A Dissertation Presented

By

ALLISON MICHELLE BAIRD

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of :

DOCTOR OF PHILOSOPHY

APRIL 26, 1996

IMMUNOLOGY

ANALYSIS OF LOW ZONE TOLERANCE IN NORMAL AND B CELL-DEFICIENT MICE

A Dissertation Presented

By

ALLISON MICHELLE BAIRD

Approved as to style and content by:

Raymond M. Welsh, Ph.D., Chair of Committee

Janet M. Stavnezer, Ph.D., Member of Committee

Dale L. Greiner, Ph.D., Member of Committee

Robert T. Woodland, Ph.D., Member of Committee

Arlene H. Sharpe, M.D., Member of Committee

David C. Parker, Ph.D., Dissertation Mentor

Thomas B. Miller, Jr., Ph.D., Dean of the Graduate School of Biomedical Sciences

Immunology

April 26, 1996

ACKNOWLEDGMENTS

I am very grateful to my advisor Dr. David Parker for his constant support and encouragement, even from great distances. I also thank Dr. Raymond Welsh for giving me the opportunity to complete the experiments for my dissertation in his laboratory after David relocated to Portland, Oregon. Many thanks to the faculty and students of University of Masachusetts Medical Center who have helped make this experience a very pleasant one. I have especially enjoyed working with the students and postdocs in the Parker and Welsh laboratories- thanks to each of you! I also want to acknowledge my family and friends who have been incredibly supportive of all my endeavors. Most importantly, I owe a great deal to my husband, Nels Carlson, who has been an enormous source of strength and who has kept me focused on this most important goal. Lastly, thanks to my two best friends, Duke and Dutchie, who were great companions at home while I was writing.

ABSTRACT

This thesis investigates the role of B cells as antigen-specific antigen-presenting cells (APC) in self tolerance to low concentrations of soluble self proteins and in acquired tolerance to low doses of soluble foreign protein antigens. Experiments were performed in normal and B cell-deficient animals, and tolerance induction was measured by T cell proliferation assays. T cell proliferation was reduced in B cell-deficient mice, indicating that B cells may be involved in efficient activation of naive T cells in response to protein antigen both *in vivo* and *in vitro*. To study acquired tolerance induced by low doses of soluble foreign protein antigen, normal and B cell-deficient adult mice were injected intravenously with repeated low doses (10 μg) of deaggregated ovalbumin (OVA), and then challenged with OVA in complete Freund's adjuvant. In animals treated with deaggregated OVA, the *in vitro* proliferative responses of LN T cells to OVA were significantly reduced, and production of the Th1 cytokine, IFN-γ, in response to OVA was lost. This occurred in both normal and B cell-deficient treated animals, indicating that B cell antigen presentation was not required for this phenomenon. B cells were also unnecessary for self tolerance of T cells to the transgenic self antigen, hen egg lysozyme (HEL), in a transgenic mouse strain with very low serum lysozyme concentration. Partial low zone tolerance induced by deaggregated, low-dose OVA was selective for the Th1 response, as measured by *in vitro* proliferation and IL-2 and IFN-γ production, because antibody responses of normal mice to this T cell-dependent antigen were largely unaffected. Both treated and untreated animals produced equivalent titers of anti-OVA antibodies, predominantly of the IgG1 and IgG2b isotypes, following challenge with OVA in complete Freund's adjuvant. Tolerance to low levels of the transgenic HEL self protein in mice expressing different MHC molecules was also addressed. Transgenic mice that were H-

$2^{b/b}$ in the class II region were not tolerant to the transgenic self protein, whereas transgenic mice of the H-$2^{b/k}$ were tolerant.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2-azino-di[3-ethylbenzthiazoline sulfonic acid]
APC	antigen presenting cell
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
cpm	counts per minute
CTL	cytotoxic T lymphocyte
EAE	experimental allergic encephalomyelitis
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HEL	hen egg lysozyme
H + L	heavy plus light chain Ig
HPLC	high pressure liquid chromatography
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneally
i.v.	intravenously
KLH	keyhole limpet hemocyanin

LCMV	lymphocytic choriomeningitis virus
LN	lymph node
μ	heavy chain of IgM
MBP	myelin basic protein
MHC	major histocompatibility complex
ML-4	transgenic mice with < 0.5 ng/ml serum HEL
ML-5	transgenic mice with 20 ng/ml serum HEL
Mls	minor lymphocyte stimulating antigen
μMT	B cell-deficient mice
M. tuberculosis	Mycobacterium tuberculosis
NOD	nonobese diabetic mice
O.D.	optical density
OVA	ovalbumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPD	purified protein derivative
s.c.	subcutaneously
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
SRBC	sheep red blood cells
TCR	T cell receptor
TH	T helper cell
Tween-20	polyoxyethelene-sorbitan monolaurate
V_β	variable region of the TCR β chain

CHAPTER I

INTRODUCTION

Immunity against infectious agents is the result of two types of host responses: the innate immune response and the adaptive or acquired immune response. The host's first line of defense against infection is the innate immune response. The adaptive immune response requires several days to become fully activated and is the second line of defense. Innate immunity is present from birth and is usually a non-specific defense against infection. It is composed of natural killer cells and also phagocytic cells, including macrophages and neutrophils, that can ingest and destroy extracellular microorganisms. Phagocytes are also able to recruit other cells of the immune response to the site of infection by initiating inflammation. The proteins of the complement cascade provide further protection against infection by binding directly to the surface of some microorganisms targeting them for uptake by phagocytes or for lysis.

The adaptive immune response is initiated only when the infection cannot be controlled by the innate immune response. Unlike innate immunity, which is present at all times, the adaptive immune response arises only after encounter with the infectious agent and is specific for the infecting organism. Adaptive immunity also provides the host with immunological memory in the event that the same pathogen initiates a second infection. The adaptive immune response can be divided into antibody-mediated and cell-mediated immunity depending on whether B lymphocytes or T lymphocytes are responsible for providing protection against the infectious agent.

Several components of the innate immune response work in conjunction with the components of the adaptive immune response to combat infection. The receptors and

response mechanisms of the innate immune system used to detect infectious agents can initiate the adaptive immune response; for example, in response to intracellular bacteria, an infected macrophage will present antigen to T cells. The intracellular bacteria provide both the peptides for presentation and induce costimulatory molecules on the macrophage, ensuring that the adaptive immune response will be productive. In addition, IFN-γ production by T cells enhances macrophage killing of intracellular bacteria, demonstrating how the adaptive immune response can also act to make various innate effector systems more efficient.

One of the most important features of the adaptive immune response is the ability of lymphocytes to recognize and respond to any foreign substance while rarely responding against self antigens. As discussed below, there are several check points that have been clearly identified during lymphocyte development, activation, and survival to ensure that B and T lymphocytes do not respond to self antigens. Other potential mechanisms for maintaining tolerance to self proteins are less well defined . The work presented in this thesis will address the role of the antigen-presenting B cell in peripheral T cell tolerance induction to self proteins as well as to foreign proteins.

A. Recognition of Antigen by B and T Lymphocytes

The adaptive immune response is initiated when the receptors on lymphocytes recognize antigen. Lymphocytes continuously recirculate from the bloodstream through the peripheral lymphoid organs, such as the lymph node (LN), spleen, and gut-associated tissues, where they encounter any number of different antigens. How does the body determine which lymphocytes will respond to an antigen? The clonal selection theory proposes that a pre-existing population of lymphocytes with the potential to respond to an

antigen, are the only cells selected to respond (1). B lymphocytes and T lymphocytes each have highly variable antigen receptors which are monospecific; thus one cell expresses a receptor that can recognize only one antigen. Cells specific for self antigens are removed during lymphocyte development and for the most part are absent from the peripheral pool of responding lymphocytes. When mature lymphocytes come in contact with an antigen they recognize, they become activated and proliferate. Thus each cell specific for an antigen can give rise to a clone of identical progeny with the same antigen specificity. These clones then develop into effector cells that can eliminate the antigen. Once the antigen is cleared, the immune response is then downregulated. Some of the activated cells will have differentiated into memory cells that can respond more rapidly upon a second encounter with same antigen, providing protection or immunity against the original antigen.

Since B and T lymphocytes are involved in different effector functions in the immune response, it is not surprising that their antigen receptors recognize antigen in different ways. The B cell antigen receptor is a cell-surface immunoglobulin molecule capable of recognizing antigen directly, and it has the same specificity as the antibody molecules secreted by clonal progeny following activation. Immunoglobulin molecules are composed of two heavy chains and two light chains (2). Each of these chains contains a variable region responsible for antigen recognition, which is encoded by variable (V), joining (J), and/or diversity (D) gene segments (3), and a constant region. The constant region of the heavy chain is responsible for engaging the effector functions of the immune system when the antibody molecule is secreted, and determines immunoglobulin class. The same variable region gene can be spliced to one of five heavy chain constant genes, μ, δ, γ, α, and ε (4) in a process referred to as class or isotype switching. Isotype switching allows antibodies with the same antigenic specificity to serve different functions in the

immune system as specified by the constant region, such as Fc receptor binding and complement fixation.

The T cell receptor (TCR) is a heterodimer composed of two chains, α and β, or γ and δ (5). Each chain of the TCR also has constant and variable regions, encoded by V, J, and/or D-like elements, which are joined together by gene rearrangement (5). However, unlike the B cell receptor, the TCR cannot recognize antigen directly. The TCR recognizes peptide fragments of antigen bound to a cell-surface molecule encoded in the major histocompatibility complex (MHC) (6-8). This phenomenon is referred to as MHC restriction. The MHC molecules must be able to bind a variety of proteins derived from many different pathogens; therefore there are several MHC genes with different specificities for binding peptide, and there are multiple alleles at each gene locus (9). Antigen processing is the mechanism that cells use to degrade proteins into peptides for presentation in MHC molecules to T cells (10). MHC class I molecules are displayed on all cells throughout the body, and they present peptides derived from intracellular proteins: self proteins, proteins from intracellular bacteria, or in the case of a viral infection, virally-encoded proteins (11). Class II MHC molecules present peptide antigens derived from exogenous proteins and are only found on antigen presenting cells, which include dendritic cells, macrophages, and B cells (10). The TCR expresses one of two MHC-specific co-receptor molecules, CD4 or CD8, that enhance the ability of T cells to engage either class II MHC molecules or class I MHC molecules, respectively (12). $CD8^+$ T cells and $CD4^+$ T cells serve different functions in the immune response. $CD8^+$ T cells, referred to as cytotoxic T cells, are able to kill infected cells expressing MHC class I molecules presenting foreign peptides. The $CD4^+$ cells provide help for cell-mediated and antibody-mediated responses and can be divided into two helper subsets, Th1 and Th2, based on the pattern of cytokines they secrete (13).

B. Lymphocyte Development

Lymphocytes develop in the central lymphoid organs, either the bone marrow or the thymus. All lymphocytes originate from stem cell precursors in the bone marrow. Stem cells exit the bone marrow and migrate to the thymus for development into T cells, whereas B lymphocytes remain in the bone marrow for the maturation process. As lymphocytes mature and begin to express their antigen receptors, the cells with receptors that recognize self antigens are either deleted or inactivated. The elimination of these self reactive B and T cells during development is referred to as central tolerance induction or clonal deletion.

The stages of B cell development can be marked by immunoglobulin gene rearrangements (14). Initially, both the heavy and light chain genes are in the germline configuration, but as the stem cell precursor proceeds into the early pro-B cell stage, the heavy chain genes, D and J, are rearranged. Next, the variable region of the heavy chain is joined to the DJ_H segment forming the late pro-B cell. As pro-B cells mature into pre-B cells, intact μ heavy chains are expressed in the cytoplasm and also at low levels on the cell surface, and the V and J light chain genes start rearranging. The immature B cell is produced when the light chain genes are rearranged and the cell expresses both light and μ heavy chains on the surface as IgM molecules. The final stage of B cell development is complete when the immature B cells express both cell-surface IgM and IgD, due to alternative RNA processing and choice of transcription termination sites in the heavy-chain transcripts (15). These mature B cells then exit the bone marrow and circulate in the periphery. B cell precursors cannot progress beyond the early pre-B cell stage if the μ heavy chain is not expressed on the cell membrane, as shown in B cell-deficient animals created by the targeted disruption of the transmembrane region of the μ heavy chain (16).

In this thesis, I took advantage of these mice to examine T cell tolerance induction in the absence of B cells.

The elimination of self-reactive B cells occurs at the immature B cell stage when the newly formed receptor has just been expressed on the cell-surface. If these IgM molecules are able to bind to multivalent cell-surface self ligands in the bone marrow, then these cells are eliminated by programmed cell death or apoptosis. This was demonstrated by Nemazee and Bürki using transgenic mice expressing immunoglobulin molecules specific for H-2K and D class I MHC molecules of the k-haplotype (17). When these mice were maintained on the H-2^d background, the anti-MHC-specific B cells could be found as responsive cells in the periphery. However, when the transgene was expressed in H-$2^{k/d}$ mice, and when the H-2^k self antigen was expressed on the cell-surface of other cells in the bone marrow, the anti-MHC-specific B cells could no longer be detected.

In contrast, immature B cells with IgM molecules specific for soluble self antigen are not eliminated, but instead can be rendered anergic or unresponsive to the self antigen. Anergic cells persist in the periphery, but are unable to expand in response to antigen. This was shown in experiments performed by Goodnow and colleagues using double-transgenic mice expressing both the soluble transgene-encoded self antigen HEL and the B cell receptor specific for HEL (18). The number of HEL-specific B cells was not reduced in these mice, as compared to mice carrying only the single immunoglobulin HEL transgene, indicating that the autoreactive B cells were able to escape clonal deletion in the presence of their self antigen HEL (18). However, the HEL-specific B cells from the double transgenic mice had reduced levels of cell-surface IgM, but not IgD, and were unable to produce anti-HEL antibody (18).

As thymocytes migrate through the thymus, they also express different cell-surface molecules during maturation (19). Initially, they do not express the TCR, CD3, or either

of the co-receptors, CD4 or CD8, and are referred to as double negative cells (20, 21). Then gene rearrangement occurs and low levels of the T cell receptor α and β chains are expressed along with both co-receptor molecules, CD4 and CD8, producing double positive cells (20, 21). It is at this point that thymocytes are screened for their ability to recognize self MHC molecules, and for whether they will react to self peptides presented on self MHC. These two events have been termed positive and negative selection, respectively.

Positive selection occurs when thymocytes encounter thymic stromal cells expressing self MHC. Thymocytes with TCRs that bind self MHC are signaled to continue their maturation, whereas thymocytes that do not recognize self MHC undergo programmed cell death. Classic experiments performed in bone marrow chimeric mice demonstrated that bone marrow cells injected into irradiated allogeneic or semiallogeneic hosts developed into T cells that "learned" to recognize antigen only on the MHC molecules expressed on the radioresistant thymic epithelial of the irradiated host (22, 23). The arrival of transgenic technology facilitated the direct demonstration of positive selection. Thymocytes bearing a transgenic TCR only developed into mature peripheral T cells when they could recognize the MHC molecules that were presenting peptide in the thymus (24-26). When these cells were unable to recognize the MHC molecules in the thymus, they died at the $CD4^+CD8^+$ stage of development. The percentage of CD4 and CD8 cells found in the periphery of the transgenic animals was also influenced by the expression of the transgenic TCR. TCR recognition of peptides on class I resulted in a predominance of peripheral $CD8^+$ T cells, whereas a higher percentage of $CD4^+$ T cells resulted from peptide recognition on class II MHC. These experiments demonstrated that the TCR, MHC, and CD4 or CD8 molecules were all involved in the process of positive selection.

The positive selection process permits survival of T cells with TCRs capable of recognizing foreign peptide on self MHC, as well as those recognizing self peptide on self MHC. The negative selection process, mediated by thymic macrophages and dendritic cells, effectively eliminates thymocytes with T cell receptors that bind with high affinity to self antigen in association with self MHC. Deletion of autoreactive thymocytes was first demonstrated in the laboratory of John Kappler using an antibody that detects $V\beta17a^+$ T cells, which react with an endogenous superantigen plus the MHC class II protein I-E, to show that these cells were present in the thymus but not in the periphery of mice expressing the I-E MHC molecule (27). Direct demonstration of negative selection was also shown using transgenic mice. Kisielow and colleagues found deletion of TCR transgenic T cells in male mice that expressed a receptor specific for the male (H-Y) antigen restricted to class I $H\text{-}2D^b$ MHC molecules (28). In other experiments, thymic presentation of the transgenic self antigen, OVA, following i.p. injection, resulted in the deletion of OVA-specific TCR transgenic $CD4^+CD8^+TCR^{lo}$ thymocytes by apoptosis (29).

Both positive and negative selection require T cell recognition of peptide presented on MHC molecules. How these two selection events differ is a very active area of research and at least three possibilities have been put forward: an affinity model, an altered ligand model, and an avidity model. The idea behind the affinity model is that high affinity interactions between the TCR and a peptide/MHC complex result in negative selection, while positive selection is the result of low affinity binding between the TCR and self MHC (30). The altered ligand hypothesis suggests that the positively selecting thymic stromal cells present a unique set of peptides, whereas the bone marrow cells responsible for negative selection present peptides that are representative of those found in the periphery. This hypothesis appears to be unlikely for two reasons: recent analysis of peptides eluted from thymic MHC molecules does not indicate that unique peptides are presented by

cortical epithelium (31), and a variety of other cell types expressing MHC molecules, such as fibroblasts or lymphoid cells, are able to induce positive selection (32, 33). Lastly, the differential avidity model proposes that positive selection is a low avidity interaction, resulting from high affinity/low density or low affinity/high density interactions between the TCR and peptide/MHC complexes. Negative selection is a high avidity interaction, resulting from high affinity/high density interactions or aggregation of the TCR upon peptide/MHC recognition. This model suggests that peptide presented above a given threshold will induce some physiological change in the TCR and will deliver an incomplete activation signal, which is sufficient to induce positive selection but inadequate to trigger apoptosis and negative selection (34, 35).

When the selection process is complete, TCR expression is upregulated and, depending on the specificity of the TCR, the expression of either CD4 or CD8 is downregulated. These mature single positive T cells then exit the thymus. Unfortunately, T cell selection in the thymus cannot eliminate every self-reactive T cell, because some self peptides may not reach the threshold of presentation that ensures tolerance induction of the developing T cells (36, 37), and other self antigens may not be presented in the thymus during the selection process. Therefore, inactivation of mature self-reactive T cells must also occur in the periphery for self antigens that are developmentally regulated or for self antigens that can only be found outside of the thymus. Before addressing the potential mechanisms of peripheral tolerance, it is important to understand how mature lymphocytes become activated by antigen.

C. *Lymphocyte Activation*

B cells become activated when they recognize and bind multivalent antigen in the periphery. Some antigens are able to activate B cells directly, and they are referred to as thymus-independent antigens or TI antigens. There are two types of TI antigens: polyclonal B cell activators which at high concentrations activate both immature and mature B cells, regardless of their specificity; and antigens that activate only mature B cells by extensive crosslinking of the cell-surface immunoglobulin molecules, due to their highly repetitive structures, such as bacterial cell wall polysaccharides (38). Thymus-dependent or TD antigens require T cell help to activate the B cells. Protein antigens are bound by B cell surface immunoglobulin molecules, internalized, processed, and then presented to helper T cells as peptides on class II MHC molecules (39). T helper cells specific for these peptides provide help for B cell antibody production in two ways: through the interaction of the cell-surface molecules CD40 ligand and CD40 expressed on T and B cells, respectively, and from T cell cytokine secretion (40). These two signals induce B cell proliferation and promote B cell differentiation into antibody-secreting cells.

T cells require two distinct signals from APCs to become fully activated (41, 42). The first signal is through the TCR upon recognition of antigen in the context of self MHC molecules. The second signal is referred to as costimulation, and it includes locally acting soluble cytokines, such as IL-1 and IL-6 (43), and cell-surface molecules found on professional antigen presenting cells, like the B7 molecules, that bind to the cell-surface molecule CD28 on T cells (44). Dendritic cells, macrophages, and B cells can all function as professional APCs, although macrophages and B cells need to be activated by antigen first before they will express the appropriate costimulatory molecules. Signal one, antigen recognition, in the absence of signal two, costimulation, results in inactivation or anergy of

mature T cells (45). Anergy is one potential way of inducing peripheral tolerance to self antigens and will be discussed more thoroughly in the next section.

Since costimulation is critical for successful T cell activation, it is an area of intense research. Recently, our understanding of T cell costimulation has become more complex with the discovery that there are two B7 family members found on APCs, B7-1 and B7-2, capable of binding the counter-receptors, CD28 and CTLA-4, expressed on the surface of T cells. The kinetics of expression of each of these molecules is unique, suggesting their expression may correlate with different roles in the induction, amplification, and downregulation of the immune response. For example, both B7-1 and B7-2 molecules are capable of inducing T cell proliferation and IL-2 production, but B7-2 is constitutively expressed (46), suggesting it may participate in the initiation of the immune response. Although B7-1 expression can be found constitutively on some APC, on others it appears 48 hours after activation (47), indicating that it may serve to amplify or regulate the immune response.

The counter-receptors are also expressed differentially. CD28 is constitutively expressed at high levels on all mouse T cells, whereas CTLA-4 expression cannot be detected until 2-3 days after T cell activation. The contribution of each of the costimulatory molecules in T cell activation has been further elucidated by the creation of mice deficient in one or more of these molecules. Examining the T cell responses of CD28 deficient animals has indicated that the major B7-binding costimulatory ligand on T cells is CD28 (48, 49). A negative regulatory role for CTLA-4 was initially suggested from experiments showing that cross-linking CTLA-4 on activated T cells would result in antigen-specific apoptosis (50). CTLA-4 has been directly implicated in the downregulation of T cell activation, because CTLA-4 deficient mice have massive lymphoproliferative disorders and die at three weeks of age (51, 52).

The APC plays a pivotal role in T cell activation, because, as mentioned above, if it does not provide the proper costimulatory signals, the T cell will be inactivated. Each type of APC, dendritic cells, macrophages, and B cells, not only varies in their expression of the B7 molecules, but has different roles in the immune response. Dendritic cells have been implicated in initiating T cell responses, because they constitutively express the costimulatory molecules B7-1 and B7-2 and also have high levels of class I and class II MHC molecules on their cell surface (53). These properties, in addition to their expression of the adhesion molecules ICAM-1, ICAM-3, and LFA-3, make them potent stimulators of naive T cells. It has been suggested that the role of the dendritic cells of the skin, epidermal Langerhans cells, in the immune response is to bring antigen from the site of infection to the lymphoid tissues. Langerhans cells, unlike mature dendritic cells, are able to pick up and process antigen efficiently, and following infection they migrate to the LN, where they then differentiate into dendritic cells. Recently, it has been shown that Langerhans cells, previously thought to be negative for costimulatory molecule expression, have B7-2 on their cell surface (54).

The highly phagocytic macrophages are the scavenger cells of the immune system. They are an important part of the innate immune response, since they express Fc and complement receptors, allowing them to engulf opsonized particles. They also possess receptors for microbial constituents, such as the macrophage mannose receptor and receptors for lipopolysaccharide, a component of the cell walls of Gram negative bacteria (55). Resting macrophages or monocytes do not express the costimulatory molecule B7-1, but they do express B7-2, and they have low levels of class II MHC molecules on their cell surface. Macrophages upregulate the expression of B7-1 and MHC class II when they engulf microorganisms or are stimulated through other receptors. Therefore, they can contribute to the adaptive immune response by activating naive T cells (56).

B cells not only contribute to the adaptive immune response by the production of antibody, but they are also very efficient APC for specific antigens because of their cell surface immunoglobulin receptors. They can pick up 10,000-fold lower concentrations of soluble antigen than other non-specific antigen presenting cells (39, 57). Resting B cells, although they constititutively express high levels of class II MHC molecules, are unable to activate naive T cells because they do not express either of the costimulatory B7 molecules. However, B7-1 and B7-2 expression can be induced on B cells by crosslinking their immunoglobulin antigen receptor, or by stimulation with the microbial component lipopolysaccharide (58), or by CD40L or cytokines.

When naive T cells become activated following antigen recognition on a professional APC, they proliferate, secrete their autocrine growth factor IL-2, and express the high affinity IL-2 receptor (59). The increased production of IL-2 is the direct result of the costimulatory signal derived from the APC, as the ligation of the CD28 molecule on the T cell both increases the transcription of IL-2 mRNA and stabilizes IL-2 mRNA (60, 61). The production of IL-2 allows T cells specific for the peptide antigen on the APCs to expand and differentiate into effector populations. There are at least three possible T cell effector populations each having different functions in the immune response: class I MHC-restricted cytotoxic $CD8^+$ T cells, and two subsets of $CD4^+$ class II MHC-restricted T helper cells, Th1 and Th2. It has been suggested that Th1 and Th2 cells arise from a common precursor cell referred to as the Th0 cell. Resting naive $CD4^+$ T cells develop into a pluripotential Th0 cell upon primary stimulation with a specific antigen. Th0 cells secrete lymphokines characteristic of both mature T helper subsets. Th1 cells secrete the cytokines IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 (62). The cytokines secreted by Th1 cells activate macrophages, mediate delayed type hypersensitivity reactions, and provide some help for B cells, with IFNγ promoting IgG2a

antibody production. Th2 cytokines are involved primarily with B cell help, as IL-4 promotes IgG1 and IgE antibody formation. IL-4 also has been implicated in the suppression of Th1 responses. Th1 and Th2 cells secrete mutually inhibitory cytokines, which cross regulate each other (63). Therefore, the development of Th1 or Th2 effector cells determines whether the resulting immune response will be cell-mediated or antibody-mediated, respectively (64). In this thesis, I show that repeated exposure to low concentrations of soluble antigen selectively inactivates the Th1 response.

D. Peripheral Tolerance

As mentioned previously, when B cells and T cells mature, precautions are taken to ensure that lymphocytes recognizing self antigen are eliminated. Because not all self antigens are expressed at high levels during the lymphocyte development, and because self reactive B and T cells can be found in the periphery of normal healthy individuals, there must be mechanisms to maintain tolerance in mature lymphocytes to self antigen encountered in the periphery. Evidence that self-reactive cells can be uncovered in the periphery has been shown in normal mice challenged with myelin basic protein (MBP). Autoreactive T cells specific for MBP are not deleted during maturation, and following challenge with MBP in adjuvant they can even be activated to induce experimental allergic encephalomyelitis (EAE), a model for multiple sclerosis (65).

For autoreactive B cells there are at least three mechanisms to keep these cells from secreting antibody that recognizes self proteins. As mentioned above, for most mature B cells to become activated they require help from T cells in the form of cell-surface interactions as well as from the cytokines produced by the helper T cell. Therefore, if the helper T cell is unresponsive or tolerant to the peripheral self antigen, the autoreactive B cell

will not be provided with the help it requires to proliferate and differentiate into an antibody-secreting cell (66). The other mechanisms for maintaining B cell tolerance to self antigens in the periphery are the same as those found during development. Membrane-bound antigens cause deletion and soluble antigens induce anergy or inactivation. Russell and colleagues demonstrated peripheral deletion of autoreactive B cells in a double-transgenic model; anti-K^b immunoglobulin transgenic B cells were present in the bone marrow but were deleted in the periphery due to liver expression of the MHC class I antigen K^b (67). However, Goodnow and colleagues found when B cells encounter high concentrations of soluble self antigen in the periphery they cannot respond to the self antigen and lose the expression of cell-surface IgM. This was shown by adoptively transferring transgenic B cells specific for HEL into an environment where their auto-antigen was expressed in the periphery, i.e., by using irradiated ML5 HEL transgenic mice, with high serum levels of HEL, as recipients (68). The same results were found by mating the HEL-specific B cell-transgenic mice to ML-4 metallothionein-HEL-transgenic mice, that express 10-fold lower levels of HEL than the ML5 mice. The transgenic B cells in these animals were not tolerant, but when the concentration of HEL was increased, by feeding the mice zinc in their drinking water, the previously nontolerant peripheral HEL-specific B cells were inactivated, as shown by downregulation of surface IgM and an inability to mount a HEL antibody response (68).

T cell tolerance to peripheral self antigens is most likely maintained by one of three mechanisms: clonal anergy or inactivation, clonal ignorance, and/or suppression. Anergy is the result of T cells recognizing antigen presented by APC in the absence of costimulation. T cells inactivated in this way will not proliferate in response to antigen even if it is subsequently presented by an APC expressing costimulatory molecules, because they are unable to produce their autocrine growth factor, IL-2, which prevents them from

proliferating and differentiating into effector cells. This may be one way of maintaining T cell tolerance to tissue antigens. Anergy was first shown using paraformaldehyde-fixed APC, which have lost the expression of costimulatory molecules, to present antigen to Th1 cell clones (69). Anergy *in vitro* has also been demonstrated using purified class II molecules on planar membranes (70), and IFN-γ-induced class II-expressing keratinocytes (71).

An *in vivo* model of T cell anergy was shown by Burkly and colleagues, in which the class II MHC molecule, I-E, was expressed only on pancreatic β-cells in mice that normally lacked I-E (72). In a mixed lymphocyte culture, the T cell proliferative responses to the I-E antigen were quite reduced in the transgenic animals. These T cells were also unresponsive to TCR-crosslinking, and it was shown that the unresponsiveness was not due to deletion, as the $V\beta17^+$ T cells specific for I-E plus superantigen could be detected in the periphery. Another *in vivo* example of anergy has been demonstrated by injecting Mls-1^a-bearing spleen cells i.v. into Mls-1^b mice. $V\beta6^+$ T cells, reactive to Mls-1^a, were not deleted in the Mls-1^b mice, but persisted as unresponsive cells that were unable to produce IL-2 (73). Recent research has shown that T cell anergy is due to an early block in the Ras signaling pathway, thus the ability to produce IL-2 is lost (74, 75).

