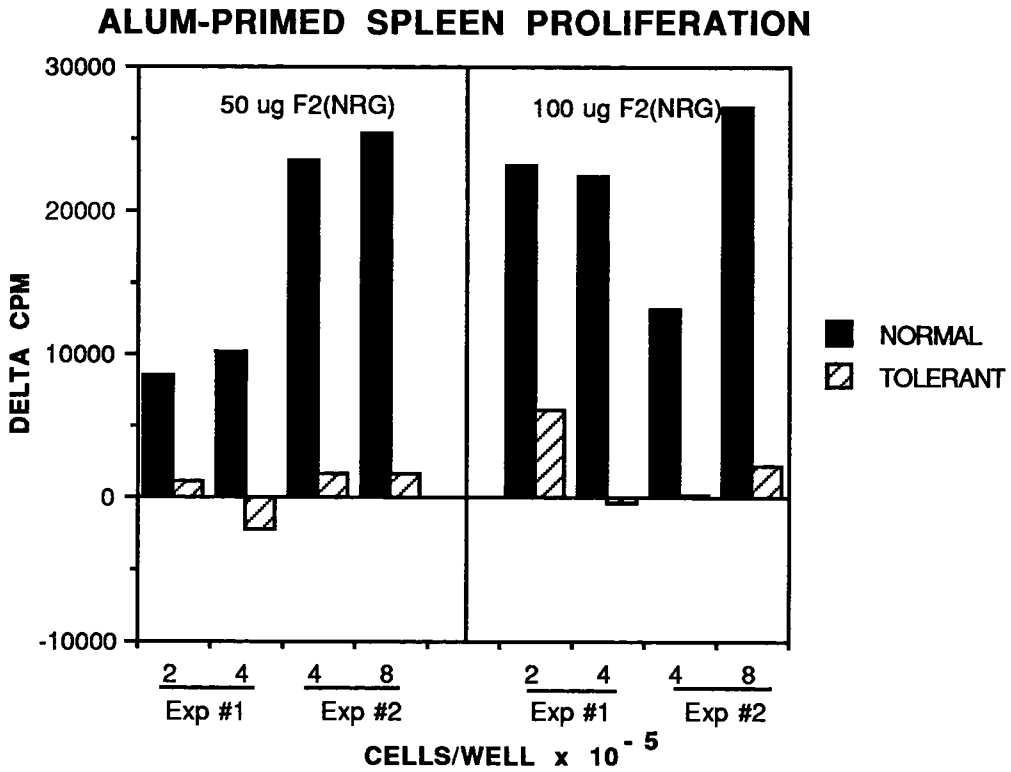


FIGURE 14. Two experiments showing in vitro proliferation as measured by cpm of whole spleen cells from alum primed mice. This figure compares normal mice (black) to mice made tolerant (hatched) by i.v. treatment with 100 μg Fab anti-δ seven days before challenge with 100 μg Fab NRG precipitated in alum. After nine days, spleens were removed and whole cells were plated at the concentrations shown with 50 (on the left) or 100 (on the right) μg F(ab)'2 NRG/ml added. The cultures were incubated for 96 hours and pulsed for the last six hours.

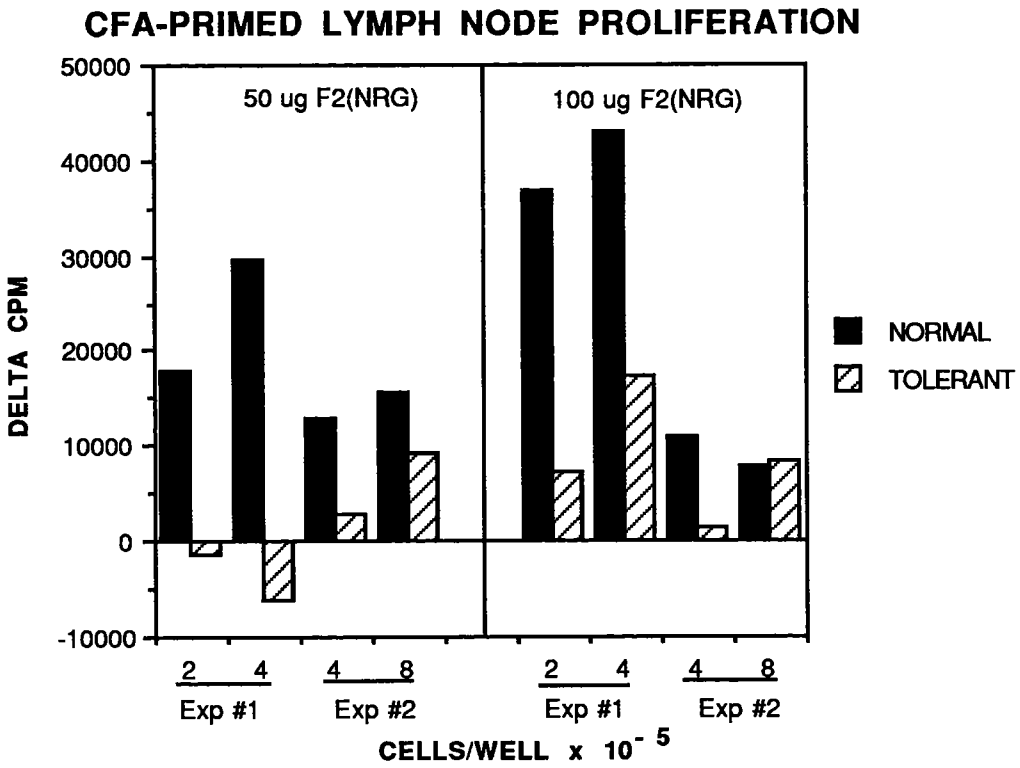


Figure 15. Two experiments showing in vitro proliferation of whole LN from CFA primed mice. Comparing normal mice (black) to mice made tolerant (hatched) as described in the previous figure. These mice were challenged with CFA s.c. All other conditions were the same as in the previous figure except that the responses of Exp#1 were done at 72 hours instead of 96 hours.

TABLE 11

IN VITRO PROLIFERATION

EXP/ CELL#/AG	NORM AG	NORM NONE	DELTA NORM	TOL AG	TOL NONE	DELTA TOL
			ALUM	SP		
#1/2/50	22205	13642	**8563**	10197	9178	**1019**
#1/4/50	45142	35028	**10114**	21890	24199	**-2309**
#2/4/50	38677	15169	**23508**	14202	12592	**1610**
#2/8/50	46877	21458	**25419**	29721	28080	**1641**
#1/2/100	36868	13642	**23226**	15219	9178	**6041**
#1/4/100	57410	35028	**22382**	23845	24199	**-354**
#2/4/100	28241	15169	**13072**	12830	12592	**238**
#2/8/100	48707	21458	**27249**	30330	28080	**2250**
			CFA LN			
#1/2/50	28232	10332	**17900**	10888	12240	**-1352**
#1/4/50	63983	34385	**29598**	20998	27218	**-6220**
#2/4/50	18083	5226	**12857**	4312	1474	**2838**
#2/8/50	26210	10711	**15499**	16891	7772	**9119**
#1/2/100	47280	10332	**36948**	19470	12240	**7230**
#1/4/100	77267	34385	**42882**	44520	27218	**17302**
#2/4/100	16205	5226	**10979**	2811	1474	**1337**
#2/8/100	18640	10711	**7929**	16095	7772	**8323**

TABLE 11. Proliferation in cpm of two experiments. Shown at the top are whole spleens from normal (untreated) or tolerant (anti-δ treated) mice, challenged with 100 μg Fab NRG precipitated in alum, with and without antigen (AG/NONE) and the Delta cpm (Normal/Tolerant). On the bottom are cpm from whole LN in groups as above but challenged with CFA s.c. In column 1 are listed: Experiment # (1 or 2) Cell # (2,4,or 8 $x10^5$ cells), and antigen concentration (50 or 100 μg $F(ab)'_2$ NRG). Shown in bold are the delta cpm which are plotted in Figures 14 and 15. All data are from cultures incubated for 96 hours except LN from experiment #1 which are at 72 hours.

without *B. pertussis* remain tolerant. CFA partially breaks tolerance, since anti-δ treated mice challenged with CFA make 18% of the mean control response (1156 μg/ml) at day 35 post challenge. This is consistent with the proliferative response of CFA primed lymph nodes from anti-δ treated mice shown above.

Since murine helper T cell subsets, which induce switching to different Ig isotypes, have been defined in vitro (119), we were interested to see if there were a different spectrum of isotypes generated in the residual antibody response of tolerant mice versus the controls. Individual isotype responses, from the sera shown in Figure 16, were measured by ELISA with goat anti-mouse isotype specific antibodies. Antibody concentrations were calculated using mouse anti-ars antibodies of different isotypes as standards. The data are presented, in Figure 17 and Table 13, as a percentage of the whole response, since the anti-δ treated mice make little antibody at this time (day 35 post challenge). There is no major difference in the isotypes of anti-rabbit Fab made in the anti-δ treated mice. Although there are differences in the isotypes produced in mice challenged with different adjuvants, the predominant isotype in all groups (whether control or anti-δ treated) was IgG1. We also tested the mice shown in Figures 2 and 6 and found no differences in the Ig isotypes produced: over 90% of the response at day 35 post challenge was IgG1 in all groups (data not shown).