The second possible mechanism for maintaining T cell tolerance to self antigens in the periphery is ignorance. In this circumstance the self antigen may be present at such a low concentration or may not bind well enough to the MHC molecules that it is not presented efficiently in the thymus, and therefore the autoreactive T cells are not deleted. Although the autoreactive T cells are present in the periphery, they do not normally respond to the self antigen, most likely because it is presented poorly, and are said to be in a state of immunological ignorance. T cell ignorance was shown by Ohashi and colleagues in double-transgenic mice that expressed the LCMV lymphocytic choriomeningitis viral

glycoprotein on the pancreatic β-cells in TCR transgenic mice in which the TCR was specific for LCMV and H-2D^b (76). The autoreactive T cells were not deleted and did not attack the pancreatic β-cells, but CD8$^+$ T cell-mediated diabetes resulted when the double-transgenic mice were infected with LCMV. This result suggests that the auto-antigen was not previously presented in a manner to allow for T cell activation, and activated T cells could enter and destroy islets.

The third mechanism for peripheral T cell tolerance is suppression. In suppression, one population of antigen-specific T cells actively suppresses the immune response of another population of T cells. This was shown by Kumar and Sercarz when they found that the injection of T cell clones, specific for a TCR peptide presented by class II MHC, could protect naive mice from MBP-induced EAE (77). Another group was able to protect rats against MBP-induced EAE by injecting a synthetic peptide representing the hypervariable region of the TCR Vβ8 molecule prior to challenge with MBP in adjuvant (78). Cytotoxic CD8$^+$ T cells may provide an additional way to suppress an immune response. For example, T cell suppression of humoral responses resulted from the ability of class II-restricted CD8$^+$ cytolytic T cells (CTL) and CD4$^+$ CTL to lyse antigen-specific B cells presenting exogenous protein antigens (79). Although suppression can be demonstrated in several experimental systems, the exact mechanisms of antigen recognition and suppression are not firmly established. It is also unknown whether suppressor cells play a role in the maintenance of self tolerance. However, recently Powrie and colleagues published evidence that CD45RB^{lo} Th2 cells were capable of suppressing CD45RB^{high} Th1 cells that mediated both a protective response against *Leishmania major* and a pathogenic response against a self antigen (80).

E. Acquired Tolerance

One way to understand the mechanisms of peripheral tolerance to self proteins is to study acquired tolerance to foreign proteins. In acquired tolerance previous exposure to antigen under certain conditions results in antigen-specific nonresponsiveness instead of priming for a secondary response. Like peripheral self tolerance, acquired tolerance involves inactivation of mature T cells. The first experiments on acquired tolerance were performed in the 1960's, when it was shown that three factors give rise to acquired tolerance: the route of antigen administration, the dose, and the form of the antigen. When soluble protein antigen is administered either i.v. or i.p., by repeated injections of low doses (10 μg) or one high dose (1 mg), in a deaggregated form, a subsequent immunogenic challenge of antigen in CFA reveals antigen-specific unresponsiveness. Several different antigens, bovine serum albumin, human gamma globulin, bovine gamma globulin, and flagellin, were found to induce acquired tolerance when given in this manner (81-86). The antigen-presenting cell responsible for acquired tolerance to soluble proteins has not been identified, but experiments performed in our lab indicated that B cells can be tolerogenic APC.

The first evidence in our lab that small resting B cells could be tolerogenic APC for T cells was demonstrated by Elizabeth Eyon in experiments where antigen was targeted to small resting B cells (87). She injected naive mice with deaggregated rabbit anti-mouse IgD Fab fragments and then challenged them with alum-precipitated Fab non-immune rabbit immunoglobulin. Antigen presentation was limited to resting B cells, because they express the IgD B cell receptor, and they can process and present Fab fragments while remaining in a resting state. Animals treated in this manner were profoundly tolerant, as shown by a significant reduction in antibody production. Victoria Yuschenkoff provided

additional evidence that resting but not activated B cells could be tolerogenic APC (88). When she injected spleen cells from transgenic mice expressing the human μ chain on the surface of mouse B cells into their nontransgenic littermates tolerance was induced, as measured by antibody production, to the human μ chain. Injection of spleen cells from double-transgenic mice, expressing both the human μ chain and the costimulatory molecule B7-1 on B cells, did not induce tolerance, indicating that only resting B cells could be tolerogenic. These findings along with those of others show that small resting B cells, which can process and present antigens but which lack costimulatory activity, can act as potent tolerizing APC for mature peripheral T cells (87-93).

Presentation of soluble antigen by antigen-specific B cells is particularly efficient at low antigen concentrations, because B cells can use their antigen receptors to capture and concentrate soluble antigen and deliver it into an antigen-processing pathway. Thus, at low antigen concentrations, the only cells capable of binding enough antigen to allow specific recognition by T cells may be the antigen-specific B cell. Therefore, I considered the possibility that rare antigen-specific B cells play a necessary role in self tolerance to low concentrations of soluble self proteins and in acquired tolerance to low doses of soluble foreign protein antigens.

This thesis examines the role of B cells in tolerance to low doses of self and foreign protein antigen by comparing tolerance induction in normal and B cell-deficient animals. In the first set of experiments, T cell proliferation assays were performed to determine whether B cells were necessary for *in vivo* priming of naive T cells. In the next experiments, I examined the effect of treatment with low doses of antigen on the resulting immune response following challenge with antigen in CFA by measuring antibody titers in untreated and treated normal animals. I also examined how low doses of antigen affected the development of $CD4^+$ T helper cell subsets by measuring cytokine production from T

cells stimulated *in vitro*. Finally, in experiments performed in two different haplotypes of transgenic animals, I addressed how the efficiency of antigen presentation can affect tolerance induction.

CHAPTER II

MATERIALS AND METHODS

Mice. C57BL/6, B10.A, and C3H mice were purchased from the National Cancer Institute (Frederick, MD). The mice were 6-20 weeks old at the start of each experiment. B cell-deficient μMT mice (16) were obtained from Dr. Charles Sidman (University of Cincinnati, OH) and were bred at the University of Massachusetts Medical Center (UMMC) animal facility. The mice were backcrossed six generations to the μMT C57BL/6 strain. The B cell-deficient mice were maintained on SulfaTrim Pediatric Suspension (Barre-National Inc., Baltimore, M.D.) containing 1280 mg sulfamethoxazole and 256 mg trimethoprim per liter of drinking water, for one week every other week. C57BL/6 mice transgenic for hen egg lysozyme (HEL) linked to the zinc-inducible mouse metallothionein promoter were kindly provided by Dr. Chris Goodnow (Stanford University, Palo Alto, CA) (94). HEL transgenic mice of the ML-4 line have a basal serum concentration of HEL of < 0.5 ng/ml, and the ML-5 HEL transgenic mice have 20 ng/ml of serum HEL (94). B cell-deficient mice carrying the HEL transgene on the H-$2^{b/b}$ background were generated by mating male mice of the ML-4 line to B cell-deficient female mice and backcrossing the HEL transgenic progeny to B cell-deficient mice. To introduce the high responder H-2^{k} haplotype, these HEL-transgenic, B cell-deficient mice were bred to H-$2^{k/k}$ mice that were heterozygous for the μMT disrupted μ heavy chain locus. The latter were (B10.BR x μMT) F2 mice given to us by Dr. Anthony Vella (National Jewish Center, Denver, CO). We screened for B cell-deficient mice by testing peripheral blood obtained from the progeny at 6 weeks of age for the absence of $B220^{+}$ cells by flow cytometry as described below. Female CBA (H-2^{k}) and B10.A (H-2^{a}) mice were bred to male mice of the HEL-

transgenic ML-4 line to create F1 ($H\text{-}2^{b/k}$ or $H\text{-}2^{b/a}$) HEL transgenic and nontransgenic mice. All animals were maintained in microisolator cages under specific pathogen-free conditions.

Antigens. HEL, OVA, and pigeon cytochrome c (PCC) were purchased from Sigma Chemical Co. (St. Louis, MO). HEL was further purified by gel filtration over a G75 Sephadex column in 5% acetic acid and by HPLC in 0.1% trifluoroacetic acid with a 60% linear gradient of acetonitrile in the Peptide Synthesis / Antibody Production core facility at UMMC. PCC peptide 81-104 and the HEL peptides (20-35, 30-43, 46-61, 74-88, 81-96, 116-129) were synthesized in the UMMC Peptide Synthesis / Antibody Production core facility. The purified protein derivative of *Mycobacterium tuberculosis* (PPD) was prepared from desiccated *M. tuberculosis* H37RA microorganisms (Difco Laboratories, Detroit, Michigan) according to a protocol from Paul Allen (personal communication). Briefly, *M. tuberculosis* organisms were suspended in PBS at 100 mg/ml, homogenized by brief sonication, and then clarified by centrifugation at 14,000 rpm using a Beckman JA-18.1 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA) for 20 minutes at 4°C. The supernatant was removed, filtered successively through 0.45μ and 0.22μ millipore filters, and stored in a freezer at -70°C. PPD was used at a final dilution of 1:200 in all assays.

Other Reagents. All LN, spleen, and purified T cell proliferation assays were performed in Click's medium (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). Cells were harvested and washed in either RPMI 1640 tissue culture medium (Gibco BRL, Gaithersburg, M.D.), or 1x Balanced Salt Solution (BSS), made in the departmental media facility, with 1.5% FBS

added. IL-2 and IL-4 sensitive cell lines were maintained in RPMI 1640 (Sigma Chemical Co.) with 10% FBS added. Additional reagents are listed below as needed.

Identification of HEL Transgenic Mice. The HEL transgene was detected by PCR. DNA was obtained from tissue derived from ear punch marking as previously described (95). Briefly, ear tissue was added to 20 µl of 50 mM Tris-HCl (pH 8.0), 2 mM NaCl, 10 mM EDTA, 1% SDS, and 1 µl of 20 mg/ml proteinase K. The mixture was incubated twice at 55°C for 15 min., with vigorous vortexing after each incubation. Distilled water was added to a final volume of 200 µl, and the mixture was heated for 5 min at 100°C and used in the PCR reaction after cooling or stored at -20°C. PCR reactions were performed in a final volume of 50 µl, consisting of 1 µl of DNA and 49 µl of a reaction mix containing 5 µl of a 10x amplification buffer (670 mM Tris-HCl pH 8.8, 160 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$), 5 µl of 50 mM 2-mercaptoethanol (ME), 20 µl of a deoxynucleotide triphosphate mix (312 µM each of dATP, dTTP, dGTP, dCTP) (Boehringer, Mannheim, Indianapolis, IN), 1 µl (1.5 units) of Taq polymerase (Boehringer, Mannheim), and 18 µl of the oligonucleotide primers (50 ng of HEL3F, 33.5 ng of HEL4R, and 30 ng of each of the endogenous Ig primers). The 50 µl reaction mix was then layered with 60 µl mineral oil (Sigma Chemical Co.). The PCR reaction was performed using two sets of primers, one set for the HEL transgene and another set for an endogenous Ig gene. The HEL primer sequences were HEL3F: 5'- GAG CGT GAA CTG CGC GAA GA-3', and HEL4R: 5'- TCG GTA CCC TTG CAG CGG TT-3' (Bio-Synthesis, Inc., Lewisville, TX) (C. Goodnow, personal communication). The endogenous immunoglobulin gene primer sequences were sense: 5'CTG GAG CCC TAG CCA AGG AT-3', and antisense: 5'-ACC ACA GAC CAG CAG GCA GA-3'. The HEL primers amplified a 160 bp transgenic band in transgenic animals only, and the Ig primers amplified a 264 bp endogenous band in all animals. PCR was performed using a Hybaid

thermal reactor (National Labnet, Co., Woodbridge, NJ) under the following conditions: initial denaturation at 94°C for 5 min., followed by 30 cycles of 1 min. at 94°C, 1 min. at 59°C, and 1.5 min. at 72°C, followed by a 9 min. elongation at 72°C. To visualize the PCR-amplified DNA, 5 µl of loading buffer (Promega, Madison, WI) was added to a 20 µl aliquot of the reaction, loaded onto a 2% agarose gel along with a 123 bp DNA ladder (Gibco BRL), and electrophoresed. The gel was then stained with ethidium bromide, and the DNA bands were visualized by ultraviolet transillumination.

Flow Cytometry. B cell-deficient HEL transgenic ($H\text{-}2^{b/b}$) and ($H\text{-}2^{b/a}$) F_1 mice were identified by testing for the absence of B cells by fluorescence staining of B220 on peripheral blood lymphocytes. Blood, 300-500 µl, was collected from the tail veins of mice into tubes containing 50 µl heparin (1000 U/ml) (Elkin-Sinn Inc., Cherry Hill, NJ) in 2.5 ml RPMI 1640 plus 1.5% FBS, and then centrifuged for 10 minutes at 1400 rpm in a Sorvall RT6000 centrifuge (Dupont, Wilmington, DE) at 4°C. Red cells were lysed with 500 µl Tris pH 7.2, NH_4Cl at 37°C for 5 min. Remaining leukocytes were washed once with medium, and centrifuged for 8 minutes. Cells were then resuspended in 50 µl FACS buffer (PBS with 1.5% FBS and 0.1% sodium azide), transferred to V-bottom tubes, and kept on ice during the staining protocol. The cells were first incubated for 30 minutes with a 1:1000 dilution of anti-B220 antibody, RA3.6B2 (Dr. Nancy Phillips, UMMC), then washed once in FACS buffer, centrifuged, and resuspended in 50 µl FACS buffer. Next, a 1:600 dilution of mouse anti-rat kappa FITC-OX12 (Serotec, Ltd., Oxford, UK) was added, the cells were incubated for 30 minutes, and then washed twice with FACS buffer. After the second wash, the cells were resuspended in 100 µl FACS buffer and fixed for 5 minutes at room temperature by adding 40 µl of 4% paraformaldehyde. The fixed samples

were washed twice with FACS buffer and resuspended in a final volume of 250 µl FACS buffer. The samples were analyzed by the UMMC Flow Cytometry Facility.

Tolerance induction. HEL or OVA were centrifuged at 160,000 g in an airfuge (Beckman Instruments, Inc.) for 60 minutes immediately before injection, and the top third of the solution was used. Ultracentrifuged HEL or OVA were diluted to a concentration of 50 µg/ml in PBS, and 0.2 ml was injected intravenously into the lateral tail vein of C57BL/6 and of homozygous µMT B cell-deficient mice three times a week for three weeks. Seven days after the last injection the mice were challenged subcutaneously in each hind footpad with 20 µl of 100 µg HEL or OVA in complete Freunds adjuvant (CFA) (Difco Laboratories).

Proliferation assays. Proliferation assays were set up with either whole LN cells, purified T cells (see below), or spleen cells. Mice were challenged with 20 µl of 100 µg HEL, OVA, or PCC emulsified in CFA in each hind footpad. Ten days later the animals were sacrificed, the popliteal lymph nodes were harvested, and single cell suspensions were made. Spleens were harvested from mice 28 days after challenge, and the red blood cells were lysed with Tris pH 7.2 NH_4Cl. Both LN and spleen cells were washed and then counted. Whole LN cultures or spleen cells from individual animals were set up at 2×10^5 or 4×10^5 cells/well in triplicate in 0.2 ml in flat-bottom 96-well plates in Click's medium (Irvine Scientific) with 10% FBS (Hyclone Laboratories) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 5×10^{-5} 2-ME added. The plates were incubated at 37°C for 96 hours in 5% CO_2 and pulsed with 1 µCi [^{3}H] thymidine (TdR) (Amersham Corporation, Arlington Heights, IL), specific activity 5 Ci/mmol, for the last 6

hours. Assays were harvested on a Wallac cell harvester (Gaithersburg, MD) and counted using a Beta-Plate counter (Wallac).

Proliferation assays with purified T cells. Mice were challenged, and LN cells were harvested as above. The LN cells were incubated at 4°C for 30 minutes with a rat anti-class II antibody, M5/114.15.2, that recognizes I-$A^{b,d,q}$ and I-$E^{d,k}$ with or without anti-B220, RA3.6B2 (Dr. Nancy Phillips). T cells were then purified by panning on rabbit anti-mouse Ig-coated plates at 4°C by the method of Wysocki and Sato (96). Spleens of naive C57BL/6 mice were used as a source of APC and were depleted of T cells as previously described (97). Briefly, the red blood cells were lysed with Tris pH 7.2, NH_4Cl and washed. An anti-T cell cocktail, consisting of anti-Thy-1, anti-CD4, and anti-CD8 antibodies, was added, and the cells were incubated on ice for 30 minutes. After a single wash, a mouse anti-rat κ antibody was added, and the cells were incubated on ice for an additional 30 minutes. Agarose-absorbed guinea pig complement was added directly, the cells were incubated for 30 minutes at 37°C, and then they were centrifuged for 10 minutes at 1400 rpm. Next, the cells were overlayed onto high density Ficoll hypaque (p = 1.09), centifuged for 20 minutes at 3000 rpm, and the interface cells were collected and washed. Six x 10^5 purified T cells were incubated with 2 x 10^5 irradiated (3000 cGy) APC. The cultures were pulsed and harvested as above.

B cell purification. Spleens were harvested from naive C57BL/6 mice and single cell suspensions were made. The red blood cells were lysed with Tris pH 7.2 NH_4Cl, washed, and then depleted of T cells as above. Following the T cell depletion the cells were washed twice, counted, washed again in RPMI 1640 without serum, and

resuspended in RPMI 1640 without serum at 1 x 10^8 cells/ml (0.1 ml cells/thigh) or 2 x 10^8 cells/ml (0.2 ml cells i.v.) for transfusion into B cell-deficient mice.

ELISA. Anti-OVA antibody titers were determined using the previously described sandwich ELISA (87). Briefly, the plates were coated with OVA, blocked overnight with PBS+1% BSA, and washed with PBS/0.05% Tween. Serial three-fold dilutions of mouse sera starting at 1:40, in PBS+1% BSA, were performed, and the plates were incubated at room temperature for 30 minutes. The plates were washed, then incubated with peroxidase-conjugated goat anti-mouse IgG + IgM (H + L chains) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), developed with 3, 3', 5, 5'-tetra-methyl-benzidine (ICN Biologicals, Lisle, IL) with H_2O_2, and stopped with 2 M H_2SO_4. The plates were read at 450 nm on a plate reader (Molecular Devices Corp., Sunnyvale, CA). The standard for these assays was an anti-OVA hyperimmune serum. The isotype of the anti-OVA antibodies was determined by using a mouse monoclonal antibody isotyping kit (Zymed, San Francisco, CA). The kit included the biotinylated anti-mouse antibodies IgG1, IgG2a, IgG2b, and IgG3, HRP-Streptavidin, and 2,2-azino-di[3-ethylbenzthiazoline sulfonic acid] (ABTS) substrate. Mouse serum was initially diluted 1:400 for IgG1 measurement, 1:5 for IgG2a measurement, 1:200 for IgG2b measurement, and 1:10 for IgG3 measurement, and further serial two-fold dilutions were performed. The plates were washed with PBS/0.05% Tween, the appropriate biotinylated antibodies were added, and then the plates were incubated at 37°C for 30 minutes and washed again. Next, HRP-Streptavidin was diluted 1:50, added to the plates, and another 30 minute incubation at 37°C was performed. The plates were developed at room temperature with ABTS, diluted 1:50 in substrate buffer containing 0.1 M citrate pH 4.2 with 0.03% H_2O_2, and read at 405 nm. Standard plates were coated with phenylarsonate-BSA, followed by purified

monoclonal mouse anti-phenylarsonate IgG1, IgG2a, IgG2b, and IgG3 antibodies, provided by Dr. Nancy E. Phillips (98). The isotype concentrations of the mouse serum samples were determined by comparing O.D. values, corresponding to the linear portion of the standard curve, with those of the standards.

IFN-γ detection by ELISA. 150μl of culture supernatant was collected at 72 hours from OVA-primed LN cells set up at 6 to 8 x 10^5 cells/well in Click's medium in 96-well plates. At the start of the *in vitro* incubation, 100 μg/ml OVA or PPD were added to the cultures. For the ELISA, Falcon microtiter plates (Becton Dickinson, Oxnard, CA) were coated overnight with 50 μl of purified mouse anti-IFN-γ (Pharmingen, San Diego, CA) at 2 μg/ml and then blocked overnight with PBS/10% FBS. The plates were washed with PBS/0.05% Tween-20, 0.2 ml of supernatant was added, serial two-fold dilutions were performed, and then the plates were kept at 4°C overnight . After washing the plates, 100 μl of biotinylated mouse anti-IFN-γ (Pharmingen) at 1 μg/ml was added, and the plates were incubated at room temperature for 45 minutes. The plates were washed again, and incubated at room temperature for 30 minutes with 100 μl of a 1:400 dilution of 1 μg/ml avidin-peroxidase (Sigma Chemical Co.). The plates were developed with the substrate ABTS (Sigma Chemical Co.) with H_2O_2 added. The concentration of IFN-γ was determined by comparing the O.D. values of the samples with the purified recombinant mouse IFN-γ standard (Pharmingen).

IL-2 and IL-4 detection by bioassay. Supernatants were collected at 24 or 48 hours from OVA-primed LN cells set up at 6 to 8 x 10^5 cells/well in Click's medium alone or with PPD or 100 μg/ml OVA. The concentration of IL-2 was determined by the proliferation of

the IL-2-dependent cell line, HT-2. HT-2 cells were set up at 2 x 10^4 cells/0.2 ml/well in 96-well flat bottom plates in duplicate with two-fold serially diluted culture supernatants. The cultures were pulsed with 1 μCi [^{3}H]TdR for the last 4 hours of the 24 hour incubation. Recombinant mouse IL-2 (Dr. Nancy E. Phillips) (99) was used as a standard in the assay. In some experiments, IL-2 activity was blocked using the monoclonal anti-IL-2 antibody (S4B6, from Dr. Tim Mosmann, University of Alberta, Edmonton, Canada), and IL-4 activity was blocked using anti-IL-4 (11B11, from Dr. William Paul, National Institutes of Health, Bethesda, MD). The concentration of IL-4 activity was determined by the proliferation of the IL-4-dependent cell line, CT-4S (provided by Dr. William Paul), as previously described (100). Briefly, serial two-fold dilutions were performed on the culture supernatants, and the assay was incubated for 24 hours and then pulsed overnight with 1 μCi [^{3}H]TdR. The IL-4 standard used in the assay was provided by Immunex Corp. (Seattle, WA). Assays were harvested and counted as above.

Statistical Analysis. Significant differences between groups were evaluated using a two-tailed Students t-test assuming equal variances. Statistical analysis was performed using a general mixed model analysis of variance (BMDP Statistical Software, Inc., Los Angeles, CA).

CHAPTER III

T CELL PROLIFERATIVE RESPONSES IN NORMAL AND B CELL-DEFICIENT ANIMALS

The goal of my experiments was to determine whether B cells were required for tolerance induction to low levels of soluble protein antigen. The most direct way of addressing the requirement of B cells in tolerance induction was to induce tolerance in the absence of B cells, using μMT B cell-deficient mice (16). I decided to measure tolerance induction in the B cell-deficient mice by T cell proliferation assays. As discussed below, the role of B cells in priming naive T cells to whole protein has been controversial in the literature, and my first aim was to determine whether I could measure T cell proliferative responses in the absence of B cells.

A. T Cell Proliferative Responses are Reduced in B Cell-Deficient Animals

Before I could examine the proliferative responses of the B cell-deficient animals, I needed to establish a protocol that measured T cell proliferative responses in normal mice. I examined many parameters for the assay: the site of injection, the draining lymph nodes with the highest number of responding cells, the day of LN harvest post-challenge, the *in vitro* cell densities necessary for an optimal response, and the antigen specificity of the response. Thus initial experiments indicated that the most effective site of injection was the hind footpads, instead of at the base of the tail. The antigen-specific T cells were confined to the draining popliteal LN, because no proliferation was observed when assays were

performed with cells from the inguinal LN. I also found that 10 days post-challenge was the optimal time for harvesting the LN T cells. Therefore, in all of the proliferation assays shown, unless otherwise noted, mice were injected with antigen in adjuvant in the hind footpads and the popliteal lymph nodes were harvested 10 days after challenge and set up *in vitro* with various antigens.

As shown in Fig. 1, I addressed the question of antigen specificity by priming normal mice with CFA alone or with OVA emulsified in CFA. Ten days later, LN cells were set up *in vitro* in medium alone, or with PPD, a component of the adjuvant, or with increasing doses of antigen. Lymph node cells both from normal mice primed with CFA or OVA in CFA proliferated to the antigen found in the adjuvant, PPD. Only LN cells from mice primed with OVA in CFA proliferated to OVA, and this proliferative response was dependent upon the dose of OVA *in vitro*. The proliferative response of both groups of animals was better at 4 x 10^5 cells/well than 2 x10^5 cells/well. The response of the LN cells from mice primed with CFA alone indicated that the OVA-specific proliferative response was due to the presence of OVA in the adjuvant, and therefore I did not continue to include the CFA control in my experiments.

After establishing the conditions of the T cell proliferation assay in normal mice, I was ready to test the proliferative responses of the B cell-deficient animals. First, I wanted to ensure that the B cell-deficient mice, created by targeted gene disruption of the transmembrane portion of the IgM molecule, did not contain any mature B cells (16). Peripheral blood from normal and B cell-deficient mice was stained for the B cell antigen B220. The results shown in Fig. 2 indicate that the peripheral blood of the B cell-deficient animals was completely negative for $B220^+$ B cells. These results confirmed the findings that B cell precursors failed to progress beyond the early pre-B stage, as shown in the initial description of the μMT B cell-deficient mice (16, 101).

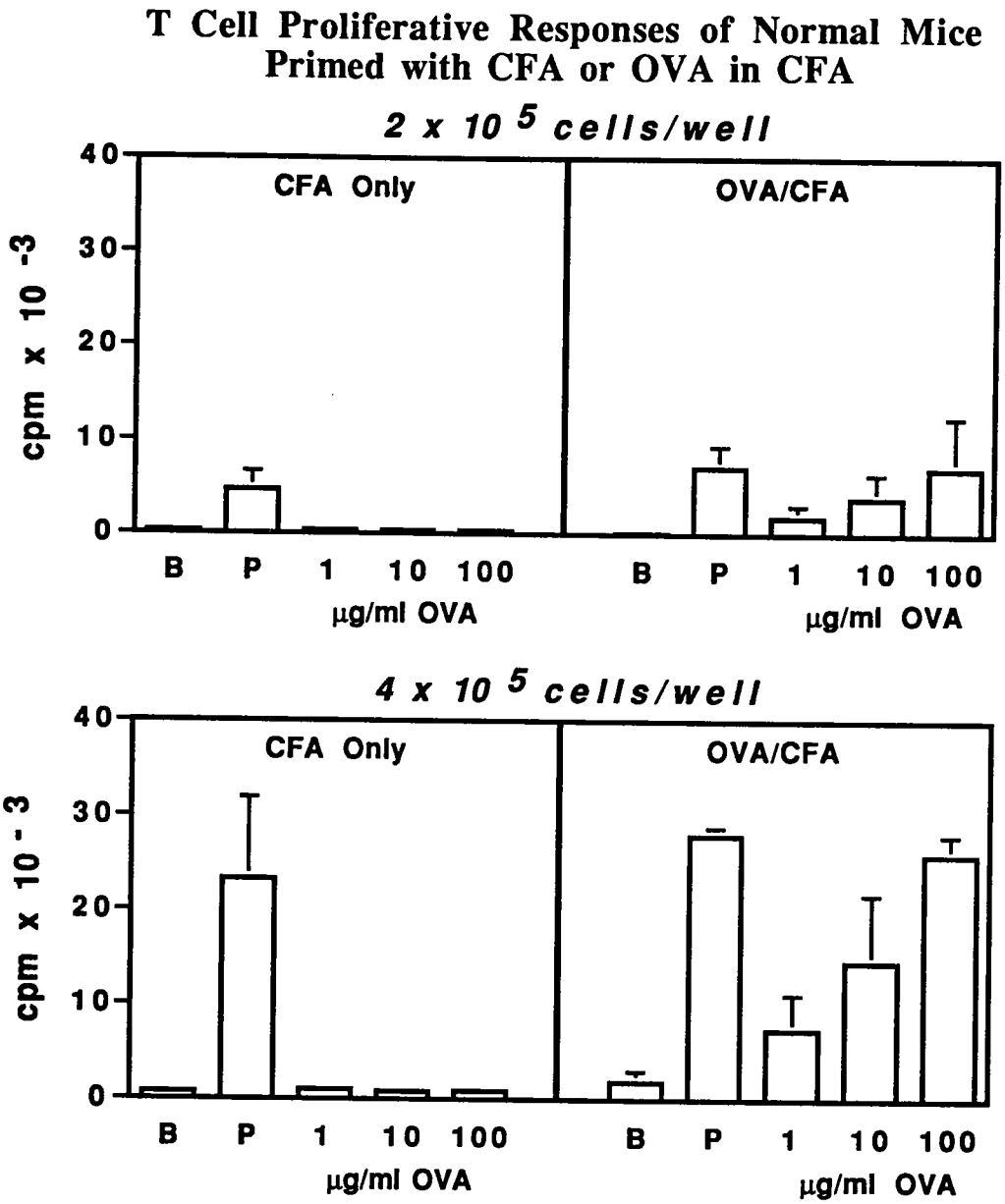

Figure 1. Two mice were primed with CFA and three mice were primed with OVA emulsified in CFA. Ten days later, the popliteal lymph nodes were harvested, and the cells were cultured in triplicate in vitro at the cell densities indicated in medium alone (B), PPD (P), or increasing doses of OVA. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Error bars represent 1 SD.

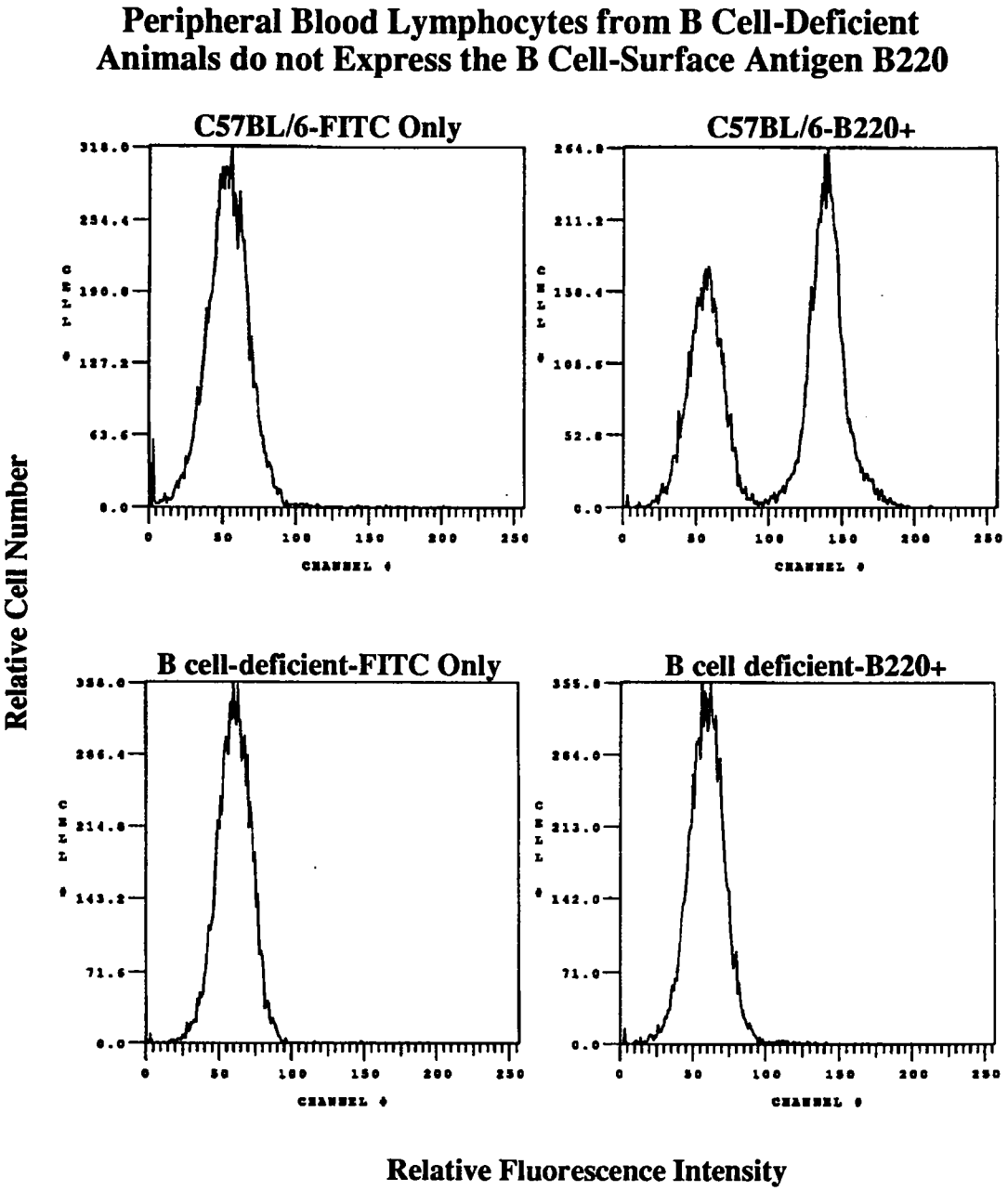

Figure 2. Expression of B220 on peripheral blood lymphocytes from C57BL/6 or B cell-deficient mice. Lymphocytes were stained with a rat anti-mouse B220 monoclonal antibody, followed by mouse anti-rat kappa FITC-OX12. The panels on the left represent lymphocytes stained with the second antibody only. The mean channel fluorescence in the C57BL/6 mice is 90.

After confirming the absence of B cells in the B cell-deficient animals, I was ready to test the T cell proliferative responses in these mice. Earlier experiments performed in animals made B cell-deficient by anti-μ treatment indicated that B cells were necessary for efficient priming of naive T cells (102-105). Other experiments performed in SCID mice reconstituted with T cells alone and performed recently in μMT mice demonstrated that T cells can be effectively primed in the absence of B cells (101, 106, 107). To test T cell proliferative responses in the absence of B cells, I primed normal mice and B cell-deficient mice with OVA in CFA and set up LN T cells with antigen 10 days post-challenge. The results of the proliferation assay shown in Fig. 3 demonstrate that the LN cells from normal animals were responsive to PPD and OVA. However, the overall proliferative response of LN T cells from B cell-deficient animals to both these antigens was 5 to 10-fold lower than the proliferative response of cells from normal animals. Since the ultimate goal of my experiments was to measure tolerance induction by T cell proliferation in individual B cell-deficient mice, the next set of experiments were designed to improve the antigen-specific response of LN cells from B cell-deficient animals.

B. B Cells Contribute to in vivo Priming and in vitro Proliferation of T Cells

The differences in the proliferative responses between the normal and B cell-deficient animals suggested that antigen presentation by B cells *in vivo* and/or *in vitro* may enhance the T cell proliferative response of the normal animals. First, I decided to investigate the role of B cells in priming T cells *in vivo*. The importance of B cell antigen presentation to T cells *in vivo* was initially reported by Ron and Sprent in experiments with mice treated with anti-μ. They found that the proliferative response of these B cell-deficient

LN Proliferative Responses are Reduced in B Cell-Deficient Animals

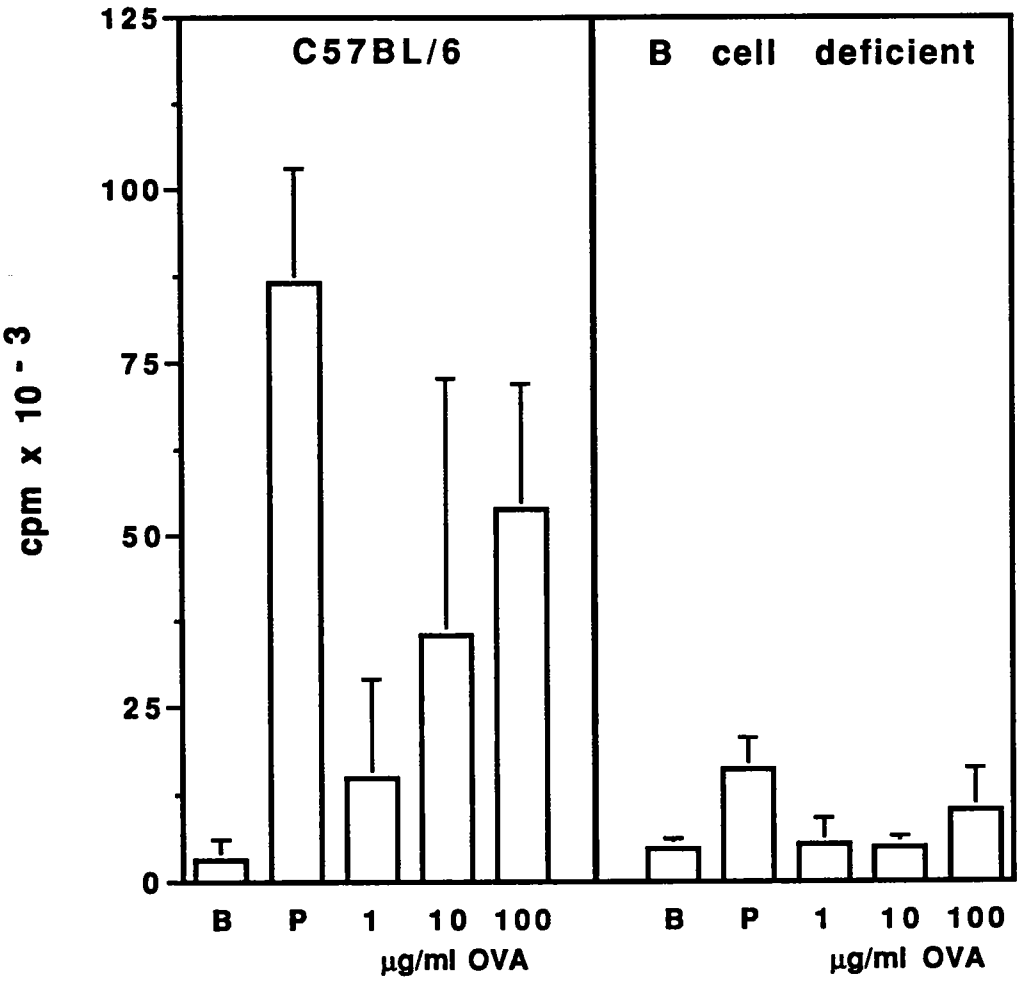

Figure 3. Six normal and six B cell-deficient mice were primed with OVA emulsified in CFA. The popliteal lymph nodes were harvested 10 days later. Cells were pooled from each group and set up in vitro at 4 x 10^5 cells/well in triplicate in medium alone (B), PPD (P), or increasing doses of OVA. The cultures were pulsed with [^{3}H]TdR for 6 hours at the end of a 96 hour incubation. Error bars represent 1 SD.

animals was restored by subcutaneous (s.c.) injection of B cells prior to s.c. injection of antigen in CFA (103). To test whether I could reproduce their findings, I injected B cells i.v. or s.c. into the μMT B cell-deficient animals prior to priming with OVA in CFA. As shown in Fig. 4, although the proliferative response of LN cells from normal animals remained much higher than the response of LN cells from the B cell-deficient animals with or without the addition of B cells *in vivo*, it appeared that the *in vitro* proliferative response of at least 3 out of 6 B cell-deficient animals was improved by the injection of B cells i.v. prior to challenge. The s.c. injection of B cells also seemed to enhance the OVA response of LN cells from 4 out of 5 B cell-deficient mice. Unfortunately, these findings were not consistently reproducible; therefore, I decided to examine the contribution of B cell antigen presentation *in vitro* to the T cell proliferative response (108).

One way to test whether B cells were important for T cell proliferative responses *in vitro* was to provide additional APCs in the *in vitro* cultures. I decided to test two different APC populations: naive APC from T-depleted spleen cells, and OVA-primed APC from T-depleted LN cells of normal mice primed with OVA in CFA in the hind footpads. Normal and B cell-deficient mice were primed with OVA in CFA, and the two different APC populations were added *in vitro* to the LN T cells. The results shown in Fig. 5 indicate that the addition of either naive APCs or OVA-primed APCs to LN cells from B cell-deficient animals did not enhance the proliferative response to antigen *in vitro*. The proliferative response of the normal animals was slightly enhanced by the addition of OVA-primed APC.

Addition of B Cells in vivo to B Cell-Deficient Mice Improves Their Proliferative Response

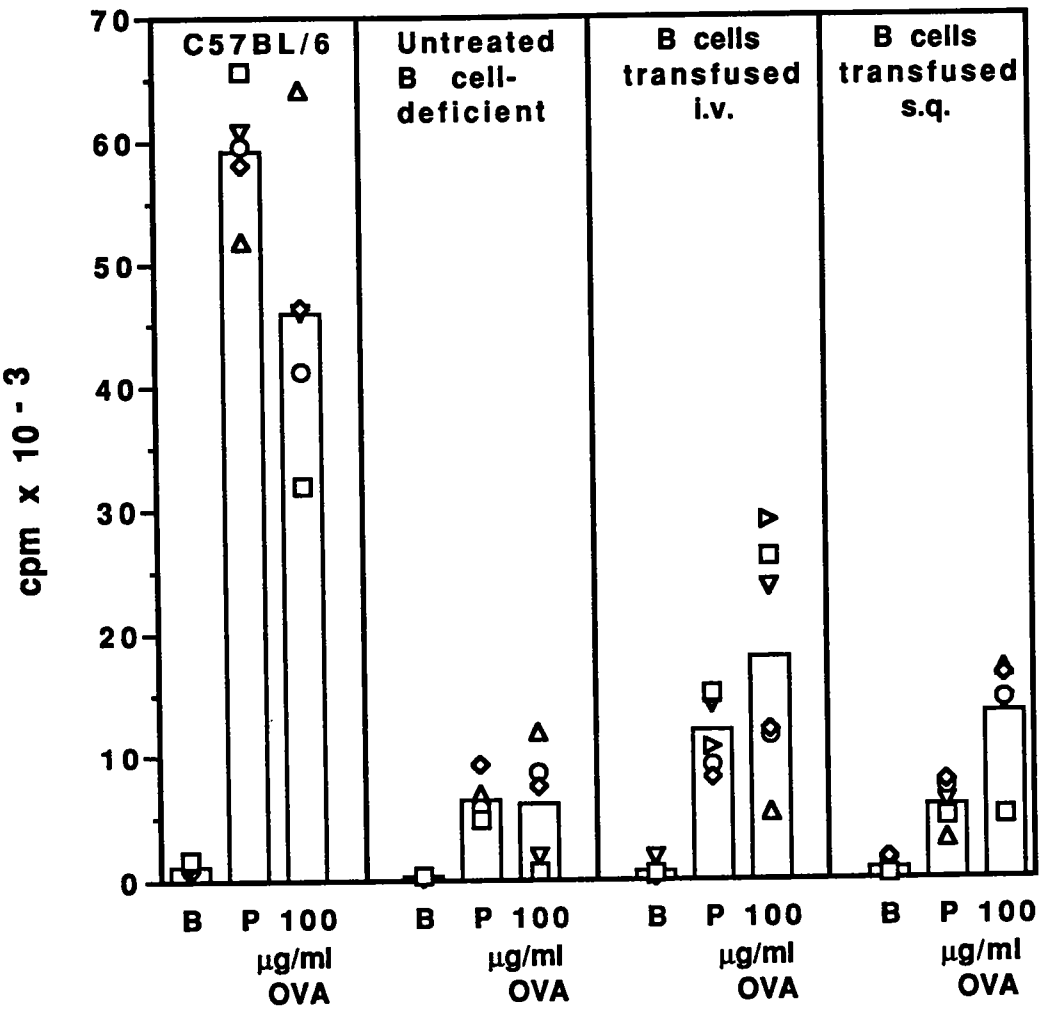

Figure 4. Six B cell-deficient mice were transfused with 40 x 10^6 B cells i.v. and 5 B cell-deficient mice were transfused with 1 x 10^7 B cells s.q. C57BL/6, untreated B cell-deficient mice, and the transfused B cell-deficient mice were primed with OVA in CFA and LN cells were harvested 10 days later. Whole LN cells from C57BL/6 mice were set up in triplicate in vitro at 4 x 10^5cells/ well and LN cells from B cell-deficient mice were set up at 2 x 10^5 cells/well in medium alone (B), PPD (P), or OVA. Symbols represent individual animals and the group average is represented by the bar. The cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation.

APC Addition in vitro does not Alter the Reduced Proliferative Response of B Cell-Deficient Mice

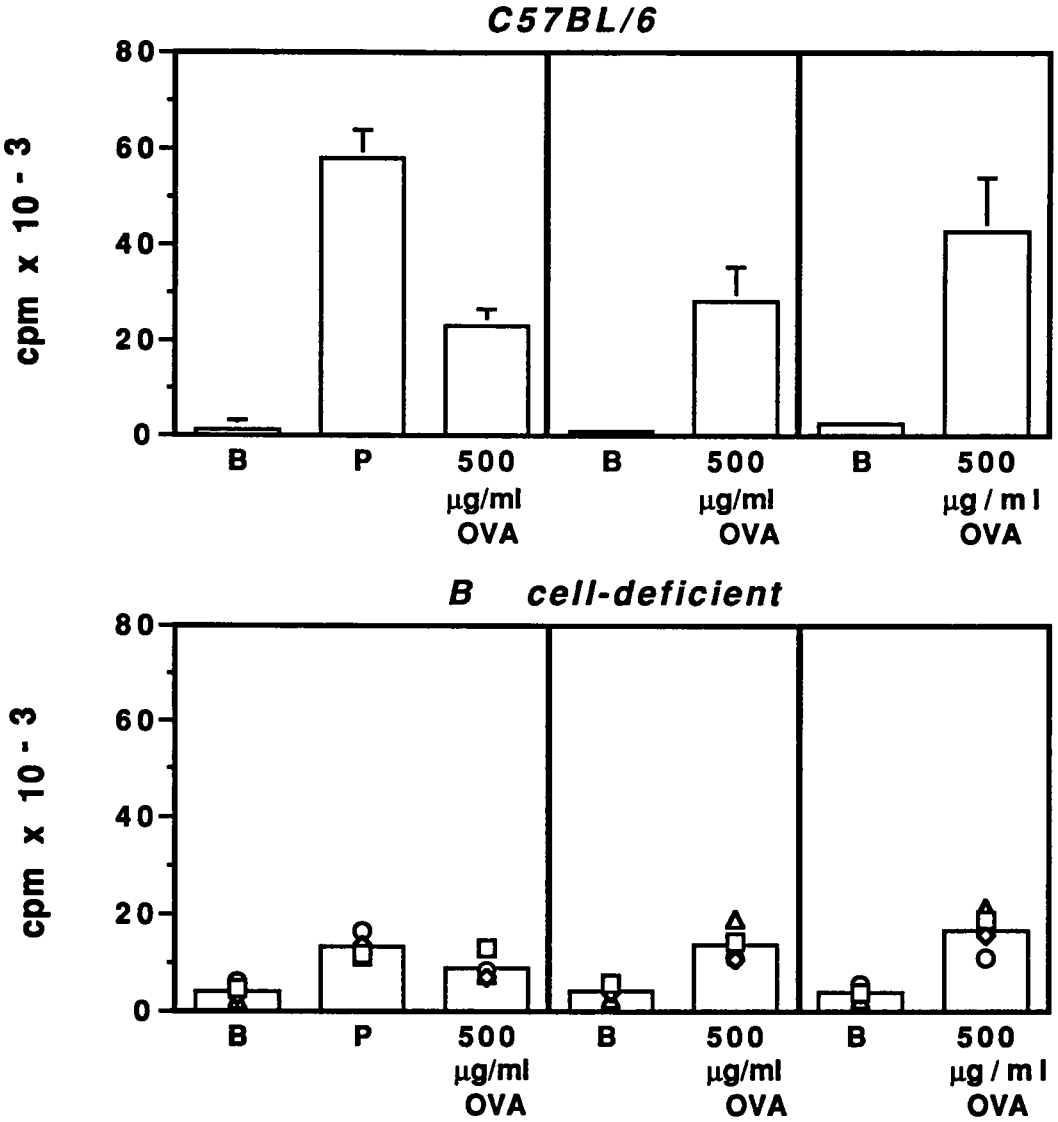

Figure 5. Three C57BL/6 and 4 B cell-deficient mice were primed with 400 µg OVA/footpad. Ten days later LN cells were harvested from each group. LN cells were set up in triplicate as shown at 2 x 10^5 cells/well in medium alone (B), PPD (P), or OVA. Spleen cells from an unprimed C57BL/6 mouse were T-depleted, irradiated, and used as APC. OVA-primed LN cells from 2 of the C57BL/6 mice were depleted of T cells, irradiated, and used as OVA-primed APC. Cultures were incubated for 96 hours and pulsed with [^{3}H]TdR for the last 6 hours. Cells were pooled from 3 mice in the top graph, symbols represent individual animals in the bottom graph, and the bars represent the mean. Error bars indicate 1 SD.

C. Proliferation Assays Performed with Purified T Cells

A more direct way to test for the contribution of B cell antigen presentation *in vitro* was to purify the T cells from the normal and B cell-deficient mice and add normal splenic APC to the proliferation assay. Normal and B cell-deficient mice were primed with OVA in CFA, and T cells were purified from lymph nodes harvested 10 days post-challenge. The purified T cells were set up *in vitro* at two different cell densities with or without additional APCs added to determine the effectiveness of the purification. As shown by the left panel of each graph in Fig. 6, there was a slight response to PPD and OVA in the absence of additional APCs, indicating that there were a few contaminating APCs remaining after the purification protocol. The T cell proliferative responses of both the normal and B cell-deficient mice increased in the presence of additional APCs, as shown on the right panel of each graph. Also, the proliferative response of T cells from B cell-deficient animals to OVA was now significantly above background ($p<0.002$ for 6×10^5 T cells, and $p<0.01$ for 4×10^5 T cells). The proliferative responses of both groups of mice were not significantly affected by the two different T cell densities tested. These results indicate that the purification of primed LN T cells reduced but did not abolish the difference between the proliferative responses of normal and B cell-deficient animals.

D. Peptide Priming in the Absence of B Cells Improves T Cell Proliferative Responses

It had been suggested that different forms of antigen, i.e. whole protein or peptide, required distinct APCs for T cell priming (109). B cells were required for presenting

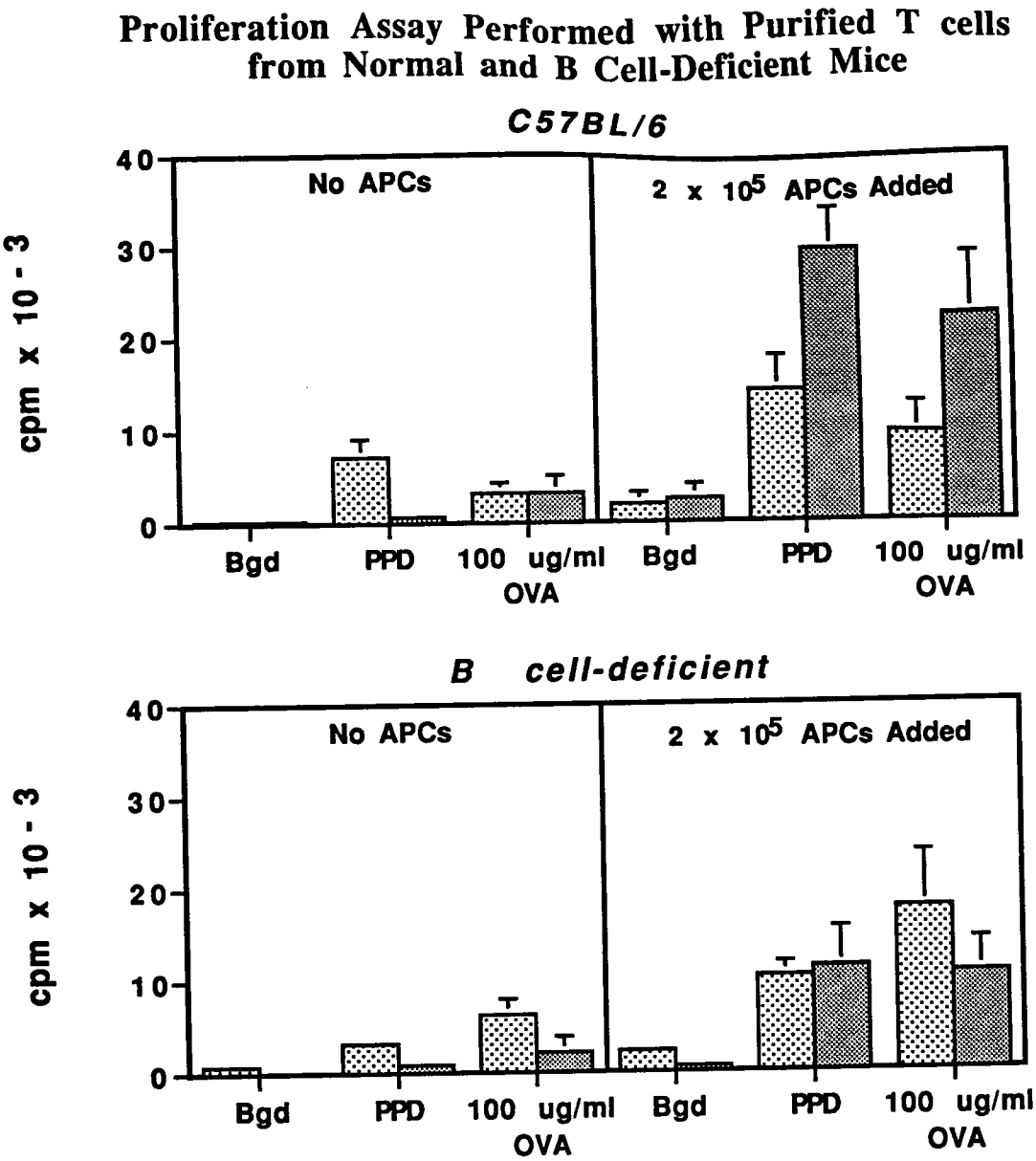

Figure 6. Seven C57BL/6 and 7 B cell-deficient mice were primed with OVA in CFA. Lymph nodes were harvested 10 days later. T cells were purified and set up in triplicate in vitro at 4 x 10^5 (dotted bars) or 6 x 10^5 (gray bars), with or without irradiated APCs added, in medium alone (Bgd), PPD, or OVA. The cultures were pulsed for 6 hours with [^{3}H]TdR at the end of a 96 hour incubation. Error bars represent 1 SD.

whole protein, whereas peptide antigen was presented most efficiently by dendritic cells. Therefore, an additional possibility for the proliferative differences observed between normal and B cell-deficient animals could have been due to the form of antigen used for T cell priming *in vivo*. I performed three experiments using either pigeon cytochrome c (PCC) whole protein in CFA or PCC peptide in CFA to prime normal and B cell-deficient mice. The results of the only experiment testing the proliferative response of purified T cells are shown in Fig. 7. In general, LN cells from mice primed with whole protein proliferated better *in vitro* to the whole protein antigen than to peptide antigen, and vice versa. In this experiment, the proliferative responses of the normal and B cell-deficient mice were comparable when the animals were primed with protein antigen. This result was not consistent with my findings from proliferation assays performed with OVA in normal and B cell-deficient animals. The differences observed in the proliferative responses between the normal and B cell-deficient mice may therefore depend on which antigen is used, that is PCC or OVA, as the proliferative response of the normal mice to the antigen PCC was markedly less than their proliferative response to OVA. Priming with PCC peptide resulted in a higher proliferative response in the B cell-deficient animals than in the normals animals, as the *in vitro* proliferative response to peptide and whole protein was greater in the T cells from peptide-primed B cell-deficient mice than in T cells from protein-primed B cell-deficient mice. In two out of three experiments I performed, the proliferative responses of B cell-deficient mice primed with peptide antigen were better than the proliferative responses observed from B cell-deficient mice primed with protein antigen.

The role of B cells in T cell priming to protein and peptide antigen remains unresolved, based on the results of my own experiments and also those of others performed in B cell-deficient mice (101, 110). There are likely to be many factors that contribute to the efficiency of T cell priming, and the requirement of B cells as APC

The Effect of Priming with Whole Protein or Peptide Antigen on the T Cell Proliferative Response

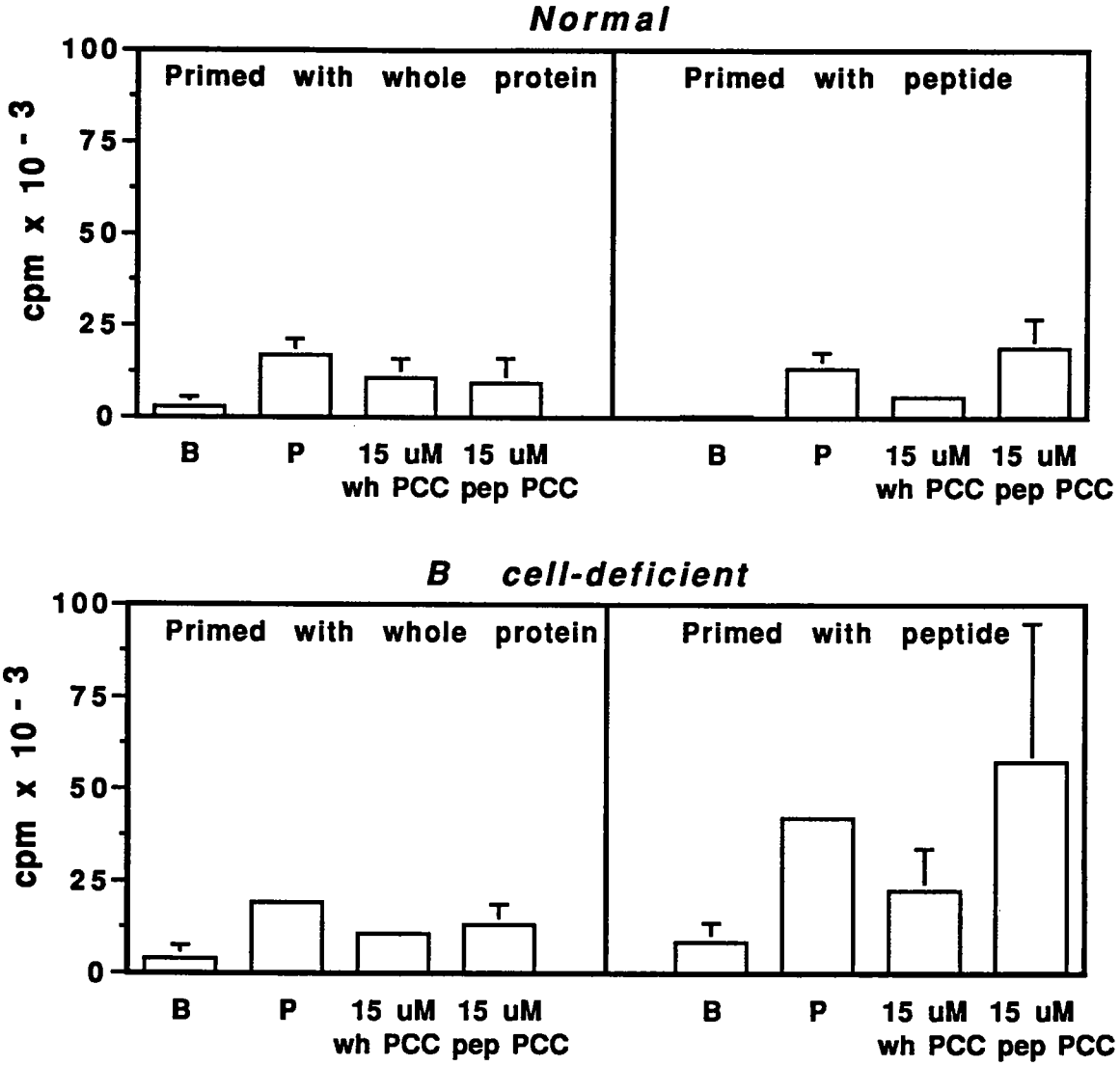

Figure 7. Four normal and 6 B cell-deficient mice were injected with 8.2 nmoles whole PCC or 31.25 nmoles PCC peptide emulsified in CFA in each hind footpad, and 10 days later LN cells were harvested. Purified T cells were set up in triplicate from each group at 4×10^5 cells/well along with 2×10^5 irradiated APC/well in medium alone (B), PPD (P), PCC whole protein, or PCC peptide. The cultures were pulsed for 6 hours with [^{3}H]TdR at the end of a 96 hour incubation. Error bars represent 1 SD.

probably depends not only on which antigens were used for the experiments, such as KLH, OVA, or PCC, but also on whether the T cells were purified or left unfractionated.