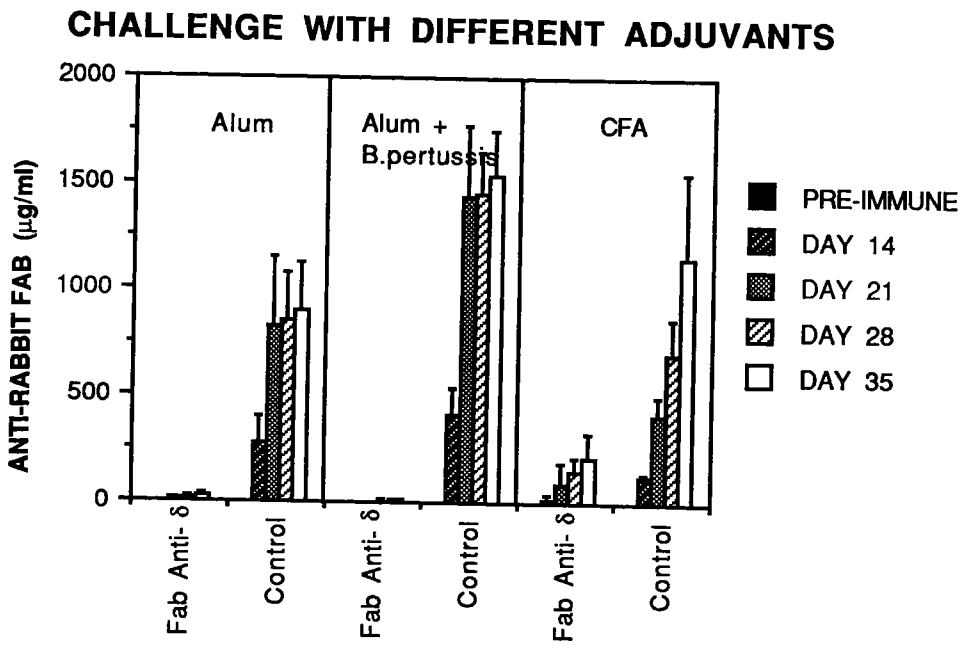


Figure 16. Mean serum anti-rabbit Fab antibody response in µg/ml from mice treated with Fab anti-δ as in Figure 1 and then challenged after 7 days with 100 µg Fab NRG either precipitated in alum alone, or with 2 x 10^8 *B. pertussis* i.p., or emulsified in CFA s.c. All anti-δ groups contain 6 mice and all control groups contain 4 mice each. Bars represent one standard deviation.

TABLE 12

CHALLENGE WITH DIFFERENT ADJUVANTS

	PRE	DAY 14	DAY 21	DAY 28	DAY 35
#1	0.7	0.7	1	1.7	0.7
#2	0.3	2.9	15	33	45
#3	0.2	1.4	6.6	14	21
#4	0.2	0.8	2.8	11	14
#5	0.3	3.4	4.4	7.4	15
#6	0.5	3.4	17	28	43
ALUM/δ	**0.37**	**2.10**	**7.80**	**15.85**	**23.12**
SD	**0.20**	**1.28**	**6.64**	**12.17**	**17.49**
#11	0.3	1.1	0.8	1.3	2.8
#12	0.2	0.3	0.3	0.5	0.9
#13	0.3	0.4	0.4	0.9	1.5
#14	0.2	1.1	2	7.9	27
#15	0.3	1.1	3	6.3	8.1
#16	0.2	1.3	1.7	2.3	3.1
PERT/δ	**0.25**	**0.88**	**1.37**	**3.20**	**7.23**
SD	**0.05**	**0.42**	**1.06**	**3.12**	**10.01**
#21	0.3	7	52	78	110
#22	0.3	60	272	168	114
#23	0.2	5.6	50	196	392
#24	0.2	1.1	53	205	231
#26	0.2	1.2	18	91	202
CFA/ δ	**0.24**	**14.98**	**89.00**	**147.60**	**209.80**
SD	**0.05**	**25.30**	**103.34**	**59.37**	**114.94**
#31	0.3	261	789	943	985
#32	0.2	441	1276	1122	1161
#33	0.3	142	501	636	807
#34	0.2	240	749	697	624
ALUM/CONT	**0.25**	**271.00**	**828.75**	**849.50**	**894.25**
SD	**0.06**	**124.64**	**324.24**	**224.97**	**230.97**
#41	0.3	550	1234	1433	1440
#42	0.2	473	1605	1611	1824
#43	0.4	360	1824	1582	1556
#44	0.2	259	1097	1152	1317
PERT/CONT	**0.28**	**410.50**	**1440.00**	**1444.50**	**1534.25**
SD	**0.10**	**127.63**	**334.05**	**210.01**	**216.42**
#51	0.2	103	357	494	909
#52	0.1	142	378	840	1712
#53	0.2	125	400	679	1149
#54	0.1	119	535	807	854
CFA/CONT	**0.15**	**122.25**	**417.50**	**705.00**	**1156.00**
SD	**0.06**	**16.11**	**80.28**	**156.87**	**392.17**

TABLE 12. Individual anti-rabbit Fab responses in μg/ml from the mice shown in Figure 16. Groups represented are: Mice challenged with 100 μg Fab NRG in alum alone, in alum plus 2 x10^8 *B. pertussis*, or emulsified in CFA. The top three groups have been treated with anti-δ as described before challenge and the bottom groups are uninjected controls. Shown in bold are the averages and standard deviation of each group, which are plotted in Figure 16.

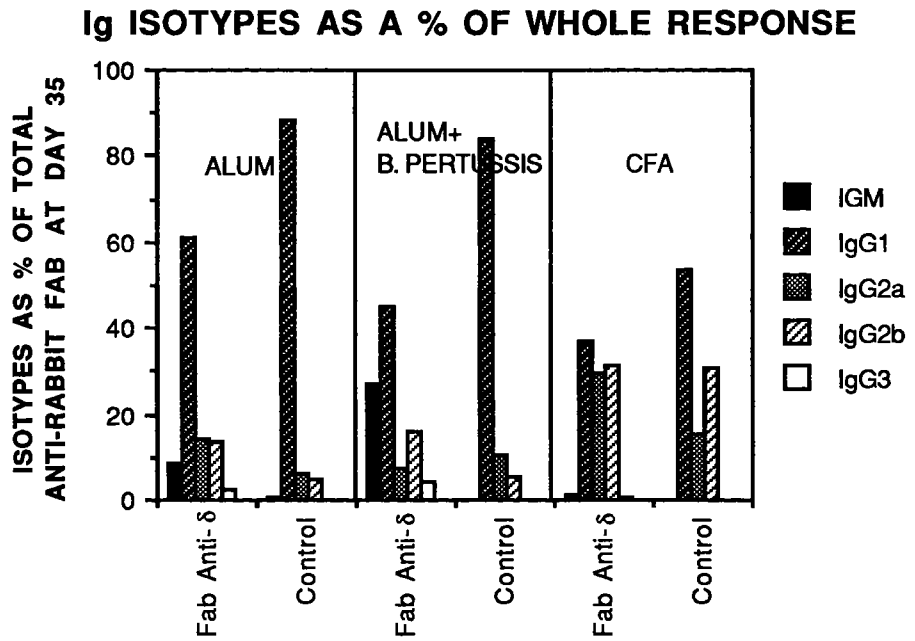


Figure 17. Mean serum anti-rabbit Fab antibody response by isotype as a percentage of the whole response at day 35 post challenge from mice shown in Figure 16. Sera were tested in ELISA using peroxidase coupled goat anti-mouse isotype antibodies.

TABLE 13

ISOTYPES GENERATED IN ADJUVANT CHALLENGED MICE

	MU	G1	G2A	G2B	G3	TOTAL
TOL/ALUM	3.43	24.72	5.7	5.45	0.97	40.27
TOL/PERT	5.07	8.37	1.43	3.05	0.78	18.7
TOL/CFA	5.82	200.67	209.5	515.33	2.33	933.65
CONT/ALUM	7.43	875	58.75	45.75	2.18	989.11
CONT/PERT	3.95	1237.75	153.25	79.25	3.05	1477.25
CONT/CFA	3.8	967.75	274	552.75	2.78	1801.08

	MU%	G1%	G2A%	G2B%	G3%	TOTAL
TOL/ALUM	8.52	61.39	14.15	13.5	2.4	99.96
TOL/PERT	27.1	44.76	7.65	16.31	4.17	99.99
TOL/CFA	0.62	21.49	22.44	55.2	0.25	100
CONT/ALUM	0.75	88.46	5.94	4.63	0.22	100
CONT/PERT	0.27	83.79	10.37	5.36	0.21	100
CONT/CFA	0.21	53.73	15.21	30.69	0.15	99.99

TABLE 13 The top panel contains the averages of anti-rabbit Fab responses by isotype in μg/ml of the mice shown in Figure 16 and Table 12. Each group contains 6 mice except Tol/CFA which has five mice. The bottom of the table contains the response as a percentage of the whole. These are plotted in Figure 17.