CHAPTER IV

THE ROLE OF B CELLS IN SELF AND ACQUIRED TOLERANCE

The next set of experiments was designed to determine whether B cells were required for self tolerance to low concentrations of soluble self proteins and for acquired tolerance to low dose soluble foreign protein antigens. We thought B cells might be necessary for tolerance induction to low concentrations of antigen because they are very efficient APC with low level antigen, and previous studies in our lab had shown that B cells, which lack costimulatory activity, can function as tolerizing APC for T cells.

I chose to use a transgenic mouse strain expressing low levels of HEL as a soluble serum protein to examine tolerance induction to a self protein. It was shown by Adelstein and colleagues that T cells from H-$2^{b/k}$ HEL transgenic mice of the ML-4 line are tolerant to HEL, although the HEL-specific B cells are not tolerized by the very low concentration of HEL in that particular transgenic line (less than 0.5ng/ml HEL in serum) (94). I was interested in determining whether HEL-specific B cells were required as HEL-specific APC to induce and maintain T cell tolerance to this transgenic self antigen, which might be present at a concentration too low to be taken up non-specifically and presented by other kinds of APC. Therefore, HEL-transgenic mice were bred with μMT B cell-deficient mice (16), and T cell tolerance to HEL was measured by T cell proliferation assays.

To follow the HEL transgene throughout the breeding scheme, mice were typed by PCR. PCR was performed with two sets of primers, one set for an endogenous Ig gene to ensure that the PCR reaction was working, and another set to screen for the HEL transgene. The results of PCR amplification of DNA from nontransgenic and transgenic littermates is shown in Fig. 8. The Ig primers amplified a 264 base pair band in both

PCR Amplification of DNA from Nontransgenic and HEL-Transgenic Mice

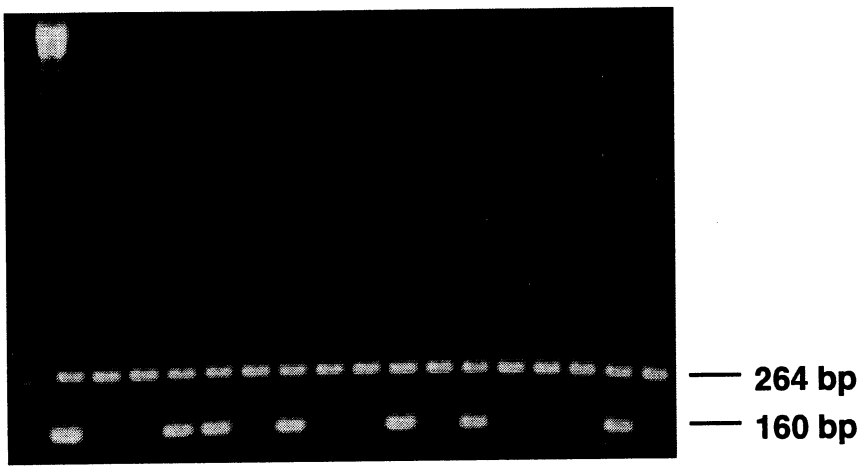

Figure 8. PCR Analysis of DNA prepared from (C57BL/6 x ML4) mice. PCR was performed with two sets of primers: the HEL primers amplified a 160 bp transgenic band in the transgenic animals only, and the Ig primers amplified a 264 bp endogenous band in all animals. A 123 bp DNA ladder is shown on the left.

nontransgenic and transgenic animals, and the HEL primers amplified a 160 bp band in the transgenic animals only. The HEL-Tg and nontransgenic animals were then tested for the presence of B cells by staining for B220 as shown in Fig. 2.

A. T Cell Tolerance to a Transgenic Soluble Self Antigen (HEL) Does Not Require B Cells as Antigen Presenting Cells

The HEL-transgenic animals were created on the C57BL/6 background, and tolerance to the transgene-encoded HEL self antigen had been shown in H-$2^{b/k}$ transgenic animals (94). Therefore, I bred H-$2^{b/k}$ F1 B cell-deficient HEL-transgenic animals to determine whether B cells were necessary to maintain self tolerance to HEL. HEL-transgenic H-$2^{b/k}$ mice and B cell-deficient HEL-transgenic animals were primed with HEL in CFA, and the T cell proliferative responses measured. The results of the LN proliferation assay in Fig. 9 demonstrate that T cells from the normal HEL-transgenic mice are compromised in their ability to proliferate to HEL, as compared to the T cell proliferative response to HEL of the nontransgenic littermates ($p<0.001$), confirming the results of Adelstein and colleagues (94). The tolerance is antigen-specific, as indicated by the response of the T cells from both the HEL-transgenic and nontransgenic littermates to PPD, a component of the adjuvant. The same pattern of results was seen in the B cell-deficient animals. T cells from the HEL-transgenic B cell-deficient mice were unresponsive to HEL, but did respond to PPD, while T cells from the nontransgenic B cell-deficient mice proliferated to the highest dose of HEL *in vitro* as well as they did to PPD. The differences observed in the T cell proliferative response to HEL between the F1 HEL-transgenic B cell-deficient mice and F1 nontransgenic B cell-deficient mice were also significant ($p<0.001$).

B Cells are not Required for Peripheral Tolerance to the Soluble Self Antigen HEL

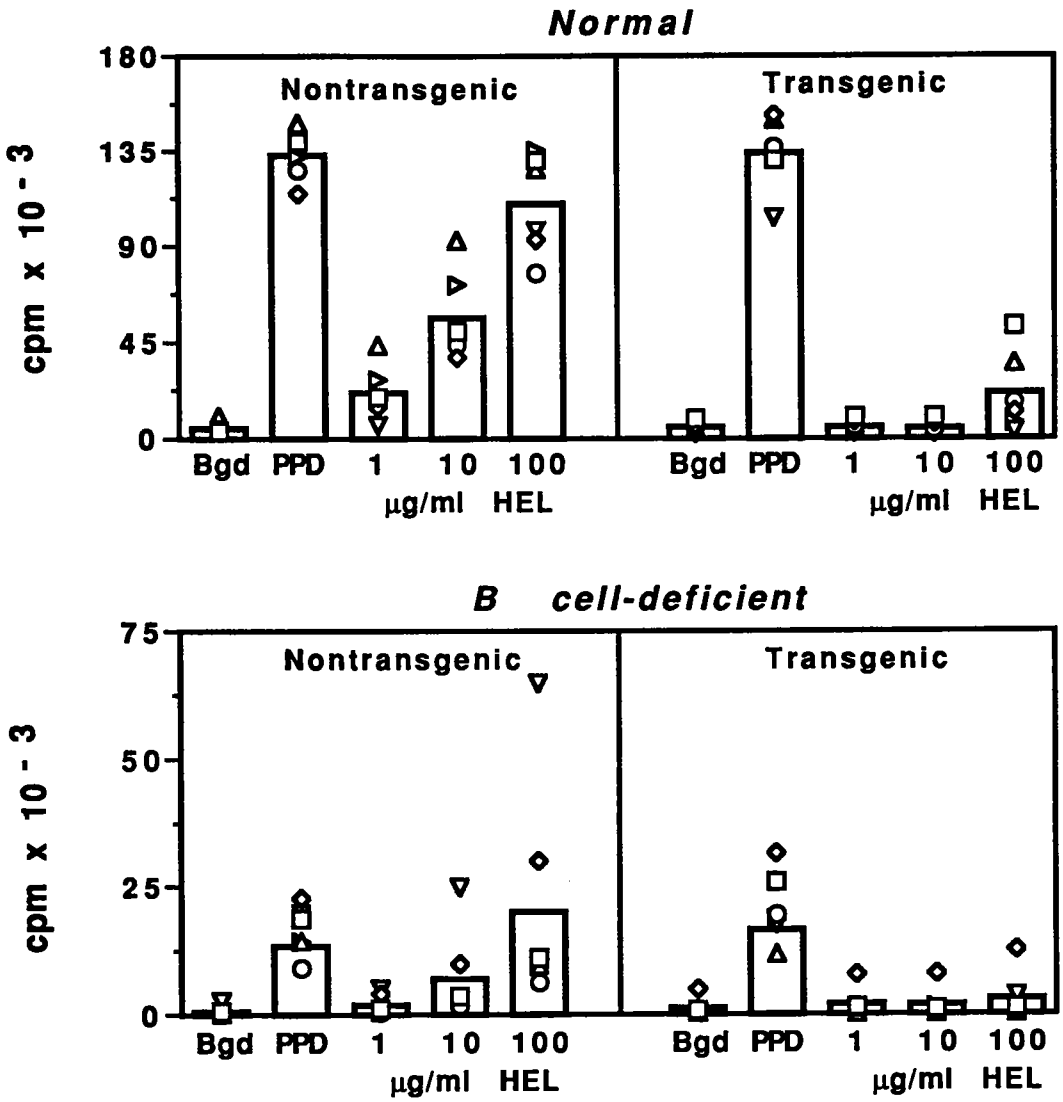

Figure 9. Six normal (H-$2^{b/k}$) mice and 6 B cell-deficient (H-$2^{b/k}$) mice were injected with 40 x 10^6 T-depleted B10.BR spleen cells i.v. and then with 100 µg HEL emulsified in CFA/hind footpad. Ten days later the draining lymph nodes were harvested. Four x 10^5 whole lymph node cells were cultured in triplicate with increasing doses of antigen and pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. The left side of each graph is the response of cells from the nontransgenic mice and on the right is the response of cells from the HEL-transgenic mice. Each symbol represents an individual animal, and the bar indicates the group average.

These results indicate that some other APC is responsible for inducing tolerance to HEL in B cell-deficient ML4 mice either in the thymus or in the periphery.

B. Reduced T Cell Proliferation caused by Repeated Injections of Low Concentrations of OVA into Mice does not Require Presentation by B Cells

To test whether B cells could act as tolerizing APC in the periphery when foreign antigen was given exogenously, I wanted to compare tolerance induction to foreign proteins in normal and B cell-deficient mice. First, to determine whether low zone tolerance induction could be measured by a reduction in T cell proliferative responses, normal mice were injected with 10 μg of deaggregated HEL i.v. three times a week for a period of three weeks. Then, the treated and untreated mice were challenged with HEL in CFA, and the LN T cell response to HEL was examined. As shown in Fig. 10, T cells from untreated normal mice responded to PPD, the control antigen, and in a dose-dependent manner to HEL. Mice treated with low doses of HEL prior to challenge were responsive to PPD, but proliferation was minimal at the highest HEL concentration. These results demonstrated that I could measure low zone tolerance induction in normal animals by *in vitro* T cell proliferation assays.

Migration of the HEL-responsive T cells out of the LN following treatment with low doses of HEL was one explanation for the reduced LN T cell proliferative responses observed in the treated animals. To address this possibility, I examined the proliferative response of splenic T cells from untreated and treated normal mice 28 days after challenge with HEL in CFA. Splenic T cells from untreated animals were able to proliferate to PPD and HEL, although this response was slightly less than the response observed 10 days

Low Zone Tolerance Can Be Measured by Reduced LN Proliferative Responses

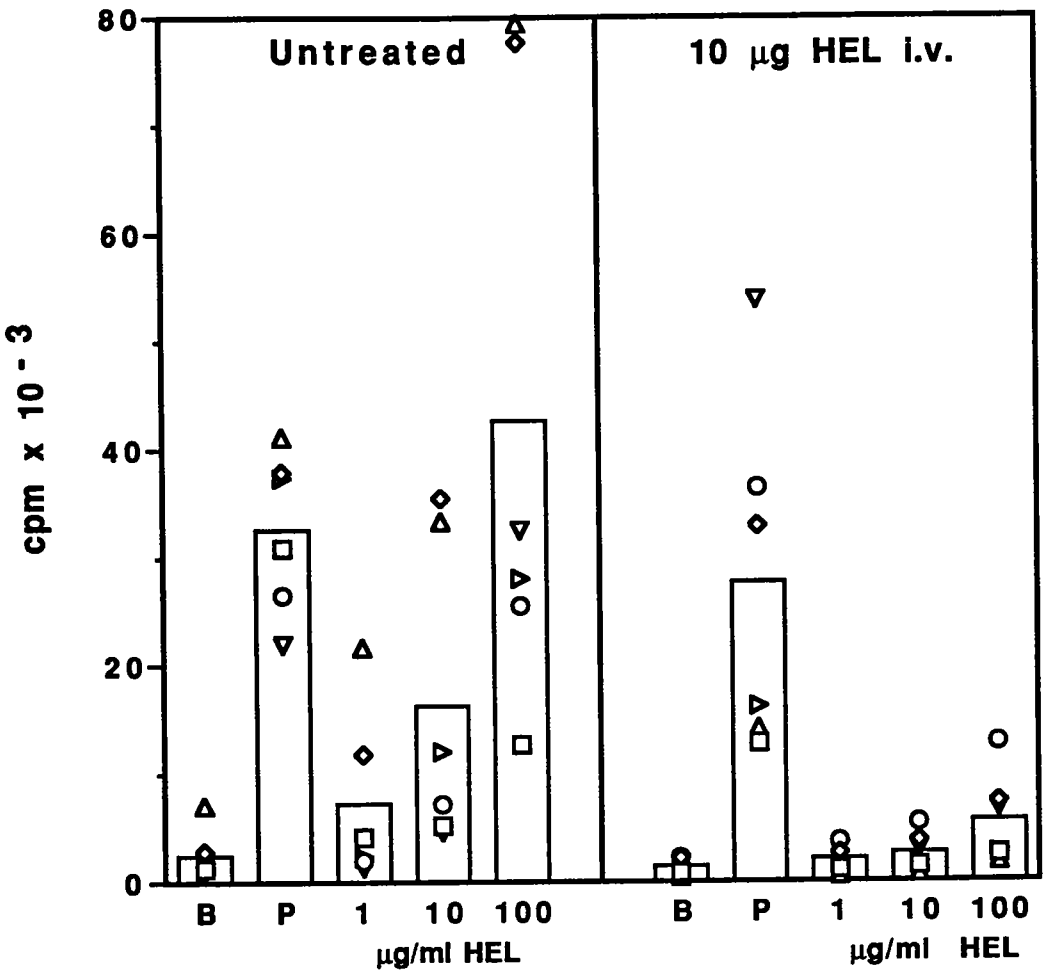

Figure 10. Six C57BL/6 mice were given 10 µg HEL i.v. 3 times a week for 3 weeks. Tolerized mice and 6 untreated controls were challenged 7 days later with HEL emulsified in CFA. Ten days post-challenge the popliteal LN were harvested. Cells were set up in triplicate in vitro at 2 x 10^5 cells/well in medium alone (B), PPD (P), or increasing doses of HEL. The cultures were pulsed with [^{3}H]TdR for the last 6 hours of a 96 hour incubation. Symbols represent individual animals, and the group mean is represented by a bar.

post-challenge from the LN T cells of the untreated animals (Figs. 10 and 11). Splenic T cells from the treated animals were compromised in their ability to proliferate to HEL, but not to PPD (Fig. 11), suggesting that the reduced LN T cell responses to HEL observed in the treated animals was not a consequence of the HEL-responsive T cells migrating to the spleen following treatment with low doses of deaggregated antigen.

After establishing that treatment with low doses of HEL resulted in antigen-specific nonresponsiveness upon subsequent challenge in normal animals, I was ready to address whether antigen presentation by B cells was required for low zone tolerance. Tolerance induction to low doses of HEL was compared in normal and B cell-deficient animals. The T cell proliferative response to HEL from treated normal animals was consistently reduced, but the results of the proliferation assays in the treated B cell-deficient animals using HEL as an antigen were inconclusive (data not shown). This may have been due to an inability to generate reproducible T cell proliferative responses to the HEL antigen in untreated B cell-deficient mice. It was also recently shown that the HEL antigen could induce B cell activation, by crosslinking the B cell antigen receptor (111). This is probably due to the ability of HEL to bind to serum proteins or cell surfaces. Because activated B cells have been shown to express costimulatory molecules and I wanted to test whether antigen presentation by resting B cells, in the absence of costimulation, would result in T cell unresponsiveness, I decided to switch to the antigen OVA to compare low zone tolerance induction in normal and B cell-deficient mice. In these experiments, mice were injected with 10 μg of deaggregated OVA i.v. three times a week for a period of three weeks. The treated and untreated mice were then challenged with OVA in CFA, and the T cell proliferative response to OVA was examined.

Figure 12 shows the results of three low zone tolerance experiments with normal and B cell-deficient mice. In the top panels of Fig. 12, T cells from normal mice injected

Low Zone Tolerance can be Measured as Reduced Proliferative Responses in the Spleen

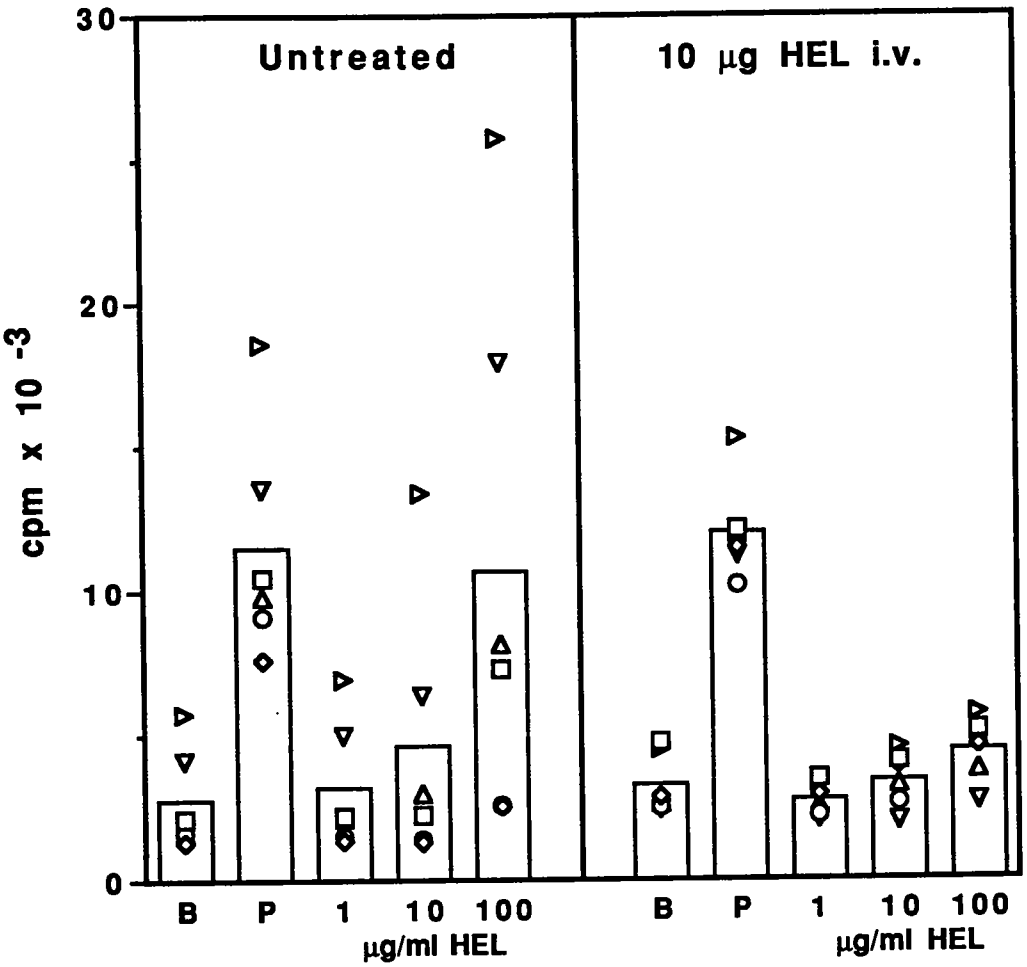

Figure 11. Six C57BL/6 mice were given 10 µg HEL i.v. 3 times a week for 3 weeks. The tolerized mice and 6 untreated controls were challenged 7 days later with HEL in CFA. Twenty-eight days post-challenge, spleens were harvested from control and tolerized mice and the cells were set up in triplicate at 4 x 10^5 cells/well in medium alone (B), PPD (P), or increasing doses of HEL. The cultures were pulsed with [^{3}H]TdR for 6 hours at the end of a 96 hour incubation. Symbols represent individual animals, and the group mean is represented by a bar.

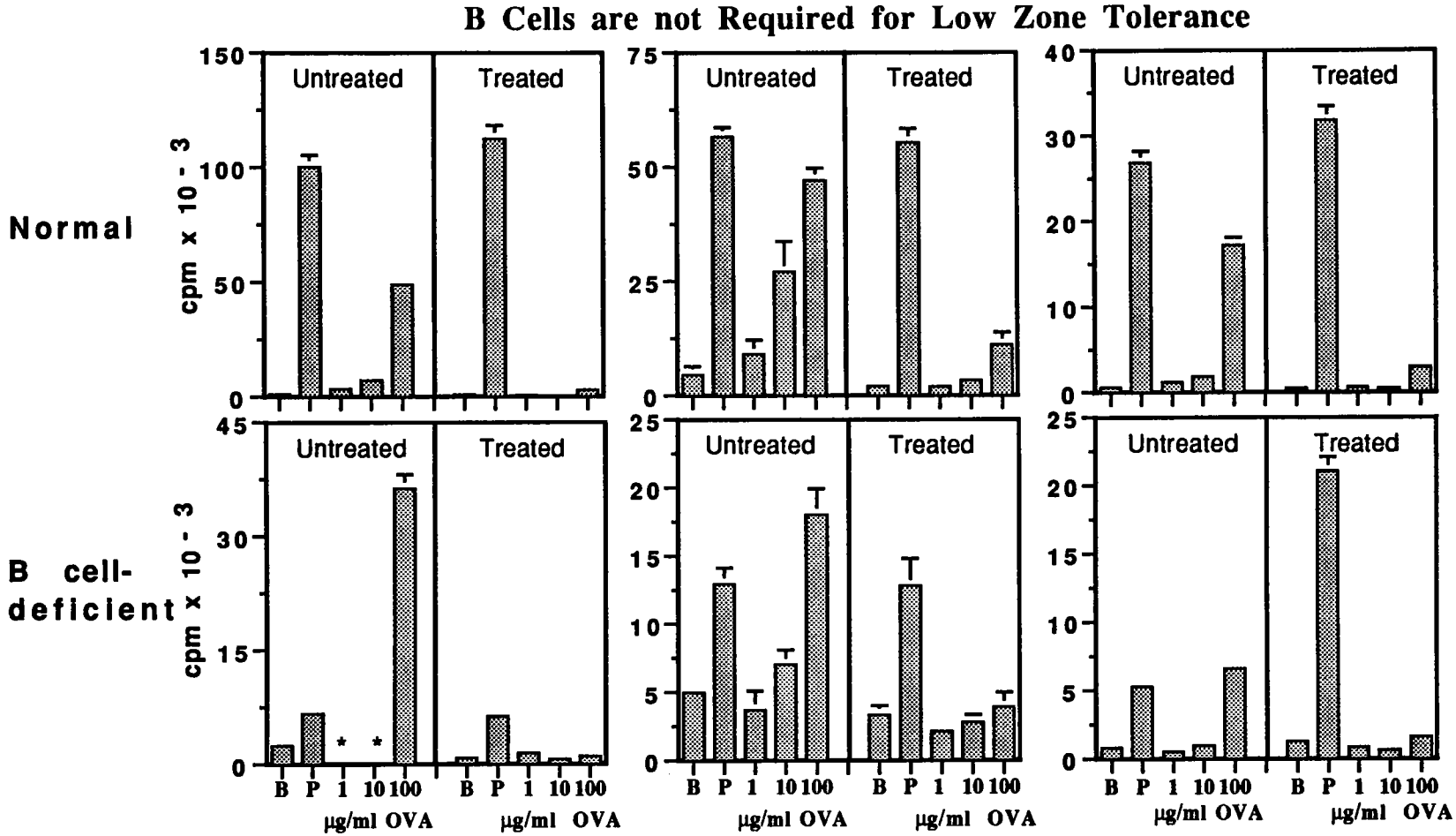

Figure 12. C57BL/6 and B cell-deficient mice were given 10 µg deaggregated OVA i.v. 3 times a week for 3 weeks and challenged along with the untreated controls with 100 µg OVA in CFA. Whole LN cells (the 4 graphs on the left) or purified T cells (2 graphs on the right) were pooled from 6 mice in each group and cultured with medium alone (B), PPD (P), or increasing doses of OVA. T cell proliferation was measured by [^{3}H]TdR incorporation and shown here as cpm. Error bars represent 1 SD. * Not tested.

with repeated low doses of OVA exhibit a marked reduction in the proliferative response to OVA, as compared to T cells from untreated normal mice (left to right $p<0.001$, $p<0.001$, $p<0.002$). The unresponsiveness was antigen-specific, because T cells from both the treated and untreated normal mice have comparable responses to PPD. As shown by the panels on the bottom of Fig. 12, when B cell-deficient mice were treated with the same protocol, the T cell proliferative response to OVA was also reduced compared with the untreated B cell-deficient mice (left to right $p<0.001$, $p<0.003$, $p<0.001$). Therefore, B cell antigen presentation was not required to induce T cell unresponsiveness following repeated injections of low concentrations of OVA.

C. The Most Effective Dose for Tolerance Induction is 10 μg OVA I.V.

Because my experiments in the B cell-deficient mice indicated that B cells were not required for low zone tolerance induction with repeated injections of 10 μg of OVA, I was interested in determining whether a requirement for B cell antigen presentation would be evident when lower doses of antigen were used to induce tolerance. Even though B cells are very efficient APC at low antigen concentrations, it remained possible that the 10 μg i.v. dose of antigen used to induce tolerance was large enough for other APC to present the antigen. Perhaps I could limit antigen presentation to B cells by reducing the dose of the antigen given i.v. First I determined whether I could induce tolerance, as measured by T cell proliferative responses, by giving lower doses of deaggregated OVA i.v. Normal mice were given repeated injections of either 10, 1.0, 0.1, or 0.01 μg OVA and then challenged a week later with OVA in CFA. The proliferative responses of the LN T cells were examined 10 days post-challenge. As shown in Fig. 13, treatment with 10 μg OVA was the only dose that resulted in antigen-specific T cell unresponsiveness, as there was only a

The Most Effective Dose for Inducing T Cell Unresponsiveness is 10 μg OVA i.v.