Mixing tolerant T cells with normal cells does not suppress the anti-rabbit Fab response. Suppression has been shown to be a mechanism for some examples of tolerance to protein antigens (89, 90). To look for suppression in the anti-δ tolerant mice, I mixed normal unprimed cells with tolerant T cells in the SCID adoptive transfer system. First, I did a titration of the T cells required for a consistent response to Fab NRG in alum plus *B. pertussis* in SCID mice with 80 x 10^6 B cells as a constant. As shown in Figure 18 and Table 14, using 16 x 10^6 normal T cells per mouse there is a consistent response in the SCID mice. I chose the lowest number of cells required to get a consistent response, since I wanted to maximize the effect of any potential suppressor cells in the tolerant T cell populations.

I had some concern that doubling the number of T cells without adding any more antigen-specific cells might lower the anti-rabbit Fab response even in the absence of any specific suppression. I wanted to have a control for that effect. Since this was a primary adoptive transfer I could not use normal cells as a control. I chose to use T cells from mice tolerant of rabbit Fab which have been reported not to use suppression as the mechanism. Oki and Sercarz (91) have shown that mice made tolerant to lysozyme by injection at birth with antigen in IFA are tolerant without suppression. So I used this as a method of making a population of T cells unresponsive for rabbit Fab, using a different mechanism. The presence of antigen from birth has not been reported to utilize suppression as a

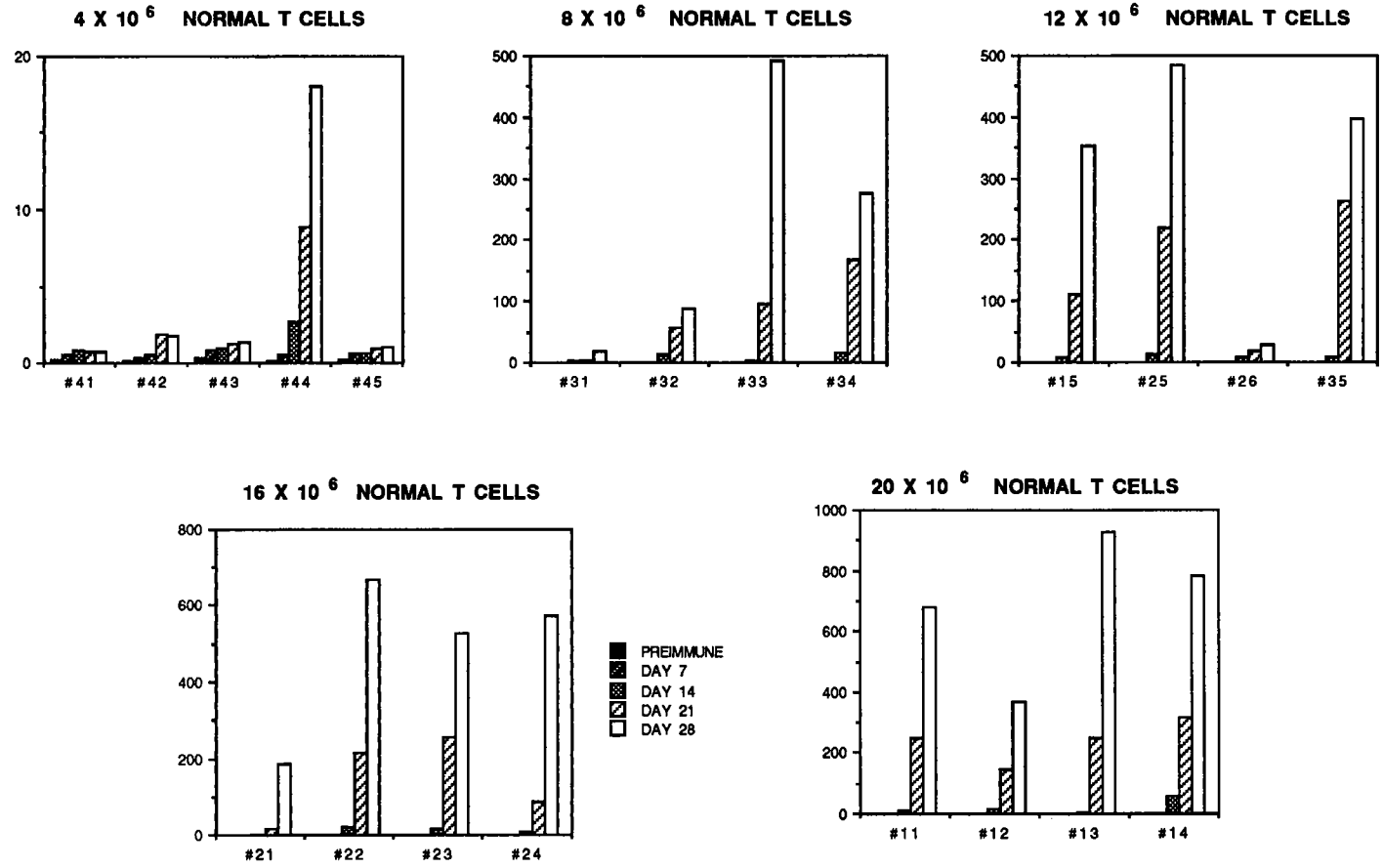


Figure 18. Individual serum anti-rabbit Fab response in μg/ml from SCID mice reconstituted with different numbers of normal T cells and a constant number (80 x 10^6) of normal B cells. Mice were challenged after two days with 100 mg Fab NRG plus 20 mg CGG precipitated in alum plus 2 x 10^8 B. pertussis. Mice were bled at the indicated times post challenge and anti-rabbit Fab measured by ELISA.

TABLE 14

ADOPTIVE TRANSFER - T CELL TITRATION

	PRE	DAY 7	DAY 14	DAY 21	DAY 28
#11	0.1	1.1	8.1	247	678
#12	0.1	0.7	13	145	370
#13	0.2	0.6	5.5	249	927
#14	0.1	0.8	57	317	780
TWENTY	**0.13**	**0.80**	**20.90**	**239.50**	**688.75**
SD	**0.05**	**0.22**	**24.27**	**70.91**	**235.80**
#21	0.1	0.4	1.4	17	186
#22	0.06	2	19	214	670
#23	0.1	0.4	16	257	528
#24	0.09	0.6	8.8	85	573
SIXTEEN	**0.09**	**0.85**	**11.30**	**143.25**	**489.25**
SD	**0.02**	**0.77**	**7.87**	**111.47**	**210.67**
#15	0.2	0.5	8.5	111	352
#25	0.1	0.3	13	219	484
#26	0.2	0.5	7.5	19	28
#35	0.2	0.5	8.8	263	396
TWELVE	**0.18**	**0.45**	**9.45**	**153.00**	**315.00**
SD	**0.05**	**0.10**	**2.43**	**109.81**	**199.05**
#31	0.2	0.7	1.8	2.5	17
#32	0.2	0.9	13	57	87
#33	0.3	0.7	2.4	95	493
#34	0.1	0.4	15	167	276
EIGHT	**0.20**	**0.68**	**8.05**	**80.38**	**218.25**
SD	**0.08**	**0.21**	**6.92**	**69.11**	**213.35**
#41	0.2	0.5	0.8	0.7	0.7
#42	0.08	0.3	0.5	1.9	1.8
#43	0.3	0.8	0.9	1.2	1.3
#44	0.09	0.5	2.7	8.9	18
#45	0.2	0.6	0.6	0.9	1
FOUR	**0.17**	**0.55**	**1.18**	**3.23**	**5.53**
SD	**0.10**	**0.21**	**1.03**	**3.81**	**8.32**

TABLE 14. Shown are individual anti-rabbit Fab responses in μg/ml from the mice shown in Figure 18. Groups shown are SCID mice receiving 20, 16, 12, 8, or 4 x 10^6 Normal T cells plus 80 x 10^6 Normal B cells. Two days after transfer the mice were challenged with 100 μg Fab NRG precipitated in alum with 2 x 108 *B. pertussis* added. Shown in bold are the averages and standard deviations of each group.

mechanism, and when antigens are present continuously the mechanism is likely to be by clonal deletion.

I made mice tolerant by two mechanisms. One group I injected CB.17 (male and female) mice with 500 μg Fab NRG emulsified in IFA i.p. within 24 hrs of birth. These mice are labelled neonatal tolerant. When these mice were 7 weeks old they were all tested for anti-rabbit Fab antibodies, and those mice making any antibody were removed (four of eighteen). In a control experiment, I took two mice which were negative for antibody at 7 weeks and challenged them with 100 μg Fab NRG in alum and these mice made no anti-rabbit Fab through day 30 post challenge (data not shown). The second group of mice, CB.17 (male and female) mice at eight weeks of age, were injected i.v. with 100 μg ultracentrifuged Fab anti-δ. These are labelled anti-δ tolerant.