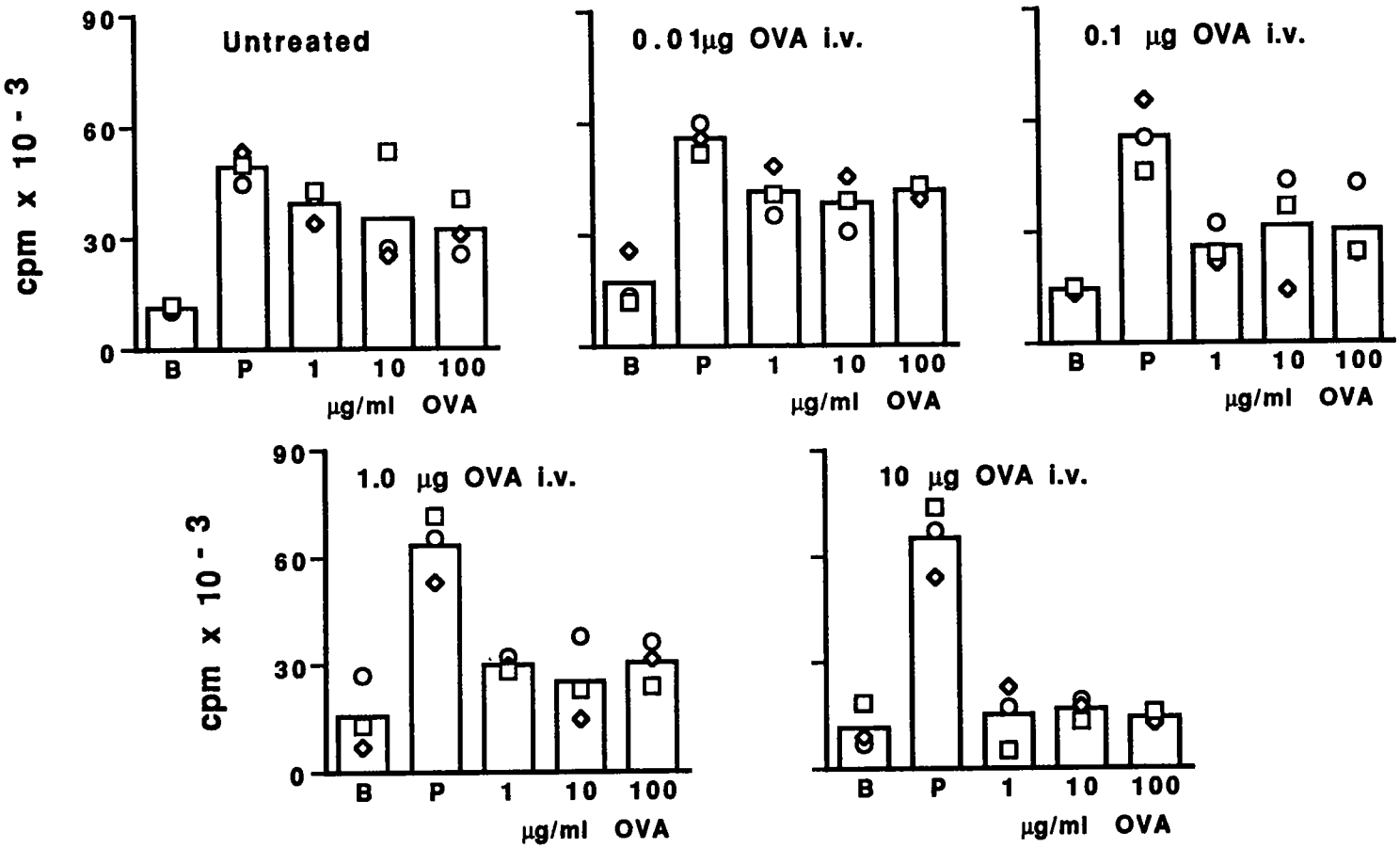

Figure 13. C57BL/6 mice were treated with 0.01 to 10 μg OVA i.v. and then challenged with the untreated mice with OVA in CFA. Ten days later, LN cells were set up at 4 x 10^5 cell/well in triplicate in medium alone (B), PPD (P), or increasing doses of OVA. Cultures were pulsed with [^{3}H]TdR for the last 6 hours of a 96 hour incubation. The symbols represent individual animals, and the bar is the group average.

small effect, if any, on the proliferative response to OVA in animals treated with repeated injections of 1 μg or less of deaggregated OVA. Since tolerance induction in normal mice served as a control for experiments performed in B cell deficient mice, and I could not measure tolerance in the normal mice with antigen doses lower than 1 μg OVA, I did not attempt to induce tolerance in the B cell deficient mice with lower doses of antigen.

CHAPTER V

SELECTIVE T CELL UNRESPONSIVENESS FOLLOWING LOW ZONE TOLERANCE INDUCTION

The previous experiments established that B cells were not required for tolerance induction to low doses of protein antigens, as demonstrated by reduced T cell proliferative responses in both normal and B cell-deficient treated animals. The aim of the next set of experiments was to define what other components of the immune response were compromised by treatment with low doses of antigen. Was the B cell compartment affected in the treated normal animals? What other T cell effector functions were compromised by the repeated low doses of antigen? I also wanted to examine the kinetics of the proliferative response in the treated animals, whether the antigen-specific T cell proliferative response of the treated animals could be rescued with IL-2, and if suppressor T cells were present in the LN cells recovered from the treated mice. The following experiments address these questions.

A. Antibody Production in Normal Mice Treated with Low Doses of Antigen is Unaffected

Mitchison defined low zone tolerance in the 1960's by showing that mice given repeated low doses of soluble BSA had significantly reduced antibody production following challenge as compared to the untreated controls (81). I decided to test whether repeated low doses of OVA, in addition to causing a reduction in the proliferative responses

of the T cells, affected anti-OVA antibody production in the normal animals. Fig. 14 shows the mean ELISA O.D. values for serum anti-OVA antibody levels in the mice at 7, 14 and or 35 days after challenge. Anti-OVA antibodies were not detected in the preimmune and prechallenge serum samples taken from the untreated and treated animals (Fig. 14 and data not shown). At day 7 the anti-OVA response of the treated mice was slightly higher than that of their untreated littermates, indicating that some of these animals may become slightly primed by the tolerance protocol. However, by day 14, the anti-OVA antibody production in the treated animals was equal to that of the untreated mice (Fig. 14). Anti-OVA antibody production was the same in the treated and untreated animals in 3 experiments and was reduced about 2-fold in a fourth experiment, indicating that the low zone tolerance protocol did not reproducibly reduce helper T cell function or affect the B cell compartment of the treated animals.

To determine whether treatment with low dose soluble antigen produced a qualitative change in T cell help, I examined the antibody isotypes produced in the untreated and treated normal mice. Normal mice were treated with the low zone tolerance protocol and then challenged with OVA in CFA along with the untreated controls. Thirty-five days post-challenge the titers of anti-OVA antibody isotypes were determined by an isotype-specific ELISA, using mouse anti-phenylarsonate antibodies of different isotypes as standards. The data presented in Fig. 15 indicate that the predominant IgG subclasses produced by both the untreated and the treated mice were IgG1 and IgG2b. Total antibody in the untreated and treated normal animals was comparable. The predominance of IgG1 antibody production and the near absence of IgG2a antibody production by both groups of normal animals suggested that the antibody response may have been due to Th2-type helper cells (112, 113).

B Cell Antibody Production is not Affected by the Low Zone Tolerance Protocol

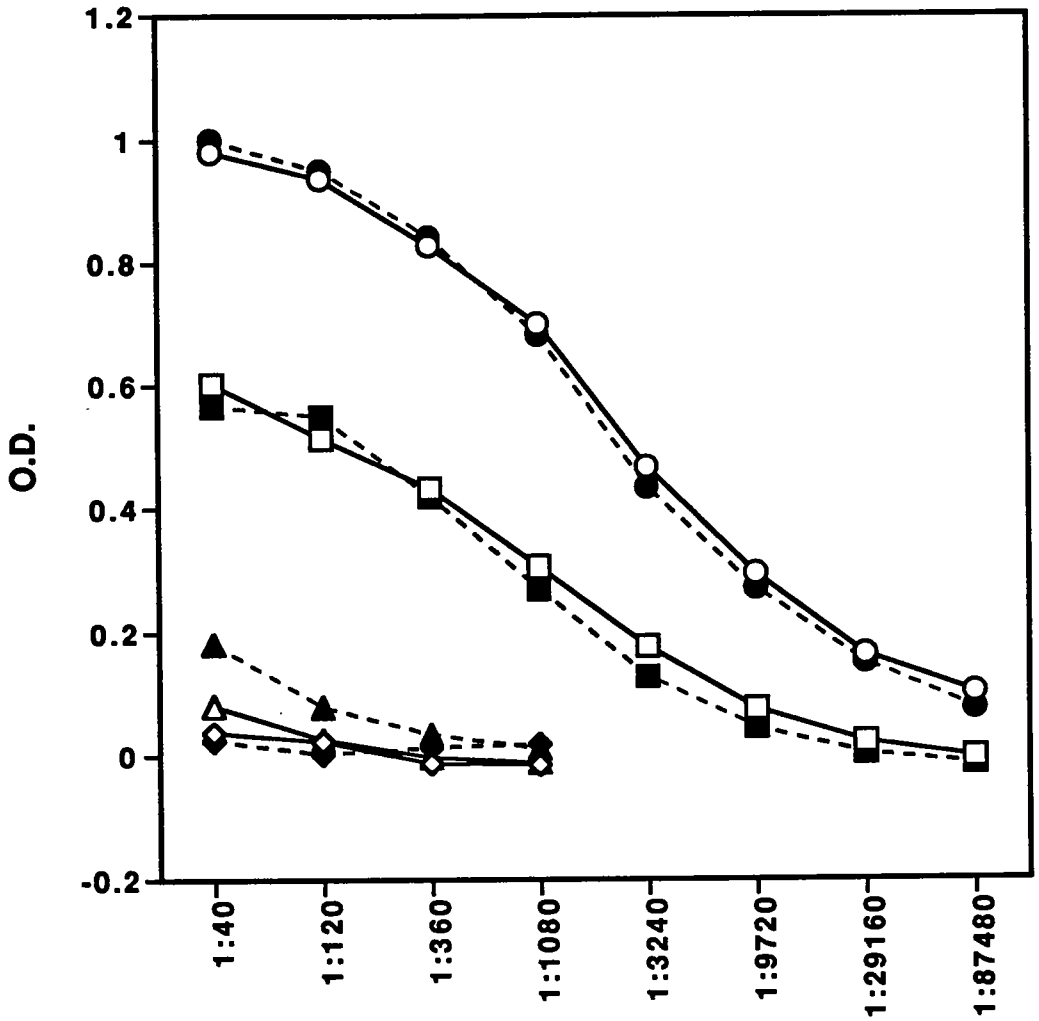

Figure 14. Six mice were treated with 10 μg OVA i.v. as in Fig. 12. The untreated controls and the treated mice were then challenged with OVA in CFA in both hind footpads. Mice were bled weekly and serum anti-OVA titers were determined by ELISA. Serum was diluted 1:40, and 3-fold titrations are shown. The open symbols represent the average of 6 individual serum samples from the untreated normal mice, and the closed symbols represent the average of 6 individual samples from the treated mice. Diamonds represent preimmune serum, triangles are from day 7, squares are from day 14, and circles are from day 35 post-challenge. The results are representative of 3 out of 4 independent experiments.

Tolerized Mice Produce the Same Anti-OVA Antibody Isotype as the Untreated Controls

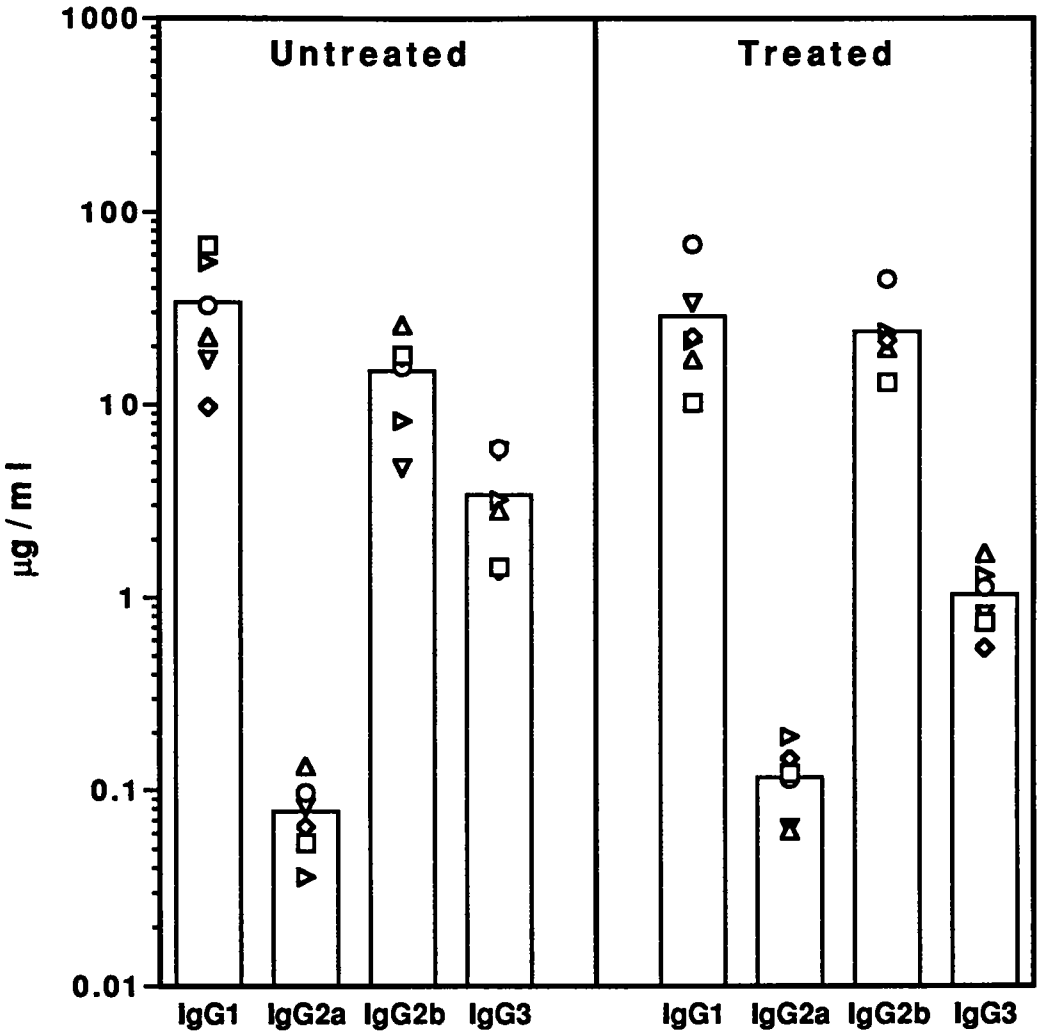

Figure 15. Six C57BL/6 mice were treated as in Fig. 12, and the treated mice and the untreated controls were challenged with OVA in CFA. Mice were bled 35 days post-challenge, and an anti-OVA isotype-specific ELISA was performed. Each symbol represents an individual animal, and the mean antibody titers are represented by bars. The results are representative of 2 independent experiments.

B. Th1 Cells are Compromised by the Low Zone Tolerance Protocol

Cytokine production by T cells from the treated animals following stimulation with OVA *in vitro* was examined in supernatants harvested at 24, 48, or 72 hours from primed LN cultures from untreated and treated normal and B cell-deficient mice. The results of an IFN-γ-specific ELISA shown in Table 1 indicated that the amount of IFN-γ produced in the presence of PPD was unchanged between the untreated and treated mice, but the amount of IFN-γ produced by cells from mice treated with deaggregated OVA was below the level of detection in both the treated normal and treated B cell-deficient mice.

The results of an IL-2 bioassay with the IL-2-dependent cell line HT-2, shown in Fig. 16, indicated that IL-2 production was also decreased in the supernatants taken from the treated animals, as expected due to the reduced proliferative responses. IL-4 was undetectable (< 0.78 U/ml) in the supernatants tested from the LN cells of either the normal or B cell-deficient untreated or treated mice using the most sensitive bioassay for IL-4 (100) (Fig. 17). Taken together, these results demonstrated that these *in vitro* proliferation assays were primarily detecting a Th1 response, and that this Th1 response was reduced in cells from the treated normal and B cell-deficient mice, while T cell help for antibody formation was largely unaffected.

C. Kinetics of the Proliferative Response in the Tolerized Animals

The peak of the T cell antigen-specific response may have been earlier in animals exposed to low doses of antigen prior to challenge, and this could result in reduced proliferative responses in these treated animals 10 days post-challenge. To rule out this

Table 1

IFN-γ (pg/ml) Production is Reduced in T Cells from Treated C57BL/6 and B Cell-Deficient Mice

Mouse strain	*in vivo* treatment[a]	Antigen *in vitro*[b] PPD	Antigen *in vitro*[b] OVA
C57BL/6	untreated	10,900 ± 1124[c]	6,881 ± 475
C57BL/6	10 μg OVA i.v.	7,798 ± 530	< 500
B cell-deficient	untreated	23,961 ± 2867	20,697 ± 3855
B cell-deficient	10 μg OVA i.v.	13,422 ± 2507	< 500

[a] C57BL/6 and B cell-deficient mice were treated with 10 μg OVA i.v. three times a week for three weeks. The untreated and treated animals were then challenged, and the draining lymph node cells were harvested five days later.

[b] Supernatants were collected from whole lymph node cells stimulated with antigen *in vitro* for 72 hrs and tested in an IFN-γ ELISA. These values were calculated on supernatants taken from the experiment shown in Figure 2A. The results are representative of 3 independent experiments, but the levels of IFN-γ from B cell-deficient mice were not consistently elevated over those from normal mice.

[c] 1 SD.

IL-2 Production is Reduced in OVA-Stimulated T Cells from Tolerized Mice

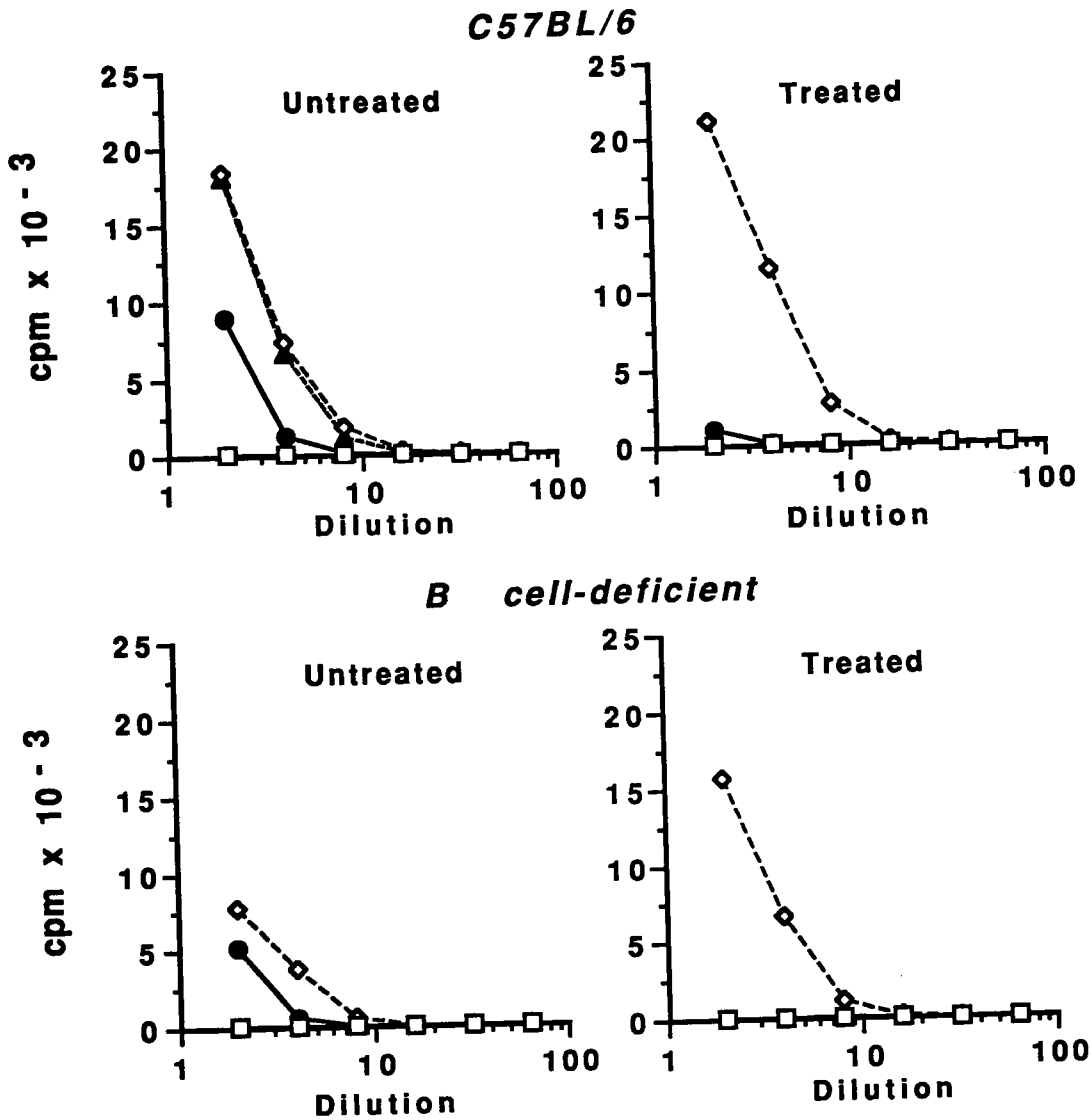

Figure 16. C57BL/6 and B cell-deficient mice were treated as in Fig. 12 and challenged with OVA in CFA along with the untreated controls. Eight x 10^5 pooled lymph node cells/200 ml were cultured with PPD (open diamonds), 100 µg/ml OVA (closed circles), or medium alone (open squares). HT-2 proliferation is shown with supernatants collected at 24 hours from normal untreated and treated mice (top) or untreated and treated B cell-deficient mice (bottom). The closed triangles are the response of the HT-2 cells to .625 U/ml IL-2.

IL-4 Production is Absent in Supernatants from in vitro Stimulated T Cells

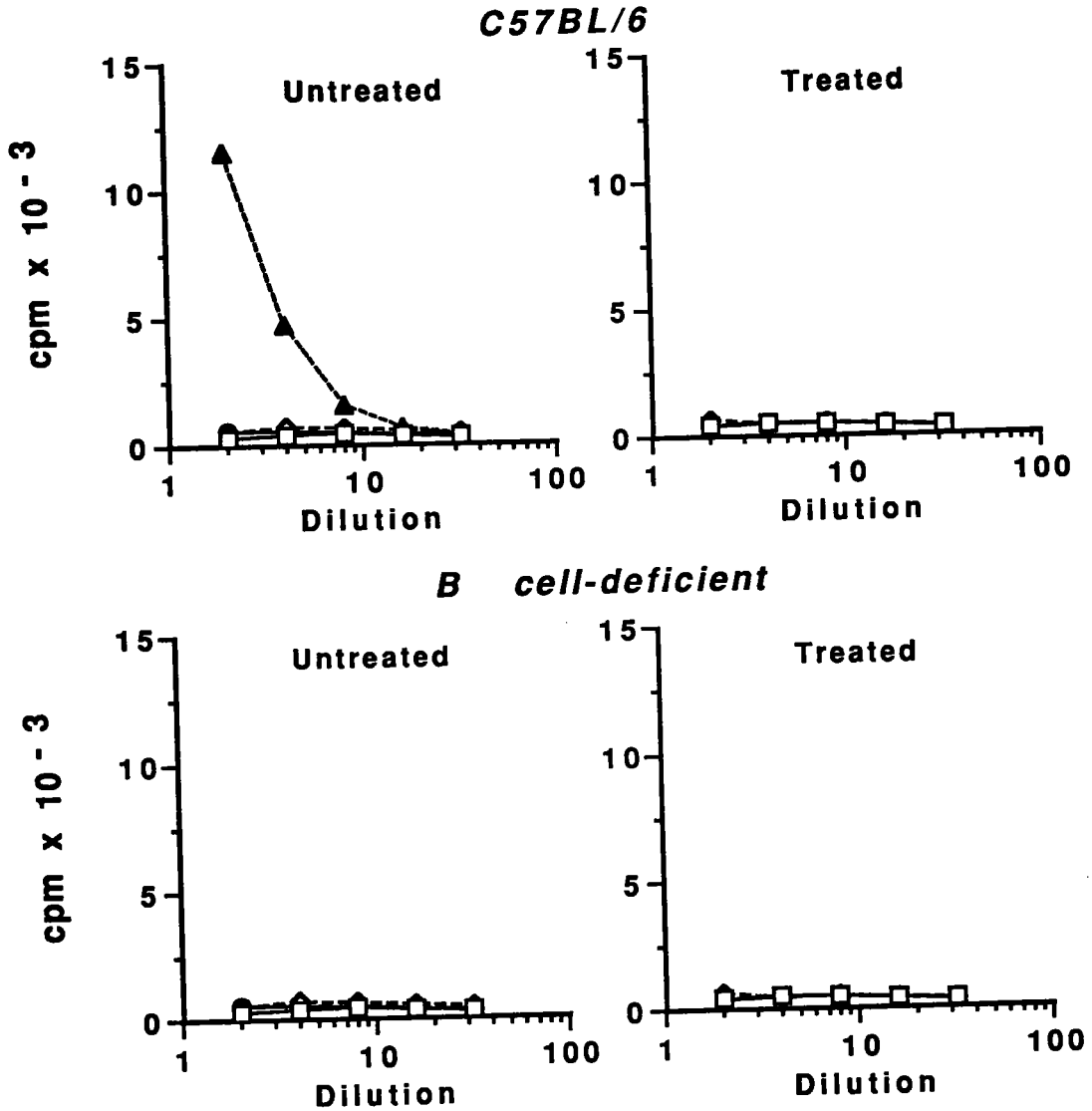

Figure 17. C57BL/6 and B cell-deficient mice were treated as in Fig. 12 and challenged with OVA in CFA along with the untreated controls. Eight x 10^5 pooled lymph node cells/200 µl were cultured with PPD (open diamonds), 100 µg/ml OVA (closed circles), or medium alone (open squares). CT-4S proliferation is shown with supernatants collected at 72 hours from normal untreated and treated mice (top) or untreated and treated B cell-deficient mice (bottom). The closed triangles are the response of the CT-4S cells to 1.56 U/ml IL-4.

possibility, I decided to examine the proliferative response of the T cells from untreated and treated animals at 5 and 7 days post-challenge. Normal mice were treated with the low zone tolerance protocol, and then the treated and untreated mice were challenged with OVA in CFA . As shown in Fig. 18, LN T cells from the untreated normal animals responded to both PPD and OVA at both day 5 and day 7 post-challenge. Interestingly, the response to the lower doses of OVA *in vitro* was not seen at the earlier time point. This may suggest that T cell affinity for antigen increases over time after challenge by selection of higher affinity T cells (114). T cells from the treated mice had reduced proliferative responses to OVA, but not to PPD, at both time points tested, indicating that the reduction in proliferation was not simply due to different kinetics in the immune response of the treated mice.

D. Addition of IL-2 in vitro Does Not Break T Cell Unresponsiveness of Cells from Treated Animals

Next, I wished to test whether the reduction in the T cell proliferation observed in treated normal and B cell-deficient mice was the result of an inability of these T cells to make their autocrine growth factor, IL-2, in the *in vitro* assay (115, 116). Addition of 1 or 10 U/ml of IL-2 to the proliferation assay increased both antigen-specific and background proliferation in cells from untreated normal and B cell-deficient animals, but failed to restore antigen-specific proliferation to the treated normal or B cell-deficient animals (Fig. 19). For some unknown reason the overall T cell proliferative responses were low in this experiment, as shown by the proliferative response in the absence of exogenous IL-2. The results from this experiment imply that the reduced proliferative response of the T cells to

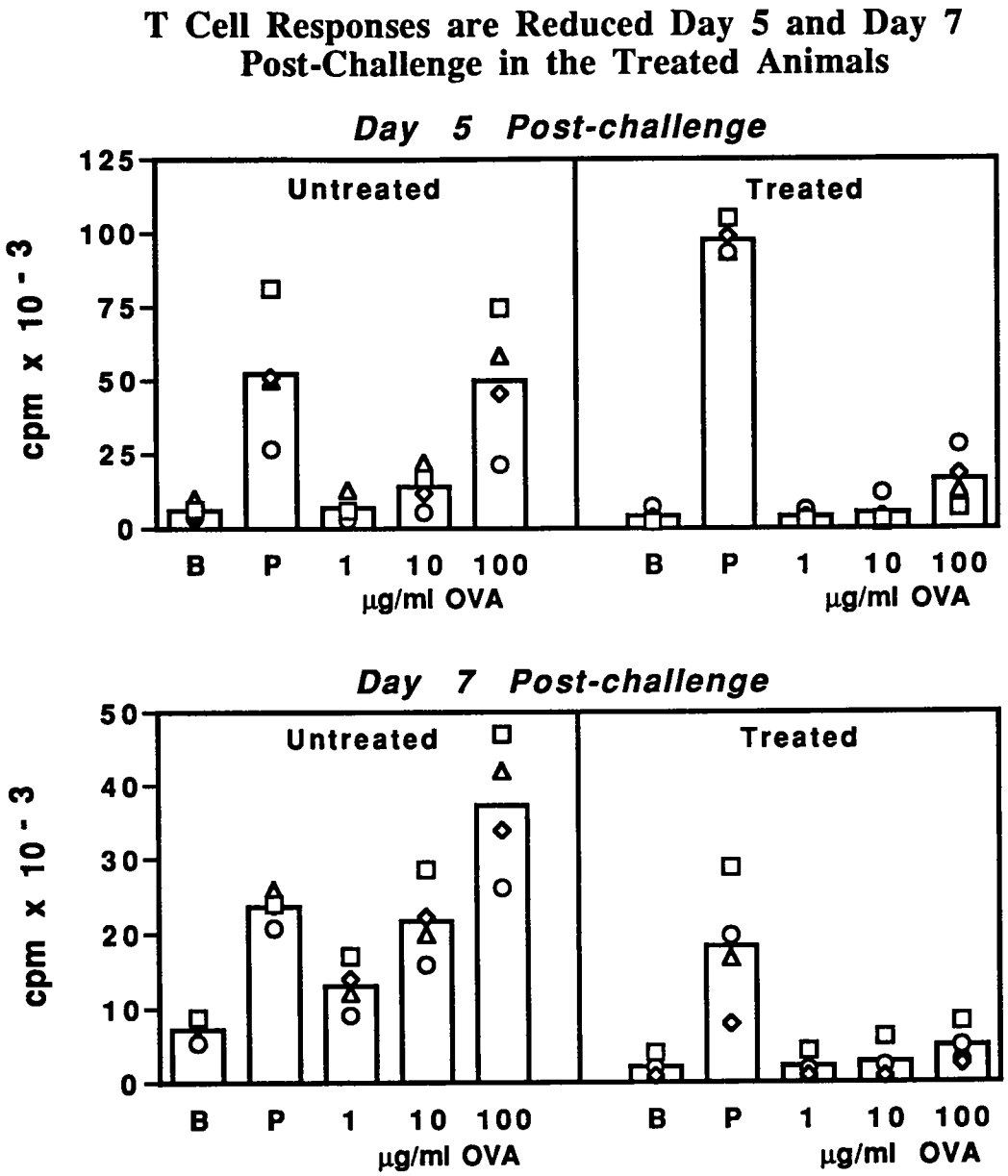

Figure 18. Normal mice were treated with 10 µg OVA i.v. and then challenged along with the untreated controls with OVA in CFA. Whole LN cells were harvested 5 or 7 days post-challenge and set up at 4 x 10^5 cells/well in triplicate in media alone (B), PPD (P), or increasing doses of OVA. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Symbols represent individual animals and the bar is the group average.

Addition of IL-2 in vitro does not Restore Antigen-Responsiveness to Tolerized T Cells

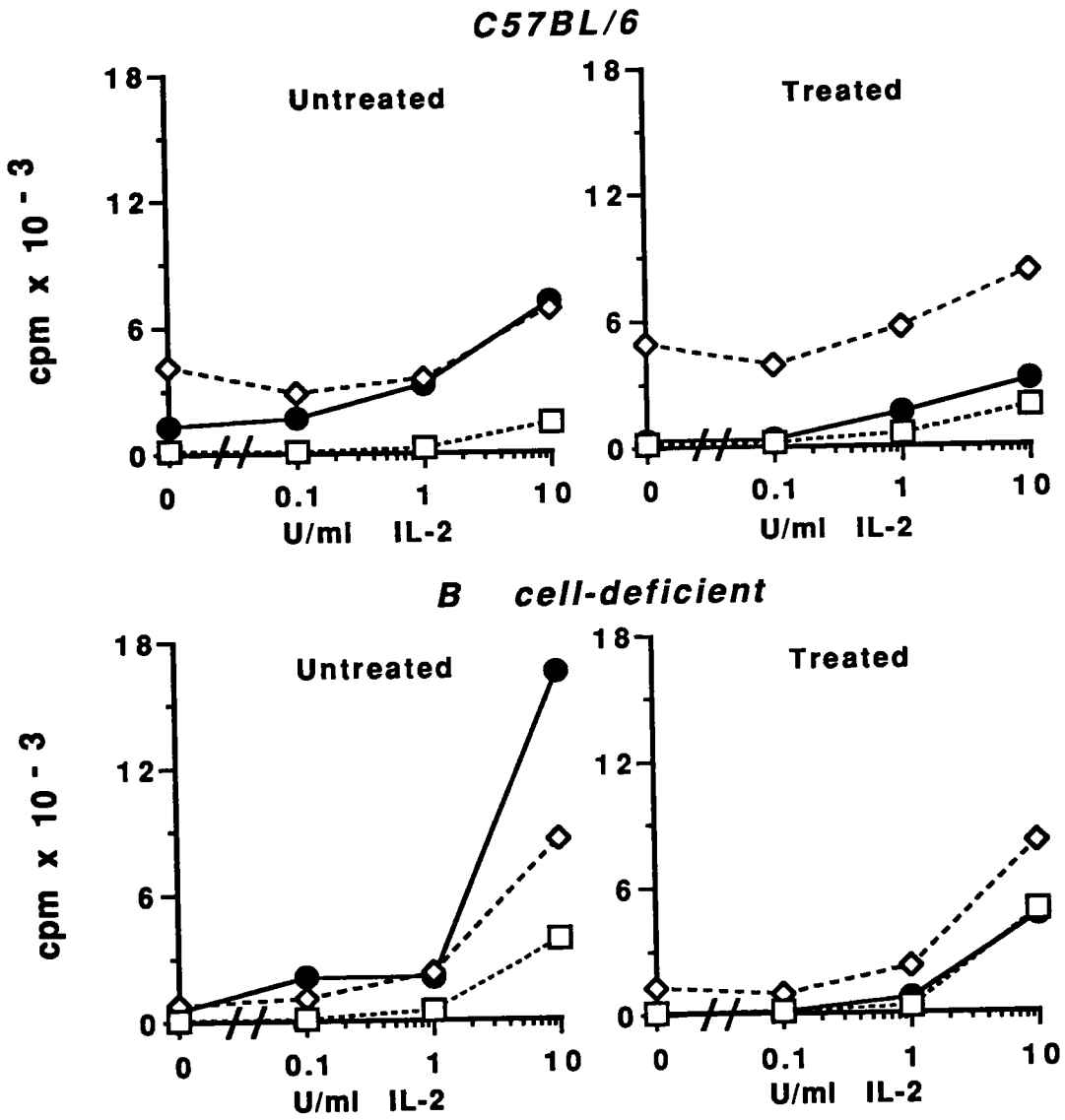

Figure 19. C57BL/6 and B cell-deficient mice were treated with 10 μg deaggregated OVA i.v. 3 times a week for 3 weeks. Untreated and treated mice were challenged with OVA in CFA. T cells were purified 10 days post-challenge and cultured in triplicate at 6 x 10^5 cells/well with increasing doses of IL-2. Open squares represent T cells cultured in medium alone, open diamonds represent T cells in PPD, and closed circles are T cells incubated with 100 μg/ml OVA. T cell proliferation was measured by [^{3}H]TdR incorporation and shown here as cpm.

OVA from the treated normal and B cell-deficient mice was not due to IL-2-reversible anergy *in vitro*. This experiment did not, however, rule out the possibility that the OVA-specific T cells became anergic *in vivo* and therefore were unable to expand properly upon challenge, resulting in fewer antigen-reactive T cells being recovered from these animals.

E. Reduced Proliferative Responses are Not Due to the Presence of Suppressor Cells in vitro

In other models of acquired tolerance, suppressor T cells have been demonstrated following treatment with soluble protein antigen (117). I wanted to determine whether the presence of suppressor T cells in the treated animals might explain the reduced T cell proliferative response to OVA. One way to test for the presence of suppressor T cells in the primed LN cells from the treated mice was to mix T cells from treated mice with equal numbers of T cells from untreated mice, in order to determine whether the proliferative response of the T cells from the untreated animals was then reduced. To control for any change in proliferation, due to only half the number of primed T cells being present, T cells from mice primed with CFA were also mixed with either untreated T cells or treated T cells. Normal and B cell-deficient mice were given the low zone tolerance protocol, and treated and untreated normal and B cell-deficient mice were challenged with OVA in CFA. At the same time a group of normal animals and B cell-deficient animals were challenged with CFA alone. Lymph node cells were harvested 10 days after challenge from all groups of mice and set up *in vitro* in the indicated combinations with PPD or OVA. The results of the mixing experiment performed on T cells from both normal and B cell-deficient mice are shown in Fig. 20. Although the proliferative response of the normal animals was

T Cell Proliferative Responses are Unchanged when Untreated and Treated Cells are Mixed

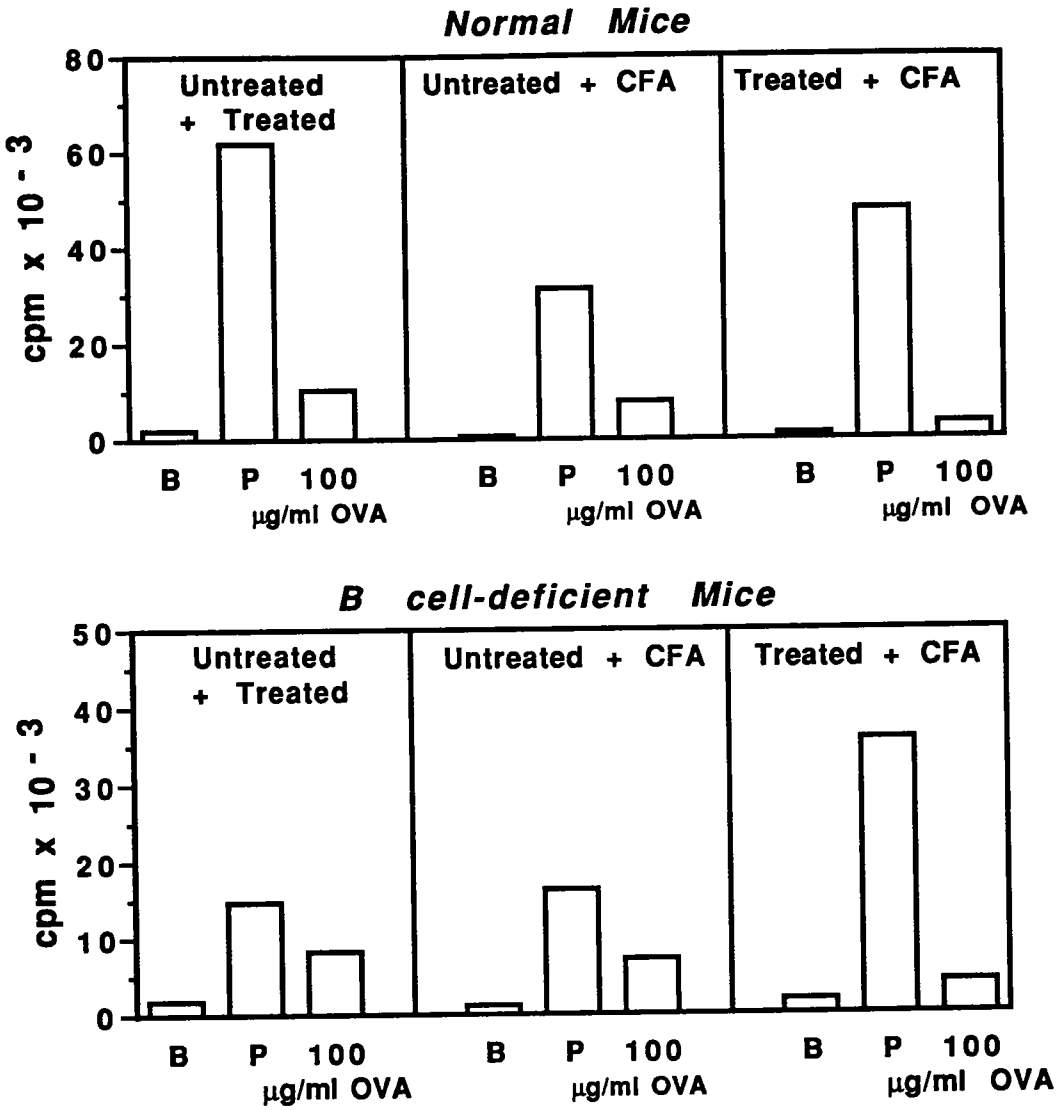

Figure 20. Normal and B cell-deficient mice were treated as in Fig.12. They were challenged 7 days later with OVA in CFA with the normal and B cell-deficient untreated mice. Another group of normal and B cell-deficient mice were challenged with CFA alone. Ten days later LN cells were harvested from all groups and set up in triplicate in medium alone (B), PPD (P), or OVA. Two x 10^5 untreated cells were mixed with 2 x 10^5 treated or CFA-primed cells, and 2 x 10^5 treated cells were mixed with 2 x 10^5 CFA-primed cells. [^{3}H]TdR was added the last 6 hours of a 96 hour incubation.

somewhat low, and tolerance in both types of animals was incomplete in this experiment, the OVA proliferative response of the untreated T cells remained the same whether equal numbers of T cells from CFA-primed or treated mice were added. Also, T cells from the treated animals mixed with T cells from CFA-primed animals remained unresponsive to OVA. The same pattern of results was observed with the different combinations of untreated and treated T cells from the B cell-deficient animals. The results from this experiment provide no evidence for suppressor T cells in either the normal or B cell-deficient treated animals.

CHAPTER VI

MHC HAPLOTYPE-SPECIFIC TOLERANCE

During my investigation into the role of B cells in self tolerance to the antigen HEL, I made the interesting observation that the induction of tolerance to the soluble self antigen HEL depended on the MHC alleles expressed by the transgenic animals. These studies were conducted with two transgenic lines of mice on the C57BL/6 H-$2^{b/b}$ background: ML-4 mice had less than 0.5 ng/ml of HEL in their serum, and ML-5 mice that had 20 ng/ml of serum HEL (94). The HEL transgene was under the control of a metallothionein promoter, and the transgene-encoded self antigen HEL was constitutively expressed in both lines of mice (94). As shown previously in Fig. 9, B cells were not required to maintain tolerance to HEL on the H-$2^{b/k}$ background, as T cells from HEL-transgenic B cell-deficient mice were unresponsive to HEL following challenge with HEL in CFA. Initially, tolerance to the HEL self antigen in the absence of B cells was examined in H-$2^{b/b}$ B cell-deficient HEL-transgenic animals (data not shown). The surprising result from those experiments was that T cells from HEL-transgenic normal and B cell-deficient animals of the H-$2^{b/b}$ background were responsive to HEL. The experiments presented here address the differences in the T cell responses of the transgenic H-$2^{b/k}$ F1 animals, which were tolerant to HEL, and the transgenic parental strain H-$2^{b/b}$, which were not tolerant.

A. HEL-Transgenic H-$2^{b/b}$ Mice are not Tolerant to HEL

The initial observation regarding the lack of tolerance to the self antigen HEL on the H-$2^{b/b}$ background was made when nontransgenic and transgenic mice of the ML-4 line

were primed with HEL in CFA, and the T cell proliferative responses were examined 10 days later. The results of four proliferation assays performed in nontransgenic and HEL-transgenic H-$2^{b/b}$ animals are shown in Fig. 21. The combined data in Figs. 21 and 22 were tested for significance using a general mixed model analysis of variance. The significance of differences between individual pairs of means were evaluated by pairwise tests for predicted cell means. I defined statistical significance as differences with a probability under the null hypothesis of 0.05 or less. To compensate for the additive error due to multiple comparisons, a Bonferonni adjustment was applied to get adjusted critical values that maintain the nominal significance. As shown in Fig. 21, the T cell proliferative response of the transgenic animals to HEL was significantly different than the response observed in medium alone, demonstrating that tolerance to HEL was not established in the transgenic H-$2^{b/b}$ animals. The difference in the T cell proliferative response to the antigen HEL between the nontransgenic and transgenic animals reached significance, even though the log means of the two groups were relatively similar, 9.94 +/- 1.31 vs. 9.10 +/- 1.41 respectively.

The results of T cell proliferation assays performed in the H-$2^{b/b}$ HEL-transgenic animals were different than those observed from T cells in the H-$2^{b/a}$ HEL-transgenic animals as shown in Fig. 22. For these experiments, B10.A (H-2^{a}) mice were bred to the ML-4 line on the (H-$2^{b/b}$) background, creating (B10.A x C57BL/6) F1 H-$2^{b/a}$ nontransgenic and HEL-transgenic animals. Mice of the H-2^{a} haplotype express H-2^{k} MHC class II molecules. Nontransgenic and transgenic mice of the H-$2^{b/a}$ haplotype were primed with HEL in CFA, and 10 days post-challenge lymph nodes were harvested. The T cell proliferative response to HEL of the transgenic F1 H-$2^{b/a}$ animals was not significantly different than the response observed in medium alone, indicating that the HEL-specific T cells from H-$2^{b/a}$ transgenic animals were tolerant to the HEL self antigen. Furthermore,

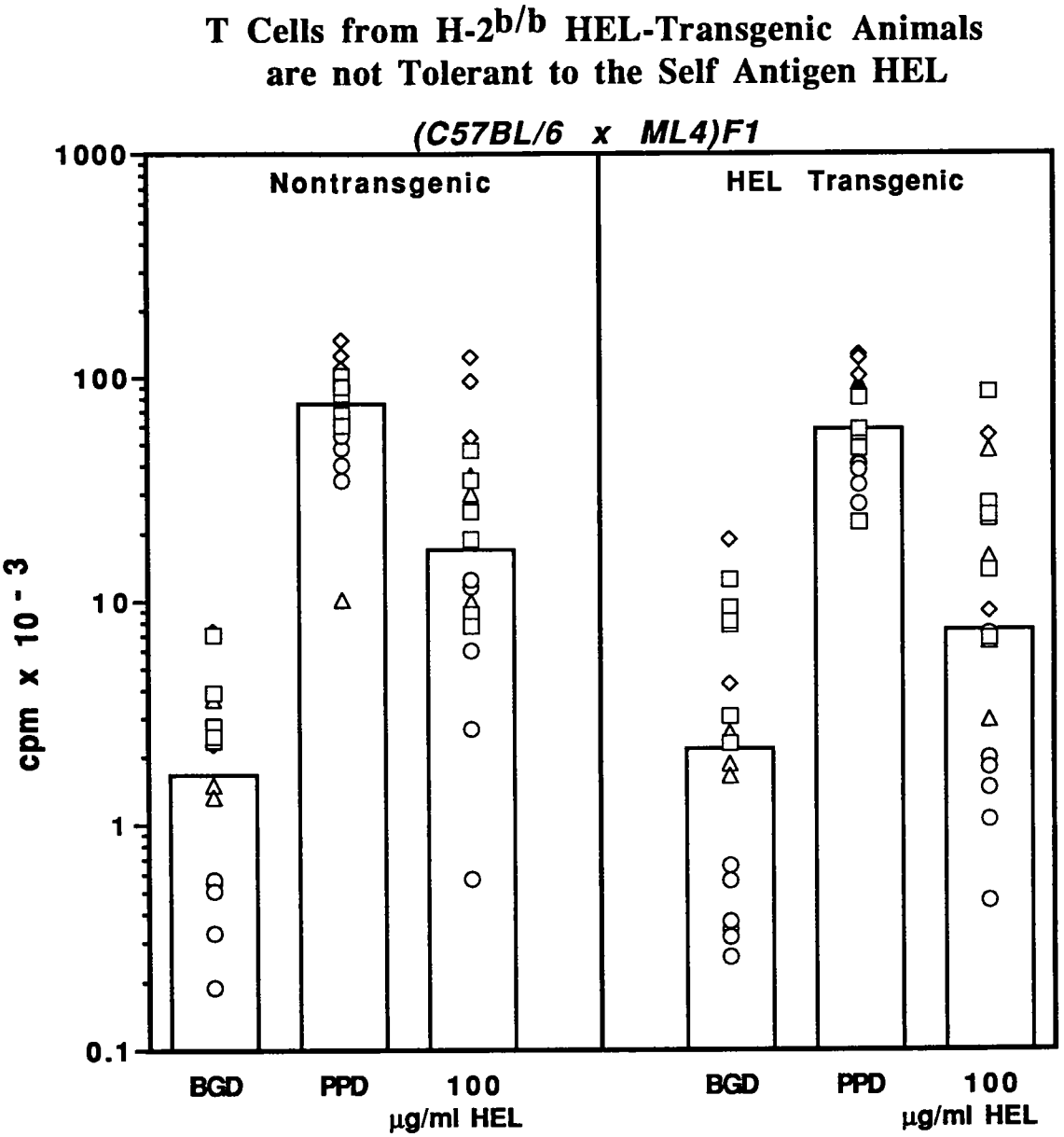

Figure 21. Twenty-one C57BL/6 nontransgenic and 22 ML-4 transgenic C57BL/6 mice were primed with HEL in CFA in each hind footpad. Ten days later the draining lymph nodes were harvested. LN cells were set up in vitro at 4 x 10^5 cells/well in triplicate in medium alone (Bgd), PPD, or HEL. Cultures were pulsed the last 6 hours of a 96 hour incubation with [^{3}H]TdR. The results of 4 experiments are shown, and individual mice within a single experiment are represented by the same symbol.

T Cells from H-$2^{b/a}$ HEL-Transgenic Animals are Tolerant to the Self Antigen HEL

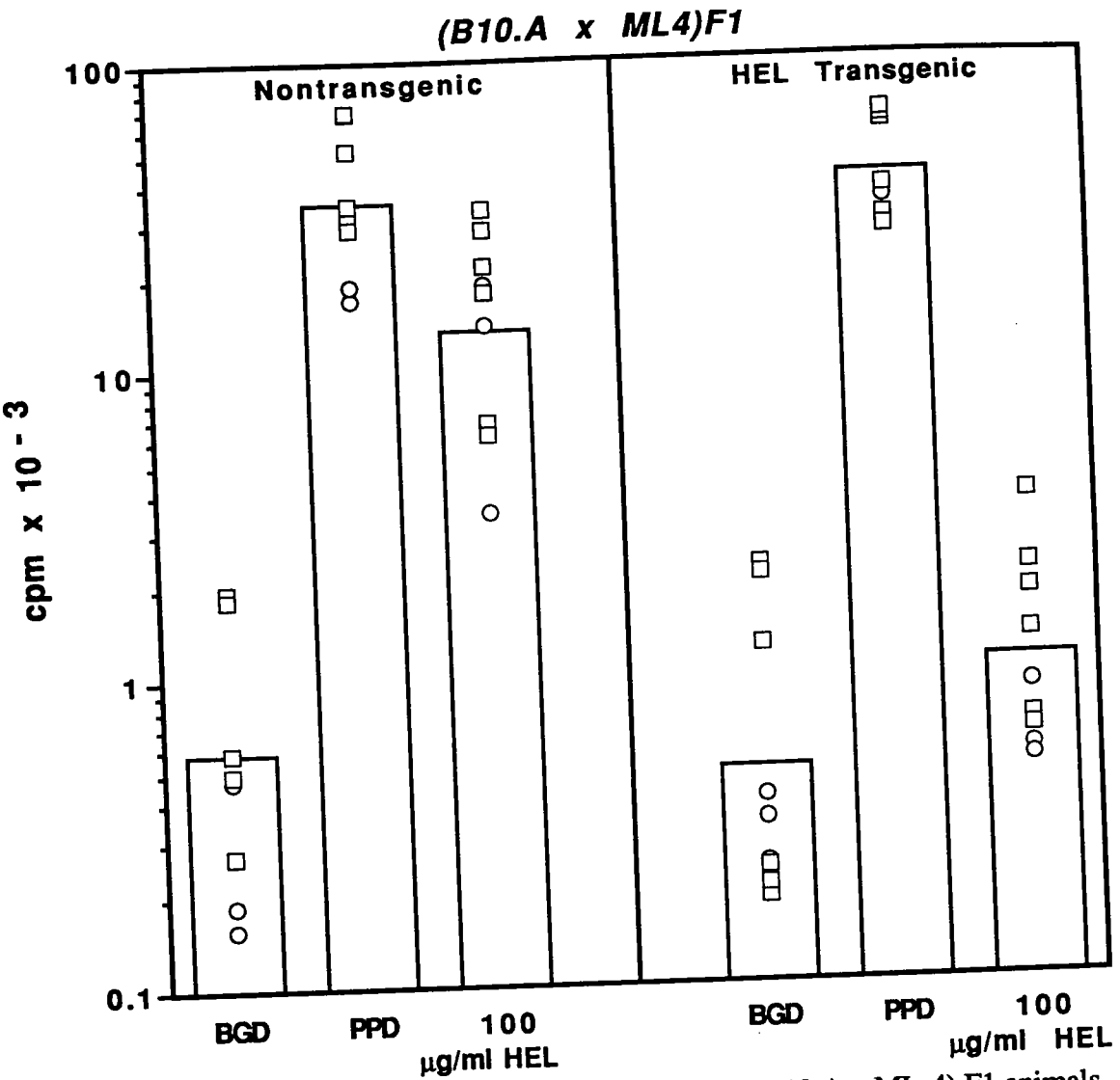

Figure 22. Nine nontransgenic and 9 HEL-transgenic (B10.A x ML-4) F1 animals were primed with HEL in CFA in each hind footpad. Ten days later the popliteal lymph nodes were harvested. LN cells were set up in vitro at 4 x 10^5 cells/well in triplicate in medium alone (Bgd), PPD, or HEL. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. The results of two experiments are shown, and individual mice within a single experiments are represented by the same symbol.

the T cell proliferative responses to HEL from nontransgenic and transgenic animals of the H-$2^{b/a}$ haplotype were also significantly different, as shown by a large difference in the log means, 9.50 +/- .77 vs. 6.99 +/- .69 respectively. The T cell responses to the control antigen PPD were comparable for both the nontransgenic and transgenic mice, demonstrating that tolerance observed in the H-$2^{b/a}$ HEL-transgenic T cells was antigen-specific.

It seems possible that the MHC molecules found in the two strains, H-$2^{b/b}$ and H-$2^{b/a}$, might have different affinities for the antigen HEL. Therefore, if the class II MHC molecules in the H-$2^{b/a}$ strain bind HEL with a higher affinity than the class II MHC molecules found in the H-$2^{b/b}$ strain, then tolerance would be induced at lower antigen levels in the H-$2^{b/a}$ strain than in the H-$2^{b/b}$ strain. As shown above, the H-$2^{b/a}$ transgenic animals were tolerant to the low levels of serum HEL, but the H-$2^{b/b}$ transgenic animals were not tolerant to HEL. I was interested in determining whether tolerance could be induced in the H-$2^{b/b}$ transgenic animals when serum levels of HEL were increased, as this might promote binding to lower affinity MHC molecules. To address this possibility, tolerance to HEL was examined in H-$2^{b/b}$ ML-5 transgenic animals, which had much higher levels of serum HEL than the ML-4 transgenic line, 20 ng/ml vs. < 0.5 ng/ml. Higher levels of HEL in the serum did not result in tolerance induction in the H-$2^{b/b}$ transgenic animals, as shown in Fig. 23, because T cells from both HEL-primed nontransgenic and ML-5 HEL-transgenic animals were responsive to HEL, even though there was more variability in the mean responses due to the small number of animals tested.

T Cells from H-$2^{b/b}$ HEL-Transgenic Mice with Higher Serum Levels of HEL are not Tolerant to HEL

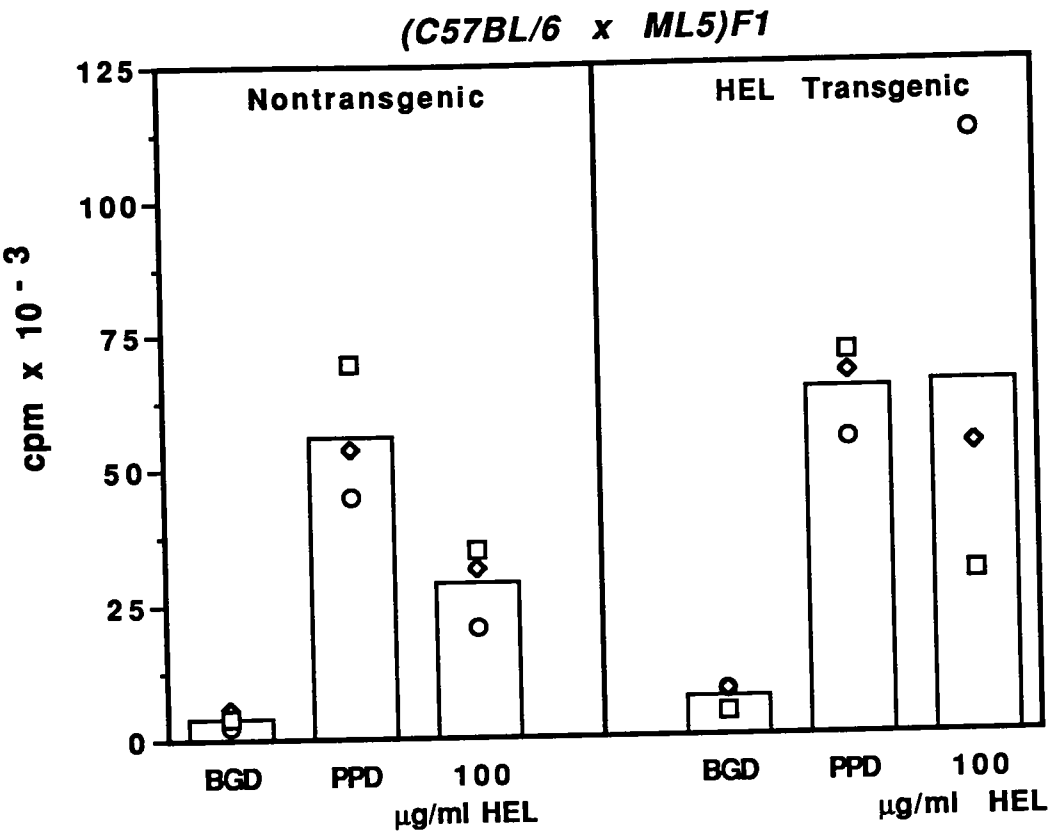

Figure 23. Three nontransgenic and 3 ML-5 HEL-transgenic C57BL/6 mice were primed with 250 µg HEL in each hind footpad. Ten days later the popliteal lymph nodes were harvested, and the cells were set up in triplicate at 4 x 10^5 cells/ well in medium alone (Bgd), PPD, or HEL. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Symbols represent individual animals.

B. The Presence of the H-2^k Allele Confers Tolerance to HEL

Experiments performed in the lab of Eli Sercarz have shown that the C57BL/6 background is defective in the ability to process HEL into an immunodominant peptide capable of binding I-A^b MHC molecules (118). This may be the reason that C57BL/6 mice have been previously characterized as low responders to the HEL antigen (119). It was unlikely that the lack of tolerance observed in the transgenic C57BL/6 mice was due to an inability to process HEL, because (B10.A x C57BL/6) F1 transgenic animals, which have similar background genes to the C57BL/6 animals, were tolerant to HEL, demonstrating that HEL had been efficiently processed and presented. It was possible that the differences in tolerance induction observed between the parental strain (H-2$^{b/b}$) and the F1 animals (H-2$^{b/a}$) were due to the presence of an MHC allele that could bind peptides of HEL with a higher affinity and present HEL peptides efficiently, thereby inducing tolerance more effectively. Adelstein and colleagues published that T cells from (CBA x C57BL/6) F1 H-2$^{b/k}$ transgenic mice were tolerant to HEL (94). As shown in Fig. 9, T cells from (B10.BR x C57BL/6) F1 transgenic animals were also tolerant to HEL. To confirm the results of Adelstein and colleagues, and to demonstrate that tolerance to the self antigen was observed when the class II MHC molecules of the H-2^k haplotype were expressed, I examined the T cell proliferative responses of nontransgenic and transgenic (CBA x C57BL/6) F1 H-2$^{b/k}$ mice. The results shown in Fig. 24 indicate that indeed T cells from both (CBA x C57BL/6) F1 and (B10.A x C57BL/6) F1 transgenic mice were unresponsive to HEL as compared to their nontransgenic littermates. These results imply that the differences observed in tolerance to the self antigen HEL between H-2$^{b/b}$ transgenic mice and H-2$^{b/k}$ transgenic mice was likely to be due to the presence of the MHC H-2^k allele.