At nine weeks of age all the tolerant mice plus normal mice were sacrificed and the T and B cell fractions were pooled and isolated as in the previous adoptive transfer. Cells were transferred into SCID mice (male and female) in the pattern shown in Table 15.

As shown in Figure 19 and Tables 16 and 17 there was no evidence of suppression in either the mixed neonatal plus normal T plus normal B cell group or the mixed anti-δ T plus normal T plus normal B cell group. The responses were variable within the groups of 5-6 mice but were consistent between the groups. In the normal T plus normal B group, there were two of six mice with very low responses (less than 10 μg/ml), three mice with intermediate

TABLE 15

MIXING ADOPTIVE TRANSFER CELL DISTRIBUTION

GROUP	ANTI-δ T CELLS	NEO T CELLS	NORMAL T CELLS	NORMAL B CELLS
B only				80×10^6
Norm T+B			16×10^6	80×10^6
Neo T		16×10^6		80×10^6
Anti-δ T	16×10^6			80×10^6
Neo Mix		16×10^6	16×10^6	80×10^6
δ Mix	16×10^6		16×10^6	80×10^6

responses (between 10 and and 70 μg/ml) and one high mouse (greater than 70 μg/ml). This pattern was true for all three groups. These individual mice are shown in Figures 20 and Tables 16 and 17.

Because of the variability of the individual responses in the normal T plus normal B control group, it is not possible to state conclusively that there is no suppression in this system. One potential reason for this variability as compared with the cell dose data shown in Figure 18 is that I neglected to add *B. pertussis* to the antigen at the time of challenge. However, this experiment shows that suppression cannot account for the lack of response in the anti-δ T plus normal B group, since there was no breakthrough as in the tolerant T plus normal T group.

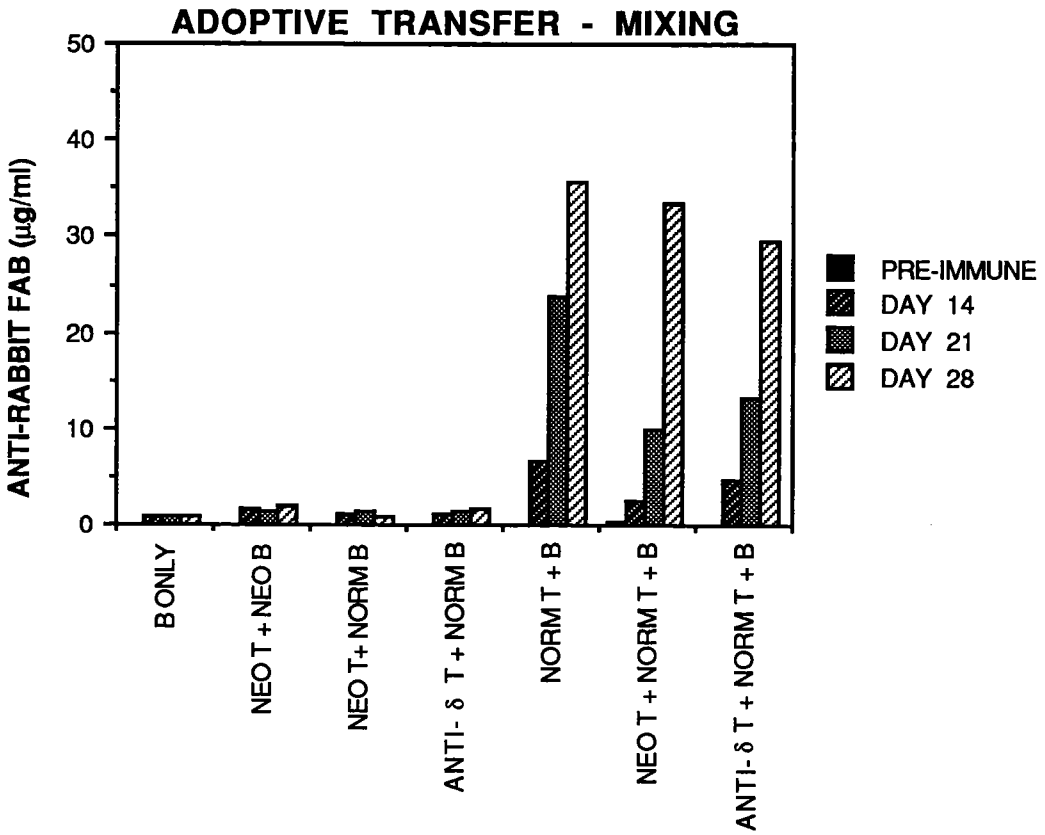


FIGURE 19. Mean anti-rabbit Fab responses in µg/ml from SCID mice reconstituted with Normal, Neonatal tolerant, or anti-δ tolerant T cells plus Normal B cells. Groups contain six mice each except Neo T plus Neo B (4) and Neo T plus Normal B (5). Cell numbers and mixtures are described in the text.

TABLE 16

ADOPTIVE TRANSFER MIXING

	PRE	DAY 14	DAY 21	DAY 28
#1	0.1	2.2	2.2	0.9
#2	0.1	1.7	1.8	12
#3	0.1	1.1	2	5.1
#11	0.2	24	111	138
#12	0.1	9.7	23	47
#13	0.1	1.5	2.1	11
NORM	**0.12**	**6.70**	**23.68**	**35.67**
SD	**0.04**	**9.08**	**43.59**	**52.75**
#24	0.1	2.3	27	105
#25	0.2	1.5	15	56
#26	0.1	5.3	2.8	4.8
#54	0.5	1.9	5	11
#55	0.1	2.6	1.5	3.2
#56	0.2	1.7	7.9	21
NEO MIX	**0.20**	**2.55**	**9.87**	**33.50**
SD	**0.15**	**1.41**	**9.67**	**40.06**
#61	0.1	1	1.7	16
#62	0.1	1.8	2.1	2.3
#63	0.1	2.6	1.9	4.2
#64	0.1	3.1	8.1	32
#7	0.3	16	51	79
#33	0.1	4.1	15	43
ANTI-δ MIX	**0.13**	**4.77**	**13.30**	**29.42**
SD	**0.08**	**5.61**	**19.19**	**28.99**

TABLE 16. Individual anti-rabbit Fab responses in μg/ml from SCID mice reconstituted with CB.17 T and B cells and shown in Figure 19. All mice received 80 x 10^6 normal B and T cells: Norm- 16 x 10^6 normal T cells, Neo Mix- 16 x 10^6 neonatal T plus 16 x 10^6 normal T cells, Anti-δ Mix- 16 x 10^6 anti-δ T plus 16 x 10^6 normal T. Shown in bold are the averages and standard deviations which are plotted in Figure 19.

TABLE 17

ADOPTIVE TRANSFER MIXING CONTROLS

	PRE	DAY 14	DAY 21	DAY 28
#4	0.1	1.3	1	0.9
#5	0.1	0.9	0.5	0.4
#6	0.1	0.8	0.9	0.8
#14	0.1	0.4	0.6	0.7
#15	0.1	0.8	1.2	1.1
#16	0.1	0.5	0.5	0.5
B ONLY	**0.10**	**0.78**	**0.78**	**0.73**
SD	**0.00**	**0.32**	**0.29**	**0.26**
#21	0.1	0.9	1.9	0.9
#22	0.1	2.6	2.4	1.6
#51	0.1	0.8	0.6	0.7
#52	0.1	0.7	0.8	0.9
#53	0.1	0.8	0.7	0.5
NEO T	**0.10**	**1.16**	**1.28**	**0.92**
SD	**0.00**	**0.81**	**0.82**	**0.41**
#31	0.1	0.7	0.9	0.8
#32	0.1	1.1	0.9	0.9
#34	0.1	3.2	2.1	5.1
#36	0.1	1.5	1.4	0.6
NEO T+B	**0.10**	**1.63**	**1.33**	**1.85**
SD	**0.00**	**1.10**	**0.57**	**2.17**
#37	0.1	0.8	0.5	0.5
#41	0.1	0.6	0.8	0.9
#42	0.1	2	2.7	4.3
#44	0.1	1.4	1.5	1.1
#45	0.1	0.7	0.8	0.8
#46	0.1	1.3	1	14
ANTI-δ	**0.10**	**1.10**	**1.26**	**1.52**
SD	**0.00**	**0.54**	**0.80**	**5.28**

TABLE 17. Individual serum anti-rabbit Fab response in μg/ml of SCID mice reconstituted with normal CB.17, neonatal tolerant, or anti-δ tolerant T and/or B cells. B only- 80 x 10^6 normal B cells only, Neo T- 16 x 10^6 neonatal T plus 80 x 10^6 normal B, Neo T+B- 16 x 10^6 neonatal T + 80 x 10^6 neonatal B, anti-δ - 16 x 10^6 anti-δ T plus 80 x 10^6 normal B. Shown in bold are the averages and standard deviations shown in Figure 19.