The H-2^k Allele Confers Tolerance to the Transgene-Encoded Self Antigen HEL

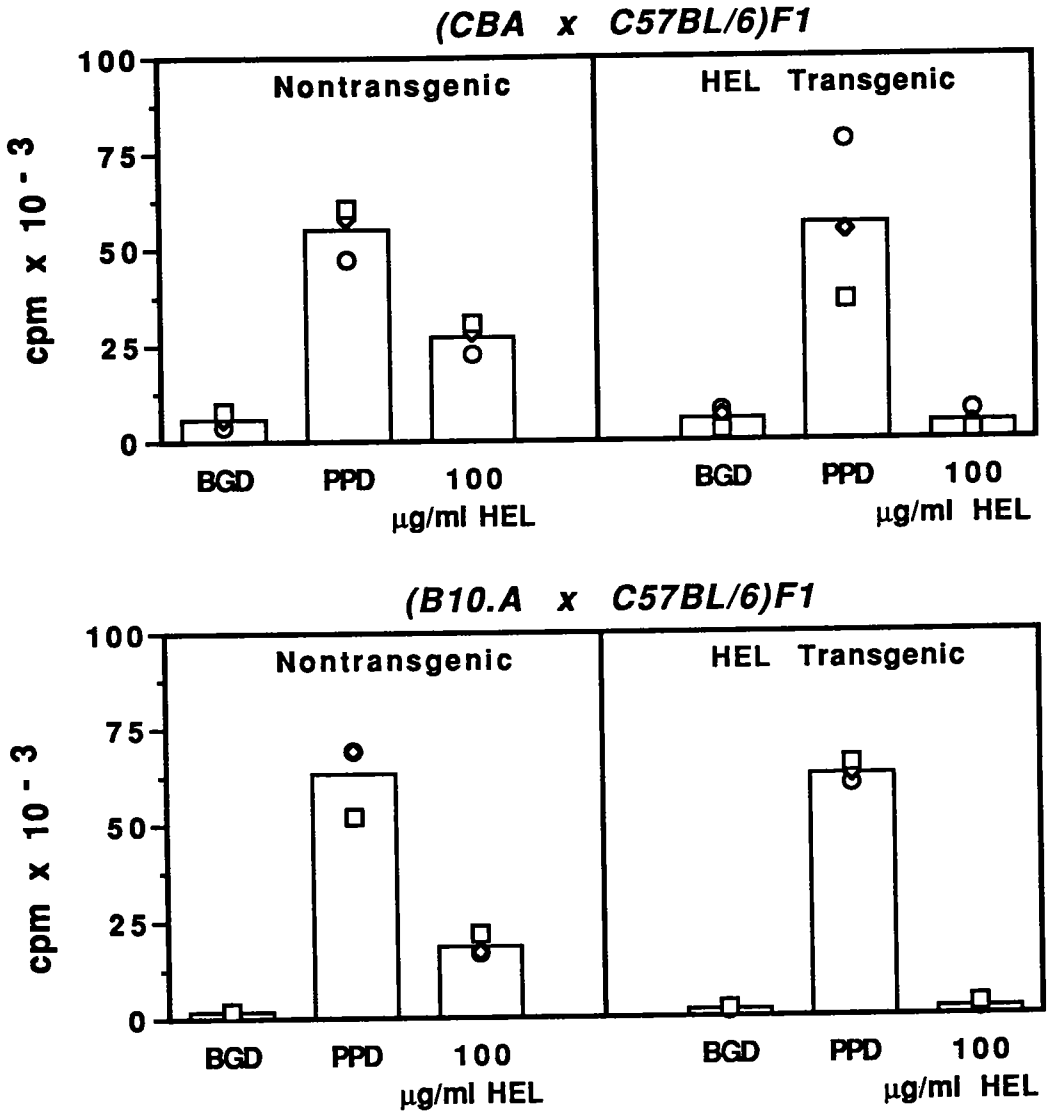

Figure 24. Three nontransgenic and 3 transgenic (CBA x C57BL/6) F1 animals and 3 nontransgenic and 3 transgenic (B10.A x C57BL/6) F1 animals were primed with 250 µg HEL in each footpad. Ten days later LN cells were harvested and set up in triplicate at 4 x 10^5 cells/well in medium alone (Bgd), PPD, or HEL. Cultures were pulsed the last 6 hours of a 96 hour incubation with [^{3}H]TdR. Symbols represent individual animals and the bar indicates the mean.

C. *H-2^k-Restricted T Cells Dominate the Response to HEL in F1 Animals*

In an F1 animal it could be assumed that there would exist HEL-specific naive T cells restricted to H-2^b, others restricted to H-2^k, and some restricted to H-2$^{b/k}$. The surprising result from my experiments in HEL-transgenic animals was that the H-2^b-restricted HEL-specific T cells from transgenic C57BL/6 mice were responsive to HEL, whereas in the F1-transgenic animal neither the H-2^b-restricted nor the H-2^k-restricted HEL-specific T cells responded to HEL. To investigate whether H-2^b-restricted HEL-specific T cells could be found in the immunized H-2$^{b/k}$ F1 animals, I decided to purify the T cells and examine the proliferative response to HEL presented by either H-2$^{b/b}$ or H-2$^{k/k}$ APCs. (B10.A x C57BL/6) F1-nontransgenic and F1-transgenic animals were primed with HEL in CFA, the lymph nodes harvested, and T cells were purified 10 days post-challenge. The results of this experiment are shown in Fig. 25. The first panel of both graphs indicates that the T cell purification protocol did not eliminate all of the APCs from the LN T cells, because T cell proliferation occurred in the presence of PPD and/or HEL in the absence of any additional APCs. However, APCs were limiting, because additional APC increased the response to PPD, and the addition of H-2$^{k/k}$ APC increased the response to HEL in the nontransgenic animals. As shown in the top right panel of Fig. 25, the H-2^k-restricted HEL-specific T cells dominate the response to HEL in the nontransgenic F1 animals, because the T cell proliferative response to HEL increased when H-2$^{k/k}$ APCs were added but did not increase when H-2$^{b/b}$ APCs were added. The H-2^b-restricted HEL-specific T cells in the H-2$^{b/k}$ nontransgenic animals did not respond *in vitro* above the level observed in the absence of additional APCs, suggesting that they probably had not expanded in the primed LN during the challenge with HEL. The T cells from F1 HEL-transgenic animals were unresponsive to HEL presented by either H-2$^{b/b}$ or H-2$^{k/k}$ APCs as

H-2^b-Restricted HEL-Specific T cells are not Detected in F1 Animals Primed with HEL

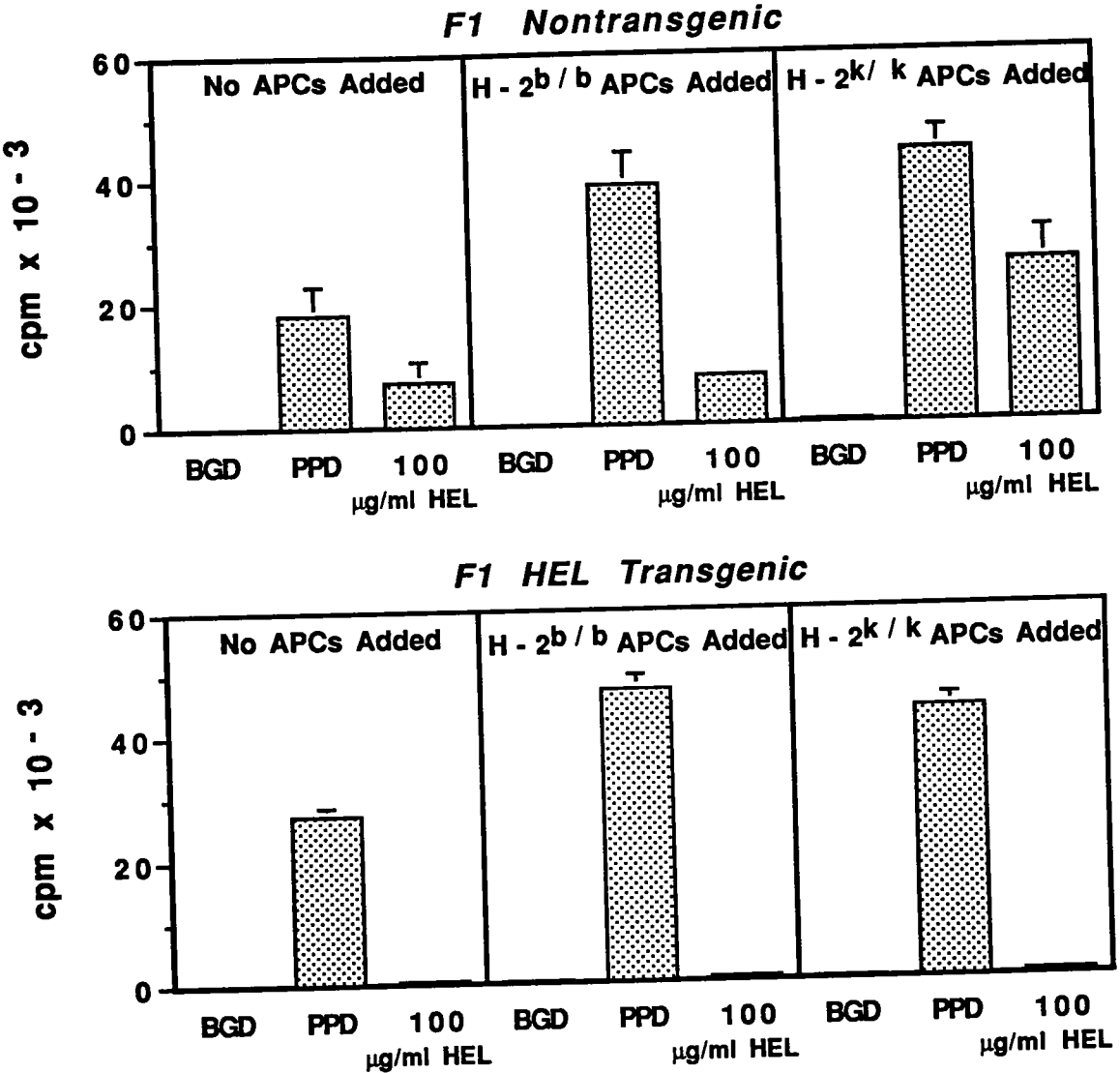

Figure 25. Five nontransgenic and 6 transgenic (B10.A x C57BL/6) F1 animals were primed with 100 µg HEL/hind footpad, and 10 days later the lymph nodes were harvested. Cells from individual animals were pooled, and the T cells were purified and set up in vitro at 4 x 10^5 cells/well. Spleen cells from naive C57BL/6 or B10.A mice were T-depleted, irradiated, and used as APC. T cells were cultured in triplicate in medium alone (Bgd), PPD, or HEL. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Error bars represent 1 SD.

expected (bottom graph, right 2 panels). These results suggest that the tolerance observed in the HEL-transgenic H-$2^{b/s}$ animals could be due to deletion of the H-2^{k}-restricted HEL-specific T cells.

The last set of experiments address whether H-2^{b}-restricted HEL-specific T cells could be detected in the transgenic F1 animals when tolerance to HEL was broken by priming animals with a larger dose of HEL in CFA. As demonstrated in the above experiment, the H-2^{k}-restricted HEL-specific T cells dominate the response to HEL in the nontransgenic F1 animals; therefore it seemed likely that these T cells would be deleted in the F1 HEL-transgenic animals. Thus, the responding T cells recovered from F1 transgenic animals primed with a large dose of HEL may be H-2^{b}-restricted HEL-specific T cells that managed to escape deletion. To address this possibility, I primed F1 nontransgenic and transgenic animals with a large dose of HEL and examined the T cell proliferative response of these animals to the whole protein and to various peptides of HEL presented by H-$2^{b/b}$, H-$2^{k/k}$, or H-$2^{b/k}$ APCs. As shown in Fig. 26, the H-2^{b}-restricted HEL-specific T cells could be detected in the transgenic F1 animals, because, when H-$2^{b/b}$ APCs were added to purified T cells from the F1-transgenic animals, the responses to HEL as well as to the H-2^{b}-restricted peptides, 30-53 and 74-88, were slightly enhanced (120). The addition of F1 APCs also enhanced the response to HEL and the peptide 74-88, but the H-$2^{k/k}$ APCs did not enhance the proliferative response of the T cells from the F1 transgenic to neither HEL nor the HEL peptides. Even though the T cell response to HEL and the HEL peptides was quite low in the F1 transgenic animals, the proliferative response to the control antigen PPD was comparable for T cells from both the nontransgenic and the transgenic animals (data not shown). These results support my idea that the H-2^{k}-restricted T cells were probably deleted in the transgenic F1 animal, because the H-2^{k} class II MHC

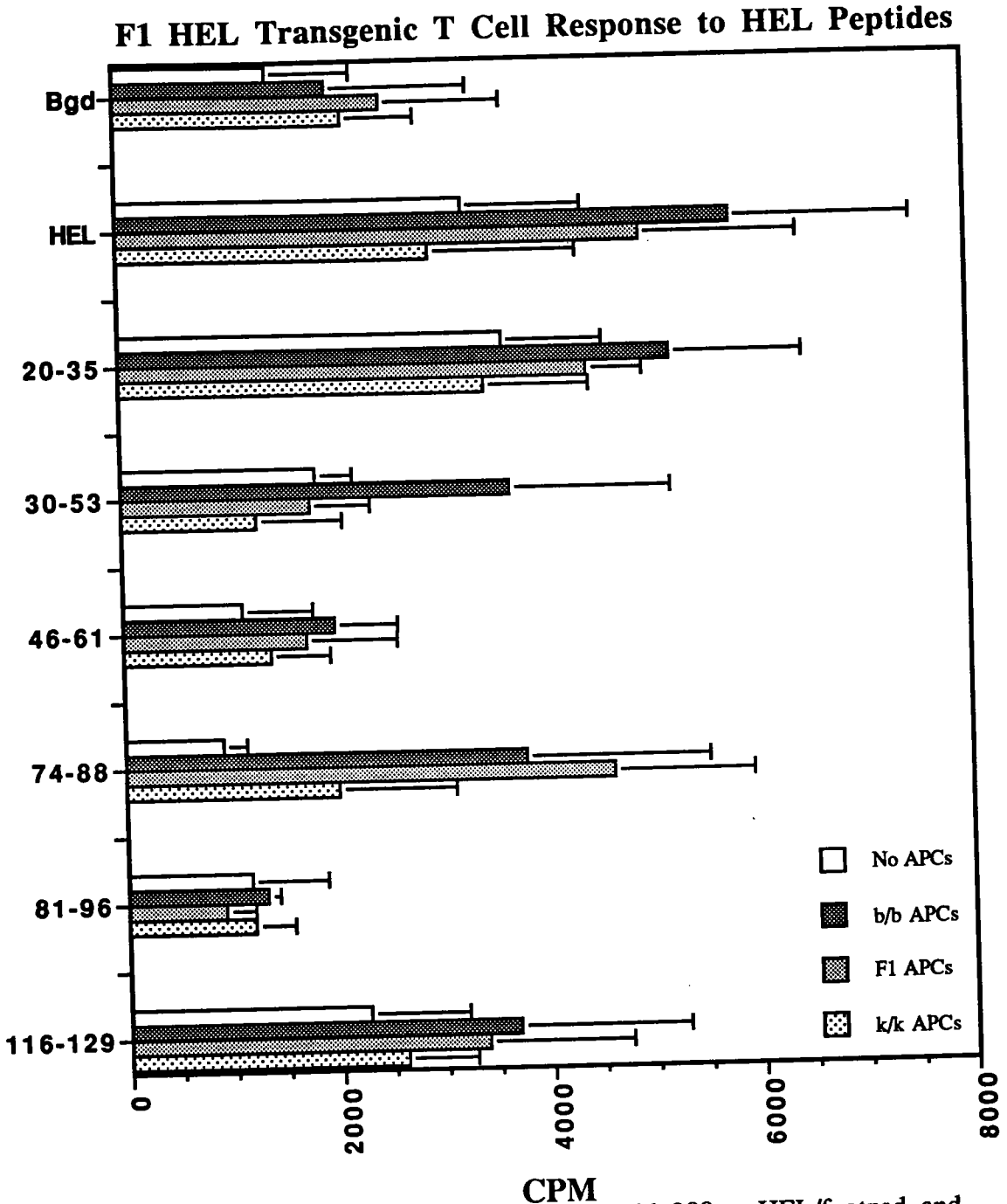

Figure 26. HEL-transgenic F1 animals were primed with 288 μg HEL/footpad, and LN cells were harvested 10 days later. Purified T cells were set up in vitro in triplicate at 4 x 10^5 cells/well with 2 x 10^5 irradiated T-depleted spleen cells as indicated. T cells were cultured in medium alone (Bgd), 288 μg/ml HEL, or 7.5 μM peptide. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Error bars represent 1 SD.

molecules were likely to bind and present HEL more efficiently than the H-2^b class II MHC molecules, while the lower affinity H-2^b-restricted T cells escaped deletion.

The T cell proliferative response of the nontransgenic F1 animals was primarily against the HEL whole protein, with the cpm ranging from 71,097 to 105,143. As shown in Fig. 27, the proliferative response of the F1-nontransgenic T cells to the HEL peptides was comparable to the T cell proliferative response observed in the F1 transgenic animals, except that the proliferative response to the HEL peptide 20-35 was much higher, especially when F1 APCs were added. Based on the results of the proliferation assay shown in Fig.25, I would have expected a greater response from the T cells of the nontransgenic animals to the H-2^k immunodominant HEL peptide 46-61 (120). Because the T cell response to this peptide was very weak, the 46-61 HEL peptide may have been synthesized incorrectly. There were two problems associated with the experiment shown in Figs. 26 and 27 that are most apparent in the HEL T cell proliferative response, as shown in the legend to Fig. 27. First, the depletion of APC in the primed LN was incomplete, as T cells from the nontransgenic animals proliferated in response to HEL in the absence of APCs. The other problem was that the addition of H-$2^{b/b}$ and H-$2^{k/k}$ APC to the purified T cells inhibited the proliferative response to the whole protein. However, it is useful to examine these experiments, because they provide an explanation for the tolerance observed in the F1-transgenic animals, and the results shown in Fig. 26 support other preliminary findings I had in the F1 HEL-transgenic animals primed with a large dose of antigen.

In summary, when F1-nontransgenic and F1-transgenic animals were primed with 100 μg HEL/footpad, it appears that only the high affinity HEL-specific T cells responded. In the nontransgenic F1 animals, the response was dominated by H-2^k-restricted HEL-specific T cells, and in the transgenic F1 animals the HEL-specific T cells were unresponsive, probably because the high affinity H-2^k-restricted HEL-specific T cells had

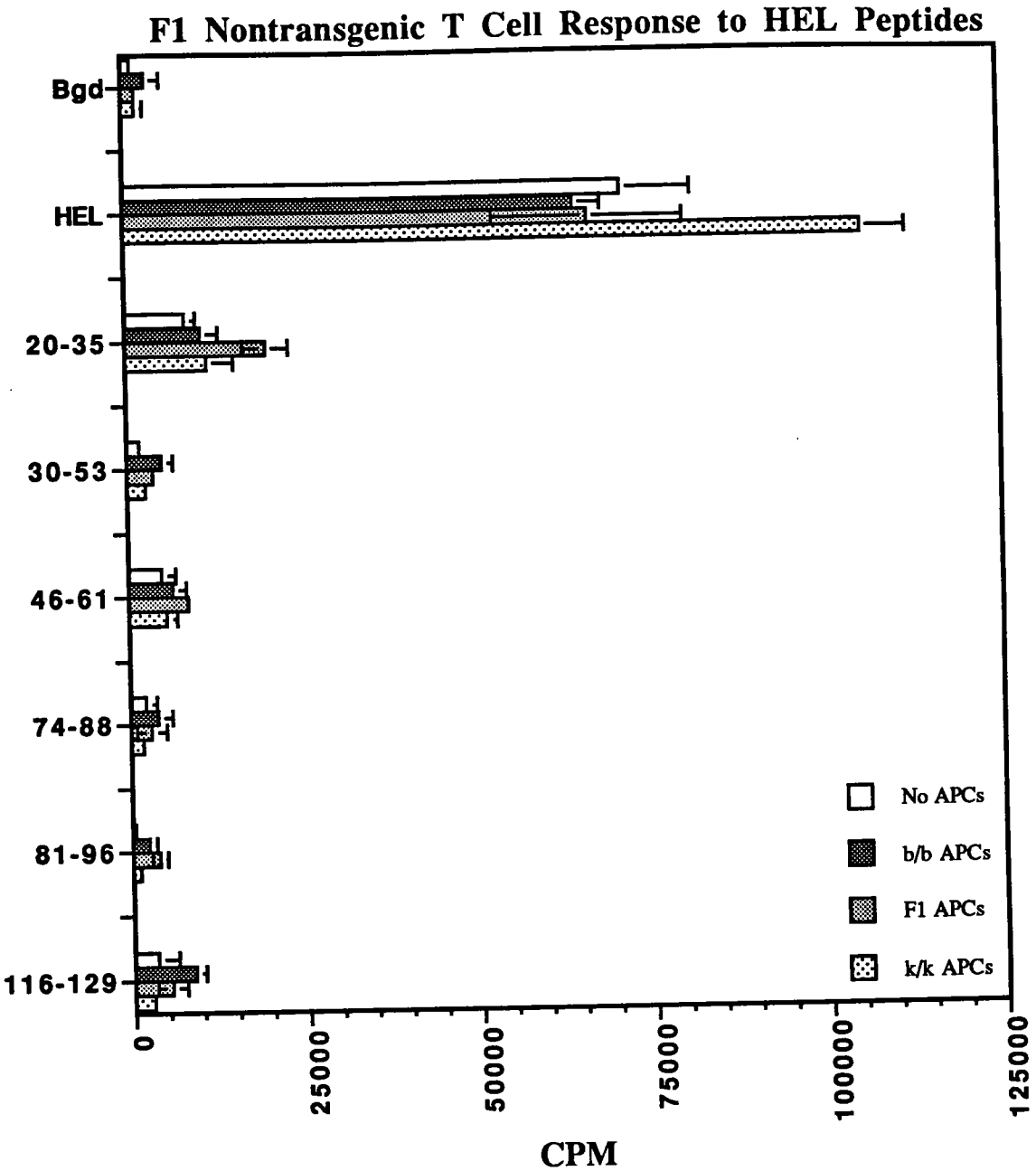

Figure 27. F1-Nontransgenic animals were primed with 288 μg HEL/footpad, and popliteal LN cells were harvested 10 days later. Purified T cells were set up in vitro in triplicate at 4 x 10^5 cells/well with 2 x 10^5 irradiated T-depleted spleen cells as indicated. T cells were cultured in medium alone (Bgd), 288 μg/ml HEL, or 7.5 μM peptide. Cultures were pulsed with [^{3}H]TdR the last 6 hours of a 96 hour incubation. Error bars represent 1 SD.

been deleted. However, when higher doses of HEL in CFA were used for T cell priming, the low affinity H-2^b-restricted HEL-specific T cells were able to expand in the F1 transgenic animal, and they were able to contribute to the *in vitro* proliferative response observed to both HEL and the peptides 30-53 and 74-88.

CHAPTER VII

DISCUSSION

The way the immune system distinguishes between self and nonself has still not been clearly defined. The experiments presented in this dissertation have addressed whether antigen presentation by B cells was required for peripheral T cell tolerance to low levels of self and foreign proteins. Experiments were performed in B cell-deficient animals, and tolerance induction was measured by T cell proliferation assays. Proliferation assays in B cell-deficient mice indicated that B cells were likely to be involved in efficient activation of naive T cells to protein antigen either *in vivo* or *in vitro*. Tolerance induction in B cell-deficient animals was as efficient as in normal animals, suggesting that antigen presentation by B cells *in vivo* was not required for tolerance to low levels of protein antigen. The effect of treatment with low doses of deaggregated antigen on the immune response was also addressed. The data presented here demonstrated that repeated exposure to low doses of deaggregated OVA before challenge inhibited the expansion and differentiation of antigen-specific T cells into Th1 effector cells in the draining lymph nodes of the treated animals but did not affect the ability of the antigen-specific T cells to provide help for antibody formation following challenge. The final set of experiments presented here suggests that the affinity of an antigen for an MHC molecule determines not only how well that antigen is presented to T cells, but also whether tolerance is induced to that antigen.

A. Tolerance Induction Depends on Efficient Antigen Presentation

During the course of my experiments, I made the interesting observation that T cells from HEL-transgenic C57BL/6 mice were not tolerant to low levels of a transgene-encoded self antigen HEL, but T cells from HEL-transgenic (B10.A x C57BL/6) F1 animals were tolerant to this self antigen, as shown in Figures 22 and 23. T cells from HEL-primed transgenic C57BL/6 mice remained responsive to HEL even when higher levels of the HEL self antigen were expressed, as in the ML-5 line (Figure 24). The lack of tolerance in the C57BL/6 transgenic animals could be due to the inability of the I-A^b molecules in the C57BL/6 mice to bind HEL determinants well enough to induce tolerance in the HEL-specific T cells. When antigen was given in adjuvant these HEL-specific T cells were revealed, because the HEL peptides were now presented at a higher concentration on APCs that also express high levels of both costimulatory and adhesion molecules, and the lower affinity T cells could be stimulated. C57BL/6 mice have been previously characterized as low responders to HEL in experiments where anti-HEL antibody responses were measured following i.p. injection of HEL, whereas B10.A mice were characterized as high responders, because they were able to produce anti-HEL antibodies under the same conditions (121). It was subsequently proposed that the T cells from HEL-primed C57BL/6 mice were unable to provide help for B cell antibody production, because these B cells could not effectively process and present HEL to T cells (122).

Experiments performed in another line of HEL-transgenic animals on the H-2^d background that express < 2 ng/ml serum HEL provide an explanation for the lack of tolerance to HEL observed in the transgenic C57BL/6 mice. T cells from the H-2^d HEL-transgenic animals were responsive to HEL *in vitro* and also responded to the HEL peptide 103-117, a 15-mer that encompasses the immunodominant determinant of HEL in this

strain, 108-116 (123). They proposed that in the transgenic animals the high affinity TCR for HEL have been eliminated while the low affinity TCR remain responsive to HEL in the periphery upon challenge. Additional evidence in favor of this explanation was subsequently provided when they demonstrated that the V_β repertoires of the nontransgenic and transgenic animals were quite different (124). In H-2^d animals the response to HEL is dominated by one peptide, 108-116, and in the nontransgenic animals a predominant TCR rearrangement was found. However, in the HEL-transgenic animals this rearrangement was absent, and each transgenic animal had unique TCR rearrangements that recognized HEL. In the nontransgenic animals, unique TCR rearrangements were also identified that varied from animal to animal, presumably recognizing determinants other than the immunodominant HEL peptide 108-116. Therefore, it is possible that in the C57BL/6 transgenic mice the high affinity HEL-specific T cells were deleted in the thymus, and the *in vitro* T cell proliferative response I observed after priming with HEL in CFA was due to low affinity HEL-specific T cells found in the periphery.

Tolerance induction to the transgene-encoded HEL antigen was found to correlate with the presence of the H-2^k allele, because T cells from transgenic animals with both H-2^b and H-2^k MHC class II molecules, (B10.A x ML-4 C57BL/6) F1 and (CBA x ML-4 C57BL/6) F1, were unresponsive to HEL. These results provide evidence that the HEL antigen was presented efficiently by H-2^k MHC molecules; therefore tolerance was established, at least for the H-2^k-restricted HEL-specific T cells. Since a concentration threshold exists for deletion of autoreactive T cells in the thymus, the haplotype of the MHC could affect the threshold for tolerance induction depending on how well it could bind certain peptides. Therefore, in the H-$2^{b/b}$ animals, the H-2^b MHC molecules might be unable to bind and present a high enough concentration of HEL to induce deletion of the autoreactive cells, whereas in the F1 animals the H-2^k MHC molecules might bind HEL

with a higher affinity and present low levels of this antigen efficiently enough to induce deletion of the HEL-specific T cells.

In light of the finding that the T cells from the transgenic C57BL/6 parental strain were responsive to HEL, it was surprising to find that the HEL-specific T cells were tolerant in the transgenic F1 animals, because in an F1 animal there should have been some HEL-specific T cells restricted to H-2^k, others restricted to H-2^b, and some restricted to H-$2^{b/k}$. If the H-2^k-restricted T cells dominated the response to HEL in the F1 nontransgenic animals, as discussed above, then in the F1 transgenic animals the H-2^k-restricted HEL-specific T cells would be deleted, and the response from the H-2^b-restricted T cells should have been more apparent. As shown in Fig. 26, the H-2^b-restricted HEL-specific T cells could be detected in the transgenic F1 animals, but only after a large dose of antigen was used for T cell priming. These results would be consistent with the idea that the HEL-specific H-2^b-restricted T cells were of lower affinity; therefore more antigen was required to elicit their response.