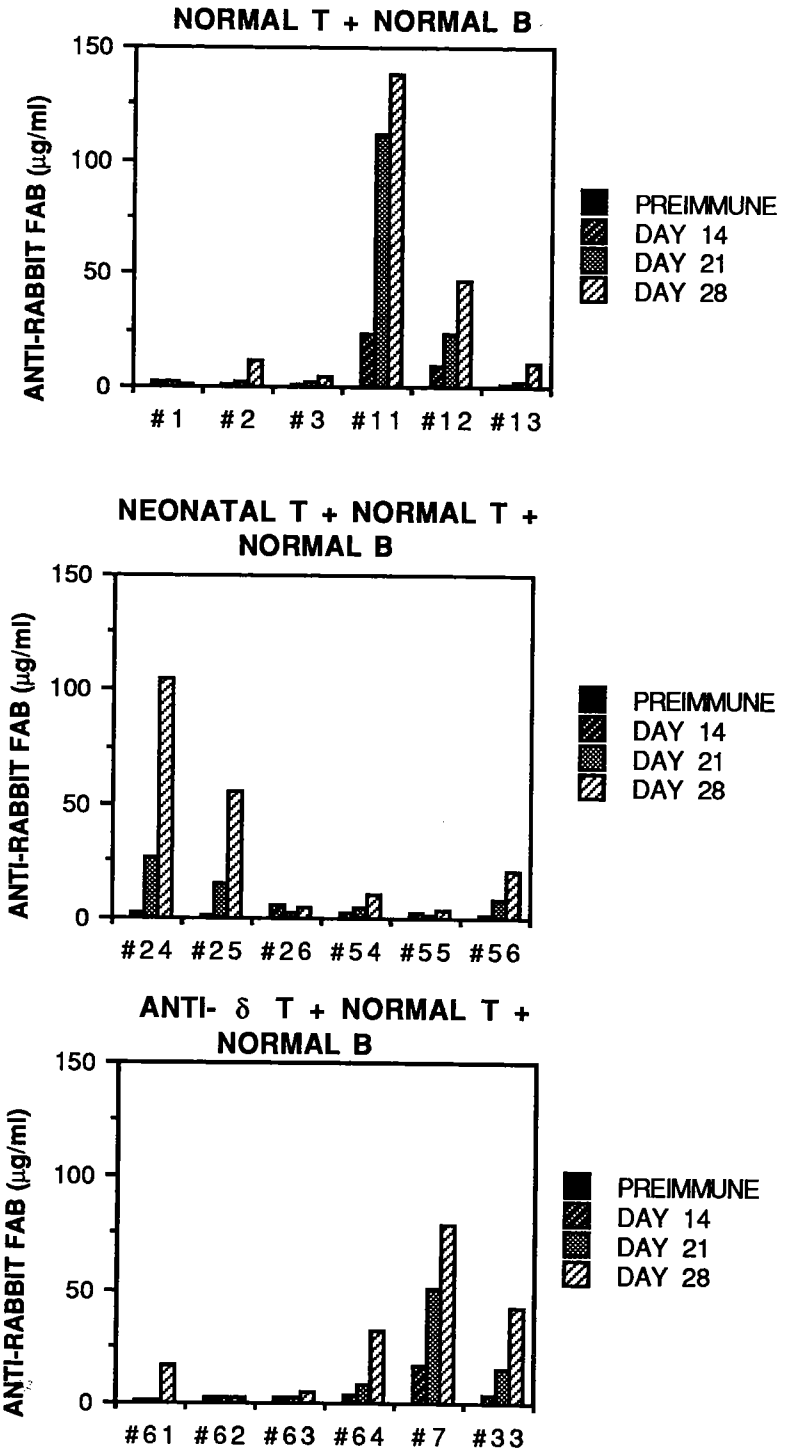


Figure 20. Individual serum anti-rabbit Fab responses in µg/ml from the mice shown in Figure 19 and Table 16.

Conclusions. In the investigation of the mechanism involved in the tolerance induced by anti-δ, I have shown that there is a definite defect in helper T cell function. A less profound effect on the antigen specific B cells was observed. The B cell defect in these mice is not global, since the chicken Ig response is not affected (see Figure 5). Since the Fab anti-δ has affinities for both IgD and anti-rabbit Fab Ig molecules on anti-rabbit Fab specific B cells, the defect may be due to a crosslinking of membrane Ig without further help. This B cell defect would not be apparent in forms of low dose tolerance (113) which cannot crosslink membrane Ig such as non anti-Ig monovalent soluble protein antigens.

In Fab anti-δ treated mice, the T cell response to rabbit Fab has not been shifted to T cells which proliferate but do not help make antibody, nor has it shifted toward helping the production of different isotypes. There is no evidence that suppression is a major cause of the T cell defect. The most probable T cell defect in this tolerance model is through loss of rabbit Fab specific T cell function through anergy or deletion of rabbit Fab specific T cell clones.

CHAPTER IV.

DISCUSSION AND FUTURE DIRECTIONS

The Small B Cell in Peripheral Tolerance

In this dissertation I have shown a role for the small B cell as an APC in peripheral tolerance induction. Small B cells as outlined in the introduction are ideally suited to be the APC involved in this type of tolerance induction. When in a resting state they can process and present antigen, but that presentation is ineffective in that it leads to tolerance in the T cell. For soluble protein antigens in the circulation the predominant MHC class II bearing APC is the small B cell. These antigen receptor bearing cells can pick up and present low concentrations of soluble proteins and inactivate the potentially autoreactive T cell clones.

The small B cell is not the only B cell which expresses membrane IgD. The $CD5^+$ B cell population also express some variable levels of membrane IgD. Most CD5+ B cells, however, are IgM^{hi} and IgD^{lo}. The IgM levels are 5-10 fold higher and the IgD levels 10-20 fold lower. Also, the $CD5^+$ subset of B cells is not depleted in mice treated with neonatal injections of anti-IgD. $CD5^+$ cells have different distribution from the normal small B cell population. These cells comprise less than 2% of the B cells in the

spleen and are not found at all in the lymph nodes, but they are approximately 20-40% of the cells in the peritoneum (120).

Considering the tissue distribution and the relatively low levels of IgD that these cells bear, it is unlikely that these cells contribute in either a positive or negative way to the tolerance induced with anti-δ. It is possible that there is some competition, for antigen, among tolerogenic and non-tolerogenic APCs. In this system, however, the tolerogenic APC outnumber by far those with the potential to activate an immune response.

Soluble and shed cell bound proteins which are sequestered, developmentally regulated, or have very low levels of expression could be the targets of this type of tolerance. These could include the components of complement (121), cytokines, hormones, and the idiotypes of the B cell Ig molecules. B cells, like all cells, will process and present endogenous molecules (25, 26). Included in the molecules processed will be its own unique Ig molecule.

While the concentration of the idiotype (the unique antigen binding site) being presented by the B cell will be extremely low, the concentration will be higher in the B cell and extremely low elsewhere. If that B cell did present that idiotype to an idiotype specific T cell, that T cell would be inactivated as a result. This inactivation would prevent the promiscuous activation of B cells to Ig secretion without any antigen stimulus, since without T cell help the B cell will not become activated. It is unlikely, due to the very low concentrations of idiotype even in the B cell, that this is the major

mechanism of unresponsiveness to idiotype in the system. It is more likely that for the most part idiotypes are invisible to the immune system due to concentration effects. However, it is valuable to have this failsafe mechanism to inactivate any idiotype reactive T cell should it encounter that idiotype with self-MHC on a small resting B cell.

Goodnow et al (122) have shown that there is a population of unresponsive B cells in the circulation of normal mice. These cells resemble those from mice which are double transgenic. The mice used by Goodnow et al were transgenic for an Ig specific for hen egg lysozyme and were also transgenic for the antigen itself, hen egg lysozyme. They have several mouse lines with different levels of expression of the lysozyme transgene (80). In these mice with low expression of the lysozyme (nanograms/ml) they find T cell tolerance but the transgenic B cells are responsive. With higher levels of lysozyme, B cells as well as T cells are tolerant (122). Some of the transgenic B cells in the tolerant mice remain in the circulation as anergic cells. These cells could also remain to present the lysozyme to emerging T cells to maintain T cell tolerance. Autoreactive B cells may persist in the periphery either as anergic cells at high self antigen concentrations (80) or as functional cells at low self antigen concentrations (122) without any T cell help. Both kinds of cells may play a role in maintenance of peripheral T cell tolerance.

Uses for the Anti-δ System

This dissertation has described a system which can be used to further explore the role of APCs in tolerance induction. This system can be exploited to answer a number of questions surrounding tolerance induction and has the potential to be useful in inducing tolerance in transplantation and in immunotherapy.

Anti-δ as a targeting agent. Enhanced immunogenicity can be shown with molecules attached to anti-Ig molecules. We can exploit this finding by coupling Fab anti-δ to immunogenic peptides with defined repertoires. This coupled antigen should induce tolerance to not only the rabbit Fab but to the protein coupled to it. Pigeon cytochrome c (PCC) is a well described system which has immunodominant peptides identified and more importantly a defined repertoire of T cell responses. The T cell receptors with affinity for a fragment of PCC have been shown to be restricted to certain Vα and Vβ regions (123). Transgenic mice have been made which bear a TCR reactive with PCC. In these mice the majority of T cells express this trangenic αβ TCR. These anti-PCC mice can be made tolerant to the peptide for which this T cell receptor is specific and then we will have a system to examine a large population of T cells for anergy.

Anti-δ in immunotherapy. Many types of immunotherapy as well as transplantation tolerance rely on the injection of monoclonal antibodies to ablate certain types of cells. These therapies must be discontinued once the patient develops antibodies reactive with these therapeutic antibodies. It may be possible to induce tolerance to monoclonal antibodies, by injecting Fab anti-δ i.v. prior to the start of the therapy.

As single gene defects which lead to disease are identified, patients may be injected with soluble proteins as replacement for the defective gene product. These patients will not be tolerant to these missing proteins. Therefore, another potential use of tolerance to proteins coupled to anti-δ would be to induce tolerance to pharmocologic agents in disease therapies.

Anti-δ tolerance with prior immunization. Fab anti-δ induced tolerance can be examined in previously immunized mice. Mice with T cells primed to rabbit Fab by s.c. injection of antigen-pulsed dendritic cells can be treated with Fab anti-δ and tolerance measured. The injection of pulsed dendritic cells has been described by Inaba et al (124) to activate T cells but not B cells, presumably because no antigen is ever available for B cell recognition and activation. Using this protocol, there will be no circulating antibody present which might interfere with induction of tolerance. If mice can be made tolerant even after T cells have been activated previously, this could have a profound effect on our understanding of

tolerance induction. If tolerance can be induced with prior activation using a B cell targeted system, then it may be useful in transplantation tolerance since many patients awaiting transplantation are already sensitized to many MHC alleles.

Our results suggest that the B cells must remain in a resting state for tolerance to be induced, but, there are unpublished results from Matzinger and Fuchs using transfer of H-Y antigen on small or activated B cells to induce tolerance to an MHC class I restricted response. These results show that tolerance is generated even when the B cells are activated by a number of methods.

Questions

I have not yet clearly defined the mechanism of this anti-δ mediated tolerance. In order to show anergy or deletion it is necessary to have a mechanism to collect the potentially unresponsive cell populations to test in vitro or in vivo by transfer into other hosts. We also have no information on the epitopes of rabbit Fab recognized or the types of T cells responding to rabbit Fab in normal mice and have no mechanism for isolating these cells in vitro.

The mechanism of tolerance induced by treatment with Fab anti-δ has yet to be determined, but my work indicates that it is likely to be deletion or anergy rather than suppression. Some of the

reasons for this belief are that suppression is more often found with a persistent antigen, like complement (121, 125) or insulin (35, 70, 72). It has also been shown that suppression decreases with time and loss of antigen (72, 125). Since Fab anti-δ is a short lived tolerogen it seems unlikely that suppression is the primary tolerance mechanism. My own results with mixing tolerant and normal cells in a primary adoptive transfer model do not indicate that suppression is the major mechanism for the tolerance induced with Fab anti-δ treatment.

Anergy seems to occur more often with cell associated molecules such as Mls (76), MHC molecules (34), or H-Y (73). However, this may be an artifact due to the mechanics of gathering enough antigen specific T cells to show anergy. Also, there are no systems yet which have tested for anergy using soluble protein antigens. With the pigeon cytochrome c transgenic mice model we may be able to determine if there are anergic cells with a soluble antigen.

Deletion in the periphery is the remaining mechanism and I feel that this is the most likely mechanism for soluble protein tolerance. However, with soluble molecules it is difficult to determine if the tolerance is occurring in the thymus or in the periphery. One way to determine if the thymus is necessary for soluble tolerance to occur is by tolerizing thymectomized mice.

Goodnow et al have recently shown (81) that transgenic B cells unresponsive for lysozyme can be reactivated with multiple injections of antigen. It will be interesting to see if T cell tolerance

utilizing anergy can be broken by multiple boosts of antigen. My work has already shown that CFA partially breaks tolerance and so it may be that a powerful enough adjuvant can reactivate anergic T cells.

To test for reactivation of anergic T cells, PCC TCR transgenic mice can be made tolerant, and if there are anergic T cells present they can be transferred into nude mice followed by immunization with PCC. Both Goodnow et al (81) in B cell, and Teh et al (73) in T cell anergy found a phenotypic change in the anergic cells they described. The anergic T cells in male mice with H-Y transgenic TCRs expressed normal levels of TCR but reduced levels of CD8 molecules. If other types of anergic cells show this phenotype then it will be possible to collect and follow these cells for recovery of function.

CHAPTER V.

REFERENCES

1. **Davis, M. M., and Bjorkman, P. J.** 1988. T-cell antigen receptor genes and T-cell recognition. Nature 334: 395.

2. **Mosmann, T. R., and Coffman, R. L.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann. Rev. Immunol. 7: 145.

3. **Braciale, T. J., Morrison, L. A., Sweetser, M. T., Sambrook, J., Gething, M.-J., and Braciale, V. L.** 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. Imm. Rev. 98: 96.

4. **Kincade, P. W., and Gimble, J. M.** 1989. B lymphocytes In *Fundamental Immunology, vol.* W.E. Paul eds. Raven Press Ltd., New York, p. 41.

5. **Kingston, R., Jenkinson, E. J., and Owen, J. J. T.** 1985. A single embryonic stem cell can recolonize an embryonic thymus, producing phenotypically distinct T-cell populations. Nature 317: 811.

6. **Moore, M. A. S., and Owen, J. J. T.** 1967. Experimental studies on the development of the thymus. J. Exp. Med. 126: 715.

7. **Paul, W. E.** 1989. The immune system: An introduction In *Fundamental Immunology, vol.* W.E. Paul eds. Raven Press Ltd., New York, p. 3.

8. **Sprent, J.** 1980. Features of cells controlling H-2-restricted presentation of antigen to T helper cells *in vivo*. J. Immunol. 125: 2089.

9. **Lassila, O., Vainio, O., and Matzinger, P.** 1988. Can B cells turn on virgin T cells? Nature 334: 253.

10. **Yancopoulos, G. D., and Alt, F. W.** 1986. Regulation of the assembly and expression of variable-region genes. Ann. Rev. Immunol. 4: 339.

11. **Miller, J. F. A. P., and Osoba, D.** 1967. Current concepts of the immunologic function of the thymus. Physiol. Rev. 47: 437.

12. **Cristanti, A., Colantoni, A., Snodgrass, R., and von Boehmer, H.** 1986. Expression of T cell receptors by thymocytes: *in situ* staining and biochemical analysis. EMBO J. 5: 2837.

13. **Sprent, J., Schaefer, M., Lo, D., and Korngold, R.** 1986. Functions of purified L3T4$^+$ and Lyt2$^+$ cells *in vitro* and *in vivo*. Imm. Rev. 91: 195.

14. **Swain, S.** 1983. T cell subsets and the recognition of MHC class. Imm. Rev. 74: 129.

15. **Klein, J.** 1986. In *Natural History of the Major Histocompatibility Complex*, John Wiley and Sons, New York,

16. **Hunkapiller, T., and Hood, L.** 1989. Diversity of the Immunoglobulin gene superfamily. Adv. Immunol. 44: 1.

17. **Doyle, C., and Strominger, J. L.** 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature 330: 256.

18. **Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H., and Littman, D. R.** 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. Nature 336: 79.

19. **Zinkernagel, R. M., and Doherty, P. C.** 1979. MHC-restricted cytotoxic T cells: Studies on the biological role of polymorphic major transplantation antigen determining T-cell restriction-specificity, function, and responsiveness. Adv. Immunol. 27: 51.

20. **Bevan, M. J.** 1977. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature 269: 417.

21. **Zinkernagel, R. M., Callahan, G. N., Althage, A., Cooper, S., Klein, P. A., and Klein, J.** 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: Evidence for dual recognition. J. Exp. Med. 147: 882.

22. **Lo, D., and Sprent, J.** 1986. Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. Nature 319: 672.

23. **Zinkernagel, R. M., Callahan, G. N., and Dennert, G.** 1978. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. Nature 271: 251.

24. **Benoist, C., and Mathis, D.** 1989. Positive selection of the T cell repertoire: Where and when does it occur? Cell 58: 1027.

25. **Rudensky, A. Y., Mazel, S. M., and Yurin, V. L.** 1990. Presentation of endogenous immunoglobulin determinant to immunoglobulin-recognizing T cell clones by the thymic cells. Eur. J. Immunol. 20: 2235.

26. **Lorenz, R. G., and Allen, P. M.** 1988. Direct evidence for functional self-protein/Ia-molecule complexes *in vivo*. Proc. Natl. Acad. Sci. USA 85: 5220.

27. **Ready, A. R., Jenkinson, E. J., Kingston, R., and Owen, J. J. T.** 1984. Successful transplantation across major histocompatibility barrier of deoxyguanosine-treated embryonic thymus expressing class II antigens. Nature 310: 231.

28. **von Boehmer, H., and Sprent, J.** 1976. T cell function in bone marrow chimeras: Absence of host-reactive T cells and cooperation of helper T cells across allogeneic barriers. Trans. Rev. 29: 3.

29. **Kappler, J. W., Roehm, N., and Marrack, P.** 1987. T cell tolerance by clonal elimination in the thymus. Cell 49: 273.

30. **Marrack, P., Lo, D., Brinster, R., Palmiter, R., Burkly, L., Flavell, R. H., and Kappler, J.** 1988. The effect of thymus environment on T cell development and tolerance. Cell 53: 627.

31. **Marrack, P., and Kappler, J.** 1987. The T cell receptor. Science 238: 1073.

32. **Lederberg, J.** 1959. Genes and Antibodies. Science 129: 1649.

33. **Weiss, A., Dazin, P. F., Shields, R., Fu, S. M., and Lanier, L. L.** 1987. Functional competency of T cell antigen receptors in human thymus. J. Immunol. 139: 3245.

34. **Lo, D., Burkly, L. C., Flavell, R. A., Palmiter, R. D., and Brinster, R. L.** 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. J. Exp. Med. 170: 87.

35. **Jensen, P. E., and Kapp, J. A.** 1985. Stimulation of helper T cells and dominant suppressor T cells that recognize autologous insulin. J. Mol. Cell. Immunol. 2: 133.

36. **Unanue, E. R., and Askonas, B. A.** 1968. The immune response of mice to antigen in macrophages. Immunology 15: 287.

37. **Unanue, E. R.** 1989. Macrophages, antigen presenting cells, and the phenomenon of antigen handling and presentation In *Fundamental Immunology, vol.* W.E. Paul eds. Raven Press Ltd., New York, p. 95.

38. **Steinman, R. M.** 1991. The dendritic cell system and its role in immunogenicity. Ann. Rev. Immunol. 9: 271.

39. **Metlay, J. P., Pure, E., and Steinman, R. M.** 1989. Distinct features of dendritic cells and anti-Ig activated B cells as stimulators of the primary mixed leukocyte reaction. J. Exp. Med. 169: 239.

40. **Hudson, L., and Hay, F. C.** 1989. In *Practical Immunology*, Blackwell Scientific Publications, Oxford

41. **Lanzavecchia, A.** 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to Class II-restricted T lymphocytes. Ann. Rev. Immunol. 8: 773.

42. **Tony, H.-P., and Parker, D. C.** 1985. Major histocompatibility complex-restricted, polyclonal B cell responses resulting from helper T cell recognition of antiimmunoglobulin presented by small B lymphocytes. J. Exp. Med. 161: 223.

43. **Rock, K. L., Benacerraf, B., and Abbas, A. K.** 1984. Antigen presentation by hapten-specific B lymphocytes I. Role of surface immunoglobulin receptors. J. Exp. Med. 160: 1102.

44. **Gosselin, E. J., Tony, H.-P., and Parker, D. C.** 1988. Characterization of antigen processing and presentation by resting B lymphocytes. J. Immunol. 140: 1408.

45. **Geppert, T. D., Davis, L. S., Gur, H., Wacholtz, M. C., and Lipsky, P. E.** 1990. Accessory cell signals involved in T cell activation. Imm. Rev. 117: 5.

46. **Bretscher, P., and Cohn, M.** 1970. A Theory of Self-Nonself Discrimination. Science 169: 1042.

47. **Lafferty, K. J., Prowse, S. J., and Simeonovic, C. J.** 1983. Immunobiology of tissue transplantation: A return to the passenger leukocyte concept. Ann. Rev. Immunol. 1: 143.

48. **Koulova, L., Clark, E. A., Shu, G., and Dupont, B.** 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of $CD4^+$ T cells. J. Exp. Med. 173: 759.

49. **Linsley, P. S., Clark, E. A., and Ledbetter, J. A.** 1990. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc. Natl. Acad. Sci. USA 87: 5031.

50. **Ashwell, J. D.** 1988. Are B lymphocytes the principal antigen-presenting cells in vivo? J. Immunol. 140: 3697.

51. **Lechler, R. I., and Batchelor, J. R.** 1982. Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells. J. Exp. Med. 155: 31.

52. **Webb, S. R., Li, J. H., Wilson, D. B., and Sprent, J.** 1985. Capacity of small B cell-enriched populations to stimulate mixed lymphocyte reactions: marked differences between irradiated *vs.* mitomycin C-treated stimulators. Eur. J. Immunol. 15: 92.

53. **Cowing, C., and Chapdelaine, J. M.** 1983. T cells discriminate between Ia antigens expressed on allogeneic accessory cells and B cells : a potential function for carbohydrate side chains on Ia molecules. Proc. Natl. Acad. Sci. USA 80: 6000.

54. **Lombardi, G., Sidhu, S., Daly, M., Batchelor, J. R., Makgoba, W., and Lechler, R. I.** 1989. Are primary alloresponses truly primary? Internatl. Immunol. 2: 9.

55. **Ryan, J. J., Gress, R. E., Hathcock, K. S., and Hodes, R. J.** 1984. Recognition and response to alloantigens *in vivo* II. Priming with accessory cell-depleted donor allogeneic splenocytes: Induction of specific unresponsiveness to foreign major histocompatibility complex determinants. J. Immunol. 133: 2343.

56. **Hori, S., Sato, S., Kitigawa, S., Azuma, T., Kokudo, S., Hamaoka, T., and Fujiwara, H.** 1989. Tolerance induction of allo-class II H-2 antigen-reactive L3T4$^+$ helper T cells and prolonged survival of the corresponding class II H-2 disparate skin graft. J. Immunol. 143: 1447.

57. **Schwartz, R. H.** 1989. Acquisition of immunologic self-tolerance. Cell 57: 1073.

58. **Jenkins, M. K., and Schwartz, R. H.** 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J. Exp. Med. 165: 302.

59. **Jenkins, M. K., Pardoll, D. M., Mizuguchi, J., Chused, T. M., and Schwartz, R. H.** 1987. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. Proc. Natl. Acad. Sci. USA 84: 5409.

60. **Jenkins, M. K., Pardoll, D. M., Mizuguchi, J., Quill, H., and Schwartz, R. H.** 1987. T-cell unresponsiveness *in vivo* and *in vitro*: fine specificity of induction and molecular characterization of the unresponsive state. Imm. Rev. 95: 113.

61. **Jenkins, M. K., Burrell, E., and Ashwell, J. D.** 1990. Antigen presentation by resting B cells Effectiveness at inducing T cell proliferation is determined by costimulatory signals, not T cell receptor occupancy. J. Immunol. 144: 1585.

62. **Mitchison, N. A.** 1964. Induction of immunological paralysis in two zones of doseage. Proc. Roy. Soc. Ser. Biol. Sci. 161: 275.

63. **Dietrich, F. M., and Weigle, W. O.** 1964. Immunologic unresponsiveness to heterologous serum proteins induced in adult mice and transfer of the unresponsive state. J. Immunol. 92: 167.

64. **Dresser, D. W.** 1962. Specific inhibition of antibody production I. Protein-overloading paralysis. Immunology 5: 161.

65. **Clayman, H. N.** 1963. Tolerance to a protein antigen in adult mice and the effect of nonspecific factors. J. Immunol. 91: 833.

66. **Shellam, G. R., and Nossal, G. J. V.** 1968. Mechanism of induction of immunological tolerance IV. The effects of ultra-low doses of flagellin. Immunology 14: 273.

67. **Parish, C. R., and Liew, F. Y.** 1972. Immune response to chemically modified flagellin III. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. J. Exp. Med. 135: 298.

68. **Chiller, J. M., and Weigle, W. O.** 1971. Cellular events during induction of immunologic unresponsiveness in adult mice. J. Immunol. 106: 1647.

69. **Gahring, L. C., and Weigle, W. O.** 1989. The induction of peripheral T cell unresponsiveness in adult mice by monomeric human γ-globulin. J. Immunol. 143: 2094.

70. **Howard, J. G., and Mitchison, N. A.** 1975. Immunological tolerance. Prog. Allergy 18: 43.

71. **Dresser, D. W.** 1969. The rates of induction and loss of immunological paralysis. Int. Arch. Allergy 35: 253.

72. **Parks, D. E., and Weigle, W. O.** 1979. Regulation of B cell unresponsiveness by suppressor cells. Imm. Rev. 43: 217.

73. **Teh, H.-S., Kishi, H., Scott, B., and von Boehmer, H.** 1989. Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules. J. Exp. Med. 169: 795.

74. **Murphy, K. M., Heimberger, A. B., and Loh, D. Y.** 1990. Induction by antigen of intrathymic apoptosis of $CD4^+$ $CD8^+$ TCR^{lo} thymocytes in vivo. Science 250: 1720.

75. **Webb, S., Morris, C., and Sprent, J.** 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 63: 1249.

76. **Rammensee, H.-G., Kroschewski, R., and Frangoulis, B.** 1989. Clonal anergy induced in mature $V\beta6^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. Nature 339: 541.

77. **Gunther, J., Haas, W., and von Boehmer, H.** 1982. Suppression of T cell responses through competition for T cell growth factor (interleukin 2). Eur. J. Immunol. 12: 247.

78. **Qin, S., Wise, M., Cobbold, S. P., Leong, L., Kong, Y.-C. M., Parnes, J. R., and Waldmann, H.** 1990. Induction of tolerance in peripheral T cells with monoclonal antibodies. Eur. J. Immunol. 20: 2737.

79. **Bandeira, A., Larsson, E.-L., Forni, L., Pereira, P., and Coutinho, A.** 1987. "*In vivo*" activated splenic T cells are refractory to interleukin 2 growth "*in vitro*". Eur. J. Immunol. 17: 901.

80. **Goodnow, C. C., Crosbie, J., Jorgensen, H., Brink, R. A., and Basten, A.** 1989. Induction of self-tolerance in mature peripheral B lymphocytes. Nature 342: 385.

81. **Goodnow, C. C., Brink, R., and Adams, E.** 1991. Breakdown of self-tolerance in anergic B lymphocytes. Nature 352: 532.

82. **Rocha, B., and von Boehmer, H.** 1991. Peripheral selection of the T cell repertoire. Science 251: 1225.

83. **Gilbert, K. M., Hoang, K. D., and Weigle, W. O.** 1990. Th1 and Th2 clones differ in their response to a tolerogenic signal. J. Immunol. 144: 2063.

84. **Lamb, J. R., Skidmore, B. J., Green, N., Chiller, J. M., and Feldmann, M.** 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. J. Exp. Med. 157: 1434.

85. **Quill, H., and Schwartz, R. H.** 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J. Immunol. 138: 3704.

86. **Gaspari, A. A., Jenkins, M. K., and Katz, S. I.** 1988. Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific TH1 clones. J. Immunol. 141: 2216.

87. **Gershon, R. K., and Kondo, K.** 1971. Infectious immunological tolerance. Immunology 21: 903.

88. **Moller, G.** 1988. Do suppressor T cells exist? Scand. J. Immunol. 27: 247.

89. **Degwert, J., Bettmann, R., Heuer, J., and Kolsch, E.** 1987. Isolation of a bovine serum albumin-specific T-suppressor cell clone and evaluation of its *in vitro* functions. Immunology 60: 345.

90. **Basten, A., Miller, J. F. A. P., Sprent, J., and Cheers, C.** 1974. Cell-to-cell interaction in the immune response X. T-cell-dependent suppression in tolerant mice. J. Exp. Med. 140: 199.

91. **Oki, A., and Sercarz, E.** 1985. T cell tolerance studied at the level of antigenic determinants I. Latent reactivity to lysozyme peptides that lack suppressogenic epitopes can be revealed in lysozyme-tolerant mice. J. Exp. Med. 161: 897.

92. **Silver, J., and Benacerraf, B.** 1974. Dissociation of T cell helper function and delayed hypersensitivity. J. Immunol 113: 1872.

93. **Otten, G. R., and N., G. R.** 1991. Split anergy in a $CD8^+$ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. Science 251: 1228.

94. **Warren, H. S., Vogel, F. R., and Chedid, L. A.** 1986. Current status of immunological adjuvants. Ann. Rev. Immunol. 4: 369.

95. **Hayakawa, K., and Hardy, R. R.** 1988. Murine $CD4^+$ T cell subsets defined. J. Exp. Med. 168: 1825.

96. **Bottomly, K.** 1988. A functional dichotomy in $CD4^+$ T lymphocytes. Immunol. Today 9: 268.

97. **Tony, H.-P., Phillips, N. E., and Parker, D. C.** 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated, major histocompatibility-restricted T cell-B cell cooperation. J. Exp. Med. 162: 1695.

98. **Bosma, G. C., Custer, R. P., and Bosma, M. J.** 1983. A severe combined immunodeficiency mutation in the mouse. Nature 301: 527.

99. **Phillips, N. E., and Parker, D. C.** 1984. Cross-linking of B lymphocyte Fcγ receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. J. Immunol. 132: 627.

100. **Good, A. H., Wofsky, L., Henry, C., and Kimura, J.** 1980. Preparation of hapten-modified protein antigens In *Selected Methods In Cellular Immunology, vol.* B.B. Mishell and S.M. Shiigi eds. W. H. Freeman and Co., San Francisco, CA, p. 343.

101. **Wysocki, L. J., and Sato, V. L.** 1978. "Panning" for lymphocytes: A method for cell selection. Proc. Natl. Acad. Sci. USA 75: 2844.

102. **Yuschenkoff, V. N., Eynon, E. E., and Parker, D. C.** 1991. Tolerance induction to a protein antigen using B cells from a transgenic mouse. J. Cell. Biochem. Supplement 15A: 245.

103. **Golub, E. S., and Weigle, W. O.** 1967. Studies on the induction of immunologic unresponsiveness II. Kinetics. J. Immunol. 99: 624.

104. **Scott, D. W., Layton, J. E., and Nossal, G. J. V.** 1977. Role of IgD in the immune response and tolerance I. Anti-δ pretreatment facilitates tolerance induction in adult B cells in vitro. J. Exp. Med. 146: 1473.

105. **Spielelberg, H. L., and Weigle, W. O.** 1965. The catabolism of homologous and heterologous 7S gamma globulin fragments. J. Exp. Med. 121: 323.

106. **Wochner, R. D., Strober, W., and Waldmann, T. A.** 1967. The role of the kidney in the catabolism of Bence Jones proteins and immunoglobulin fragments. J. Exp. Med. 126: 207.

107. **Golub, E. S., and Weigle, W. O.** 1967. Studies on the induction of immunologic unresponsiveness I. Effects of endotoxin and phytohemagglutinin. J. Immunol. 98: 1241.

108. **Finkelman, F. D., Scher, I., Mond, J. J., Kung, J. T., and Metcalf, E. S.** 1982. Polyclonal activation of the murine immune system by an antibody to IgD I. Increase in cell size and DNA synthesis. J. Immunol. 129: 629.

109. **Finkelman, F. D., Woods, V. L., Berning, A., and Scher, I.** 1979. Demonstration of mouse serum IgD. J. Immunol. 123: 1253.

110. **Sigal, N. H., and Klinman, N. R.** 1978. The B-cell clonotype repertoire. Adv. Immunol. 26: 255.

111. **Kappler, J. W., Staerz, U., White, J., and Marrack, P. C.** 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature 332: 35.

112. **Ramsdell, F., Lantz, T., and Fowlkes, B. J.** 1989. A nondeletional mechanism of thymic self tolerance. Science 246: 1038.

113. **Chiller, J. M., Habicht, G. S., and Weigle, W. O.** 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. Science 171: 813.

114. **Mosmann, T. R., and Coffman, R. L.** 1987. Two types of mouse helper T-cell clone Implications for immune regulation. Immunol. Today 8: 223.

115. **Parish, C. R.** 1972. The relationship between humoral and cell-mediated immunity. Transplant. Rev. 13: 35.

116. **Whiteley, P. J., Poindexter, N. J., Landon, C., and Kapp, J. A.** 1990. A peripheral mechanism preserves self-tolerance to a secreted protein in transgenic mice. J. Immunol. 145: 1376.

117. **Grun, J. L., and Maurer, P. H.** 1989. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles : The role of endogenous interleukin 1 in proliferative responses. Cellular Immunol. 121: 134.

118. **Street, N. E., Schumacher, J. H., Fong, T. A. T., Bass, H., Fiorentino, D. F., Leverah, J. A., and Mosmann, T. R.** 1990. Heterogeneity of mouse helper T cells Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J. Immunol. 144: 1629.

119. **Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F. J., Finkelman, F. D., Bond, M. W., and Mosmann, T. R.** 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. Imm. Rev. 102: 5.

120. **Herzenberg, L. A., Stall, A. M., Lalor, P. A., Sidman, C., Moore, W. A., Parks, D. R., and Herzenberg, L. A.** 1986. The Ly-1 B cell lineage. Imm. Rev. 93:81-102.

121. **Cairns, L., Rosen, F. S., and Borel, Y.** 1986. Mice naturally tolerant to C5 have T cells that suppress the response to this antigen. Eur. J. Immunol. 16: 1277.

122. **Adelstein, S., Pritchard-Briscoe, H., Anderson, T. A., Crosbie, J., Gammon, G., Loblay, R. L., Basten, A., and Goodnow, C. C.** 1991. Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen. Science 251: 1223.

123. **Winoto, A., Urban, N. C., Lan, J., Goverman, J., Hood, L., and Hanburg, D.** 1986. Predominant use of a Vα gene segment in mouse T cell receptors for cytochrome c. Nature 324: 679.

124. **Inaba, K., Metlay, J. P., Crowley, M. T., and Steinman, R. M.** 1990. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J. Exp. Med. 172: 631.

125. **Harris, D. E., Cairns, L., Rosen, F. S., and Borel, Y.** 1982. A natural model of immunologic tolerance. Tolerance to murine C5 is mediated by T cells, and antigen is required to maintain unresponsiveness. J. Exp. Med. 156: 567.