There are at least three possibilities to explain why the H-2^b-restricted HEL-specific T cells do not respond to the normal antigen dose in the transgenic F1 animals, but do respond in the H-$2^{b/b}$ animals. The first possibility has been referred to as determinant snatching. If the H-2^k MHC molecules have a higher affinity for HEL than H-2^b MHC molecules, then HEL peptides may not be presented to H-2^b-restricted HEL-specific T cells in the F1 animals, because HEL preferentially binds and is processed on H-2^k MHC molecules. Support for determinant capture as a mechanism of determinant selection has come from the laboratory of Eli Sercarz after analyzing the presentation of HEL in nonobese diabetic (NOD), (NOD x Balb/c) F1, and E_α^d transgenic NOD mice (125). They found that the presence of the E^d molecule, which binds the dominant determinant 108-116, in the F1 animals eliminated the response to the adjoining peptide determinant 95-102,

which is normally seen as a subdominant determinant in NOD mice. Another explanation for the tolerance to HEL observed in the F1 transgenic animals is that the presence of H-2^k may have an effect on the H-2^b T cell repertoire. It is possible that a self peptide presented on H-2^k MHC molecules could cause deletion of the H-2^b-restricted HEL-specific T cells. An additional possibility is that when negative selection of the high affinity H-2^k-restricted HEL-specific T cells occurs, very low affinity H-2^k-restricted HEL-specific T cells might escape deletion. These low affinity H-2^k-restricted HEL-specific T cells could develop into Th2 effector cells upon encountering HEL presented by APC following challenge with HEL in CFA, as it has been shown that peptide/MHC class II complexes that interact weakly with the TCR favor priming of Th-2-like cells (126). These low affinity H-2^k-restricted HEL-specific Th2 cells would not proliferate *in vitro* upon antigen stimulation, but could suppress the response of the H-2^b-restricted HEL-specific T cells.

B. Are B Cells Necessary for in vivo Priming of Naive T Cells?

The first set of experiments presented in this thesis addressed whether activated B cells could stimulate unprimed naive LN T cells. These experiments were performed in B cell-deficient animals, created by the targeted disruption of the μ heavy chain, and the findings indicated that there was a defect in the ability of T cells to proliferate *in vitro* following priming *in vivo* in the absence of B cells. I found that the overall proliferative response of whole LN cells from the B cell-deficient animals was consistently 3 to 10-fold less than in normal animals (Figure 3). This defect was first reported in animals made B cell-deficient by anti-μ treatment (102). Over the years, many investigators have addressed the role of B cells in stimulating T cell responses. Several possibilities have been suggested: B cells may be required for priming naive T cells *in vivo* (102-105), B cells may

contribute *in vitro* as antigen-specific APC for T cells (101, 108), the antibody produced by B cells may contribute to macrophage priming of T cells (105), the form of the antigen may be important in determining which APC is required to initiate T cell responses (127), and finally non-B APC may initiate T cells responses, but B cells may be necessary for T cell clonal expansion (103, 105). After noticing the decreased ability of T cells from B cell-deficient mice to proliferate *in vitro* following antigen priming, I also became interested in determining which, if any, of these possibilities were correct.

The ability of activated B cells to stimulate naive T cells has been very controversial. As mentioned above, the basis for the idea that B cells were important for initiating T cell proliferative responses stemmed from the finding that T cells from protein primed anti-μ treated mice proliferated poorly when restimulated *in vitro* with antigen. Although my own experiments supported this finding, experiments performed in *SCID* mice demonstrated that B cells were not required for efficient T cell priming. Sunshine and colleagues found that when *SCID* mice were transfused with adult thymocytes, and subsequently primed with the protein antigen KLH, the T cells proliferated and secreted IL-2 (106). Ronchese and Hausmann also demonstrated that T cells could be efficiently primed in the absence of B cells by reconstituting SCID mice (H-2^d) with either F1 (H-$2^{b/d}$) T cells alone or F1 T and B cells (107). They found that the MHC restriction of all the responding T cells was H-2^d, indicating that the T cells had been primed by the dendritic cells of the host, not the F1 B cells.

However, additional evidence in support of the idea that B cells were involved in initiating the immune response in the LN was demonstrated by Ron and Sprent when they reported that the addition of B cells subcutaneously into anti-μ treated mice, prior to injection of antigen, restored the T cell proliferative response (103). This was also shown by Kurt-Jones and colleagues when hapten-primed B cells were injected into anti-μ treated

mice together with haptenated antigen in CFA (105). As shown in Figure 4, when I injected T-depleted spleen cells into B cell-deficient animals, the T cell proliferative responses were partially restored in only some of the animals. The difference between my findings and those of Ron and Sprent could have been the source of B cells, because I used T-depleted spleen cells, and they used B cells purified from lymph nodes. Alternatively, antigen-specific B cells may enhance the T cell proliferative response by presenting antigen more efficiently *in vitro* than other APC.

Indeed, evidence that B cells contribute *in vitro* to the T cell proliferative response as antigen-specific APC was reported by Malynn and colleagues after discovering that SRBC-specific T cell proliferation required the presence of primed SRBC-specific B cells in the *in vitro* cultures (108). Furthermore, Epstein and colleagues (101) found that the T cell proliferative response of normal and B cell-deficient animals was equivalent only when B cells were rigorously removed from the primed T cells by including anti-B220 in their purification scheme, suggesting that contaminating B cells in the T cell preparation could have contributed to the proliferative response by acting as antigen-specific APC *in vitro*. In my own experiments, the proliferative response of OVA-primed T cells from B cell-deficient mice was not enhanced by the addition of either OVA-primed B cells or non-specific B cells, as shown in Figure 5. However, the results shown in Figure 6 indicate that when T cells were purified from both the normal and the B cell-deficient mice, the T cell proliferative responses of the normal and B cell-deficient mice were more comparable when the T cells were set up at lower cell densities *in vitro*. In other experiments, B cell depletion *in vitro* did not bring proliferative responses of normal mice down to the levels of B cell-deficient mice, but it is possible that my B cell depletion was not always as complete as that of Epstein and colleagues, even when I used anti-B220 to deplete.

There is also experimental evidence suggesting that circulating immunoglobulin may enable macrophages to function as antigen-specific APC. Kurt-Jones and colleagues reconstituted anti-μ-treated F1 animals with parental FITC-specific B cells from normal mice, primed either 2 weeks or 4 months earlier, and examined the MHC-restriction of the recipient T cells after challenge with FITC-OVA in CFA (105). The primed T cells in the anti-μ treated animals reconstituted with FITC-specific B cells transferred 4 months after donor priming were restricted to recognizing antigen on APC of the same haplotype as the transferred B cells. However, when FITC-specific B cells were transferred two weeks after donor priming, and therefore could probably continue to produce antibody, the responding T cells were able to recognize antigen presented by both the F1 APC as well as the parental APC. Thus, the antibody produced by the donor B cells after challenge in the recipient anti-μ treated animals may have been bound by the host macrophages, enabling them to present antigen to the T cells.

Recent experiments performed in the lab of Kim Bottomly have indicated that the form of the antigen dictates which APC preferentially primes $CD4^+$ T cells. On the basis of experiments using mice that express I-E on different APC or mice that are B cell-deficient, they concluded that dendritic cells were the most efficient APC for priming T cells to peptide antigen, whereas B cells were necessary for T cell priming to whole protein (109, 127). My own experiments were not entirely consistent with their findings. The results shown in Figure 7, comparing priming with PCC peptide vs. PCC whole protein indicate that in the absence of B cells, priming with peptide was more efficient, but this finding may depend more on which antigen was used. In three experiments I performed, the T cell proliferative response of the normal animals to the protein antigen PCC was consistently lower than their response to the protein antigen OVA (Figures 3, 7, and data not shown). Furthermore, Constant and colleagues showed that there was a greater difference between

the T cell proliferative responses of normal and B cell-deficient mice primed with PCC protein (6-fold difference) than primed with OVA protein (less than 2-fold difference) (127). Therefore, it may appear that peptide priming results in better T cell responses when B cells are absent, but this may just depend on which protein was used in the experiment, PCC or OVA. The best way to address whether dendritic cells or B cells preferentially present peptide or protein antigen, respectively, would be to prime normal and B cell-deficient mice with a variety of different antigens in the form of peptide antigen and protein antigen and then examine the T cell response *in vitro*.

The results of my own experiments, as well as those of others, have not completely resolved the role of B cells in priming naive T cells to antigen. Currently, the general thinking is that dendritic cells, due to their constitutive expression of high levels of costimulatory activity, probably initiate T cell responses, while activated B cells can drive T cell clonal expansion (128). Evidence that dendritic cells stimulate naive T cells has been shown *in vitro* using naive T cells from transgenic animals. Transgenic T cells that recognize a peptide of PCC were found to proliferate and produce IL-2 when antigen was presented by either dendritic cells or activated B cells, but at lower cell numbers dendritic cells were better stimulators of naive T cells (129). Dendritic cells might be superior APC for naive T cells at lower cell numbers, because they express higher levels of costimulatory activity than activated B cells (130).

The idea that B cells can promote T cell clonal expansion and diversification of the T cell response once the T cell has been initially activated by dendritic cells correlates with B cells' unique antigen-specific APC capability. A single protein antigen could be captured by a variety of B cells with different antigenic specificities; thus a unique array of peptides, including dominant and subdominant determinants, would be generated in each B cell and presented very efficiently, depending upon where the binding site of the immunoglobulin

molecule was located on that protein (131). Support for this idea comes from experiments in which the injection of a single immunodominant peptide from the autoantigen MBP not only resulted in the development of EAE but was also able to generate T cells that were responsive to other peptides within MBP (132). Similar experiments performed in B cell-deficient mice should demonstrate definitively whether B cells are required for expansion and diversification of the immune response.

C. B Cells as Tolerizing APC to Low Doses of Antigen

The next set of experiments presented in this thesis addressed whether B cells were required as antigen-specific APC in tolerance to low concentrations of soluble self proteins or in acquired tolerance to low doses of a foreign protein antigens. There are several reasons for proposing that B cells would be involved in tolerance induction to low concentrations of soluble protein antigens. B cells are very efficient APC, due to their cell-surface immunoglobulin receptors, and can pick up antigen at 10,000-fold lower concentrations than other non-specific APC (39, 57, 133, 134). Therefore, antigen-specific B cells might be the only APC in the periphery that could capture enough of a low dose antigen to affect a T cell response. Also, B cells have been shown to process and present monovalent antigen while remaining in a resting state (135). It has been well established that T cells require two signals from an APC to become fully activated, antigen recognition and costimulation, and since resting B cells lack costimulatory activity it seemed likely that they would present antigen in a tolerogenic manner to a naive T cell. Furthermore, several studies have shown that naive T cells cannot be activated by resting B cells (129, 136, 137, 138). In addition, our laboratory and others have shown that small resting B cells can act

as potent tolerizing APC for mature peripheral T cells (87-93). Therefore, I used B cell-deficient animals to directly test whether B cells were involved in tolerance to low concentrations of soluble self proteins and in acquired tolerance to low dose soluble foreign protein antigens.

To look at tolerance to soluble self proteins in the absence of B cells, B cell-deficient mice were created that expressed a transgene-encoded self antigen, HEL, at low levels in the serum (Figure 8, and 94). I found that self tolerance, as measured by a reduction in HEL-specific T cell proliferation, was intact in the ML-4 HEL-transgenic, B cell-deficient animals, as shown in Figure 9. There are at least two possible explanations for this result. Perhaps some other APC can present HEL sufficiently well to inactivate the HEL-specific T cells in the periphery, despite the extremely low serum concentration of HEL. Alternatively, the HEL self antigen may have been presented in the thymus resulting in the deletion of the self-reactive T cells during development. Although my own experiments could not distinguish between tolerance induction occurring in the thymus or the periphery, in H-$2^{b/k}$ ML-5 mice, which express higher levels of HEL in the serum than ML-4 mice, tolerance to HEL was shown to result from thymic deletion of a high affinity HEL-specific TCR (111 and C. Goodnow, personal communication).

The second system I used to examine tolerance induction by B cells was acquired tolerance. In acquired tolerance, prior exposure to an antigen under certain conditions will result in antigen-specific nonresponsiveness when the antigen is later given in an immunogenic form. The experiments on acquired tolerance in the 1960's demonstrated that deaggregated antigen, given either i.v. or i.p. in repeated low doses or one high dose, resulted in tolerance induction using antibody formation as a readout. I found that low zone acquired tolerance could also be measured by T cell proliferation assays. As shown in Figures 10 and 12, normal mice given repeated injections of deaggregated antigen i.v. were

found to have reduced T cell proliferative responses following challenge with antigen in CFA. The results in Figure 13 suggested that the antigen dose used for tolerance induction was important, as T cell responses were not inhibited in animals pretreated with doses of antigen of 1 μg or less.

The results shown in Figure 12 demonstrated that acquired tolerance to low dose foreign proteins also was shown to occur in the absence of B cells, as both normal and B cell-deficient animals treated with low doses of OVA exhibited a reduction in antigen-specific T cell proliferation and IL-2 production and in a loss of antigen-specific IFN-γ production following challenge. Recently, B cell-deficient mice have been used to show that B cells are also not required for high zone tolerance to protein or peptide antigen or for superantigen-induced T cell unresponsiveness (139-141). These findings on self and acquired tolerance in B cell-deficient mice do not rule out the possibility that there may be other conditions under which antigen-specific B cells are required for tolerance induction. Instead, they indicate that other APC in the periphery must be able to present both high and low concentration antigen to immunocompetent T cells in a tolerogenic manner. Recently, macrophages that have differentiated in the presence of macrophage colony stimulating factor have been shown to be able to induce antigen-specific apoptosis of peripheral T cells (142). It has also been shown that even dendritic cells, normally thought to be constitutively high for the expression of costimulatory molecules, can be tolerogenic APC for mature peripheral T cells when antigen is targeted to them (F. Finkelman, submitted).

D. What is the Effect of Low Doses of Antigen on the Resulting Immune Response?

In the course of these experiments, I found that partial low zone tolerance appears to have a selective effect on the Th1 response, as measured by a reduction in antigen-specific proliferation and loss of IFN-γ production *in vitro*, without a corresponding loss in the ability to make a high titer IgG1 and IgG2b antibody response to this T-dependent antigen following challenge with antigen in CFA. Acquired tolerance to low doses of foreign protein antigen was originally defined by Mitchison (81) by measuring antibody formation to BSA. He was unsuccessful at inducing low zone tolerance, measured by antibody formation, with the antigens OVA or HEL, and found instead that these antigens immunized the treated animals at very low doses, down to 0.1 μg (143). As shown in Figure 14, I confirmed that low doses of deaggregated OVA do not prevent antibody responses following challenge with OVA in adjuvant, although none of my mice made an antibody response before challenge. Instead, I found that Mitchison's low zone tolerance protocol with OVA resulted in T cell unresponsiveness in the LN proliferation assay and loss of IFN-γ production.

Because the treated mice made the same amounts of the same IgG subclasses as the controls in most experiments (Figure 15), it seems likely that both the B cell compartment and T cell help for antibody formation were not affected by treatment with deaggregated OVA. The lack of an effect on the B cell compartment was expected. There are different dose requirements for inducing tolerance in the B and T cell compartments: lower doses of antigen compromise the T cell compartment, but higher doses of antigen are needed to inactivate the B cells (94, 144). The selective loss of the LN T cell proliferation response is harder to explain. It may reflect merely a difference in sensitivity of T cell proliferation

versus help for antibody formation; perhaps T cell help is not limiting for antibody formation at the level of T cell tolerance that was achieved. A more interesting possibility is that exposure to low dose antigen in the absence of adjuvant reduced the ability of OVA-specific T cells to differentiate into Th1 effector cells capable of making IFN-γ or the large amounts of IL-2 required for the LN proliferation assay. Since neither IL-2 nor IFN-γ are necessary for T-dependent antibody formation (145, 146), the antibody response was relatively unaffected. Attenuation of a future Th1 response, particularly production of the pro-inflammatory cytokine, IFN-γ, could be an appropriate response to low dose antigen in the absence of infection or tissue damage in order to limit immunopathological responses to products of commensal microorganisms or environmental antigens.

The attenuated Th1 response could result from a shift in production from Th1 to Th2 cytokines in the low dose OVA-treated animals. Guéry and colleagues recently reported that continuous administration of low dose soluble proteins to Balb/c mice using mini-osmotic pumps suppresses subsequent Th1 cytokine production while priming strongly for Th2 cytokines (147). Enhancement of Th2 cytokines was previously reported in partial high zone tolerance to human IgG (148-152). Th2 cells would be expected to retain helper function for the antibody response, but lose the ability to proliferate vigorously and make IFN-γ upon restimulation *in vitro*. Th1 and Th2 cells secrete mutually inhibitory cytokines which cross regulate each other (63). This cross regulation tends to tip the immune response towards cell-mediated or humoral immunity, respectively (13, 153). Recently published experiments have shown that stimulation of naive T cells with low or very high doses of antigenic peptide *in vitro* promotes the development of Th2-like cells, regardless of APC type (154, 155). However, in my experiments, I was unable to detect production of the Th2 cytokine, IL-4, by LN cells from treated animals or untreated controls. This may be due to the C57BL/6 background, which has been

associated with low IL-4 production (147). On the other hand, high levels of IgG1, which are characteristic of antibody responses driven by Th2 cells (113), were produced both in animals treated with low doses of antigen and in the untreated controls. Therefore, I have no evidence for or against an effect of low dose, deaggregated antigen on a subsequent Th2 response. It is not clear from the high zone tolerance experiments mentioned above that Th2 cytokines are necessary for inhibition of the Th1 response (149). It may be just that the Th1 response is more sensitive to tolerizing treatments, so that partial tolerance spares the Th2 response (156). In fact, Romball and Weigle have shown that treatment with a high dose of deaggregated human IgG, under conditions which induce more complete tolerance, as measured by antibody formation, results in tolerance of both T helper subsets, measured by IL-2 and IL-4 production (156).

It should be noted that none of my mice made antibody before challenge in response to repeated injections of 10 μg of deaggregated OVA, and only 6 of 16 mice showed barely detectable priming for an earlier antibody response following challenge with OVA in adjuvant. Therefore, the selective loss in the ability of treated mice to mount a Th1 response occurs silently, and seems unlikely to be accompanied by a lot of clonal expansion and production of memory or effector cells.

E. What is the Mechanism of Tolerance Induction in Selective Low Zone Tolerance?

As shown in Figure 18, the T cell proliferative responses of the treated animals were also reduced at earlier time points post-challenge, at day 5 and day 7, suggesting that the unresponsiveness observed 10 days post-challenge was not simply due to a change in the kinetics of the response in the treated animals. I also found no evidence that the

antigen-reactive cells had migrated into the spleen following i.v. administration of antigen, as the splenic T cell proliferative response of the treated animals were also reduced (Figure 11). Due to the low frequency of antigen-specific T cells in my animals, I am unable to follow the fate of the antigen-specific T cells in the periphery after the treatment with low doses of antigen. However, Kearney and colleagues used TCR transgenic T cells adoptively transferred into syngeneic recipients to follow the fate of a few antigen-reactive T cells when antigen was given s.c., i.p., or i.v. (157). They found that antigen administered s.c. in CFA led to the proliferation and persistence of the antigen-specific T cells in the LN, and these cells remained responsive to restimulation with antigen several weeks later. Antigen given i.v. or i.p. also caused initial proliferation of antigen-reactive T cells, but then most of these cells disappeared from the lymphoid tissues, and the remaining cells were unresponsive. They proposed that the antigen-specific T cells could not be detected in the LN, because they had migrated to the gut-associated lymphoid system where they died, or they may have undergone activation-induced cell death in the LN, or perhaps they were unable to produce adequate levels of IL-2 which resulted in apoptosis induced by growth factor withdrawal.

There are at least two additional mechanisms that may account for the reduced T cell proliferative responses following treatment with low doses of deaggregated antigen: suppression or anergy. Suppression has previously been implicated in high zone tolerance to human γ globulin. Spleen cells from tolerant mice adoptively transferred into irradiated recipients were shown to suppress an antibody response (158-160). However, Gahring and Weigle reported that suppressor cells were not responsible for the reduced T cell proliferative responses they found in mice that were tolerant to human γ globulin (161). I also found no evidence for suppressor T cells in the treated, challenged animals, because

the normal proliferative response to OVA was not reduced when equal numbers of T cells from the treated and untreated animals were added together (Figure 20).

There are two interactions that have been reported to give rise to anergy. The first has been well established using Th1 clones, and was shown to result from T cell receptor recognition of antigen in the absence of costimulation (45). If exogenous costimulation was provided during the initial TCR recognition of antigen, then anergy was prevented. Costimulation promotes the production of the T cell autocrine growth factor IL-2 (60, 61). When T cells become unresponsive, due to the induction of anergy, they are unable to produce IL-2. Furthermore, it has been reported that addition of exogenous IL-2 to the unresponsive T cells will reverse T cell clonal anergy. Jacobs and colleagues found that high zone tolerance, as measured by T cell proliferation, could be reversed by addition of exogenous IL-2 *in vitro*, suggesting that the mechanism of tolerance induction in their model was anergy of the antigen-specific T cells (116). However, as shown in Figure 19, I found that the addition of exogenous IL-2 *in vitro* failed to reveal an expanded population of anergic, antigen-responsive cells in the challenged LN of the treated animals. One possibility for the difference in our findings could be due to the amount of IL-2 added to our *in vitro* cultures. Exogenous IL-2 may have been a limiting factor in my experiments, as the T cell proliferative response in the presence of 10 U/ml IL-2 was still not at a plateau. Therefore, I cannot completely rule out anergy as the mechanism for the T cell unresponsiveness in low zone tolerance because the T cell response may have been restored if higher concentrations of IL-2 were added *in vitro*. It is also possible that the OVA-specific T cells had become anergic *in vivo*, but did not expand upon challenge, which would result in fewer antigen-reactive T cells being recovered from these animals.

Experiments performed in the lab of Paul Allen have established the second interaction that will result in T cell anergy. Their experiments demonstrated that the

presentation of altered peptide ligands to T cell clones would result in antigen-specific unresponsiveness to subsequent stimulation with the immunogenic peptide (162). Altered peptide ligands differ from immunogenic peptide ligands in the residues that interact with the TCR. They propose that when T cells recognize the altered peptides on MHC molecules that they become partially activated. These altered peptide ligands may interact with lower affinity with the TCR than the normal immunogenic peptide, resulting in only a partial signal being transduced from the TCR. This may be due to an inability of the peptide/MHC complex to cause a change in the TCR. Perhaps the MHC/peptide/TCR interaction is short-lived, or there may be no association of required surface molecules with the TCR. Unlike the T cell anergy that is induced when peptide is recognized in the absence of costimulation, anergy induced by this interaction cannot be prevented by the addition of exogenous costimulation (163). They studied the presentation of altered peptide ligands to both Th1 and Th2 clones and found that anergy could be induced in both $CD4^+$ T cell helper subsets (163, 164). Th1 clones that had been exposed to altered peptide ligands failed to proliferate and were unable to produce IL-2 or IFN-γ upon stimulation with the immunogenic peptide (163). Anergy induced in Th2 clones by this partial activation also resulted in a loss of proliferation, but unlike anergic Th1 cells, the anergic Th2 cells retained their ability to produce cytokines upon restimulation (164).

It is interesting to propose that treatment with low doses of deaggregated antigen, in the absence of adjuvant, may affect the responding T cells in a manner similar to an altered peptide ligand. In addition to the affinity of the TCR for its peptide ligand, perhaps there is also a concentration threshold so that the total number of peptide/MHC complexes interacting with the TCR is also critical for a normal signal to be delivered upon TCR engagement. Even though there might be fewer peptide/MHC complexes formed on an APC when low doses of antigen are given in adjuvant, an immune response would be

induced, because costimulatory molecules and adhesion molecules would be upregulated allowing a productive and long lasting interaction to occur between the APC and T cell. In the absence of adjuvant, the level of cell-surface costimulatory and adhesion molecules on the APC would be less; therefore the T cell may only get a partial signal from antigen recognition because it dissociates more rapidly from the APC.

My findings in mice treated with low doses of peptide are similar to the findings observed when T helper clones are presented with altered peptide ligands. Treatment with low doses of antigen followed by challenge with an immunogenic form of antigen resulted in reduced proliferative responses and a reduction in IL-2 and IFN-γ production. Although I was unable to detect IL-4 production *in vitro*, the presence of OVA-specific IgG1 in the treated animals suggested that the Th2 cells were not compromised in their ability to secrete IL-4. Therefore, perhaps the phenomenon of low zone tolerance cannot be explained by a special APC that would present antigen in the absence of costimulation, but instead directly correlates with the antigen dose, and the way in which the resulting peptide/MHC complexes that are formed eventually interact with the TCR.

F. Summary and Future Directions

This thesis provides evidence that tolerance induction to low levels of antigen can occur in the absence of B cells. Self tolerance to a transgenic self antigen, HEL, in a strain with very low levels of serum HEL was found in both normal and B cell-deficient transgenic animals. Also, normal and B-cell deficient animals treated with repeated low doses of deaggregated OVA had reduced *in vitro* T cell proliferative responses upon challenge with OVA in CFA. The *in vitro* T cell proliferation assays were primarily detecting a Th1 response, and tolerance following treatment with deaggregated low dose

OVA was selective for this response, because the production of IL-2 and IFN-γ was reduced in cells from the treated normal and B cell-deficient mice. The antibody response of B cell-sufficient mice to this T cell-dependent antigen was largely unaffected by the treatment, as both treated and untreated animals produced equivalent titers of anti-OVA antibody, predominantly of the IgG1 and IgG2b isotypes, following challenge with OVA in CFA. In experiments performed in HEL-transgenic animals of two different haplotypes, the efficiency of antigen presentation was also shown to be important in tolerance induction.

My results contribute to the evidence in the literature that antigen dose is intimately involved in the development of either Th1 and Th2 effector cells (154, 155). Antigen dose probably relates to the level of peptide/MHC complexes presented on an APC, and it would be interesting to determine the relative number of peptide/class II MHC complexes on APC following antigen given i.v., i.p., or s.c. in adjuvant. Also, because IL-10 has been shown to downregulate Th1 responses (63), detection of this cytokine following *in vitro* stimulation of T cells from the treated animals would provide an explanation for the reduced proliferative responses and the Th1 cytokines observed in animals given low doses of antigen. In addition, Th2 cells have been implicated in the suppression of Th1 responses (80); therefore it might be worthwhile to try to generate Th1 and Th2 clones from the treated animals and then determine how they would affect a response to an immunogenic challenge when injected i.v. into syngeneic recipients.

Additional questions also remain regarding the lack of tolerance to a transgenic self antigen observed in the C57BL/6 strain. Is tolerance induced by negative selection in the high affinity T cells, while the low affinity T cells escape deletion? This could be examined in the F1-nontransgenic and transgenic animals using an antibody to MHC class II to block the proliferative response to antigen. If the response observed in the nontransgenic animals

is mostly due to high affinity T cells and in the transgenic animals it is due to low affinity T cells, then more antibody would be required to block the T cell proliferative response from the nontransgenic animals than from the transgenic animals. There is also evidence in the literature that C57BL/6 mice cannot present an immunodominant determinant of HEL, 46-61, because they are unable to cleave the arginine residue found at position 61, an event which would enable the I-A^b MHC molecules to bind this determinant (118). However, C3H.SW mice that possess identical MHC molecules as the C57BL/6 mice are able to cleave HEL from 46-61 to 46-60 and therefore respond to this determinant. It would be interesting to breed transgenic C57BL/6 mice to C3H.SW mice and test the proliferative responses of the F1-transgenic animals to determine whether the presence of a high affinity dominant determinant of HEL would result in more efficient tolerance induction.

REFERENCES

1. Burnett, F. M. 1957. A modification of Jerne's theory of antibody production using the concept of clonal selection. *Aust. J. Sci.* 20:67-69.

2. Edelman, G. M., B. A. Cunningham, W. E. Gall, P. D. Gottlieb, U. Rutishauser, and M. J. Waxdal. 1969. The covalent structure of an entire γG immunoglobulin molecule. *Proc. Natl. Acad. Sci. USA* 63:78-85.

3. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature.* 302:575-581.

4. Kataoka, T., T. Kawakami, N. Takahashi, and T. Honjo. 1980. Rearrangement of immunoglobulin γ1-chain gene and mechanism for heavy-chain class switch. *Proc. Natl. Acad. Sci. USA* 77:919-923.

5. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature* 334:395-402.

6. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248:701-702.

7. Rosenthal, A. S., and E. M. Shevach. 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J. Exp. Med.* 138:1194-1212.

8. Katz, D. H., T. Hamaoka, and B. J. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J. Exp. Med.* 137:1405-1418.

9. Carbone, F. R., and M. J. Bevan. 1989. Major histocompatibility complex control of T cell recognition. *In* Fundamental Immunology. W. M. Paul. Raven Press Ltd., New York. 541-567.

10. Harding, C. V., F. Leyva-Cobian, and E. R. Unanue. 1988. Mechanisms of antigen processing. *Immunol. Rev.* 106:77-92.

11. Townsend, S., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601-624.

12. Janeway, C. A. 1992. The T-cell receptor as a multicomponent signaling machine - CD4/CD8 coreceptors and CD45 in T-cell activation. *Annu. Rev. Immunol.* 10:645-674.

13. Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145-173.

14. Hardy, R. R., C. E. Carmack, S. A. Shinton, J. D. Kemp, and K. Hayakawa. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J. Exp. Med.* 173:1213-1225.

15. Cheng, H.-L., F. R. Blattner, L. Fitzmaurice, J. F. Mushinski, and P. W. Tucker. 1982. Structure of genes for membrane and secreted murine IgD heavy chains. *Nature* 296:410-415.

16. Kitamura, D., J. Roes, R. Kühn, and K. Rajewsky. 1991. A B cell deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature* 350:423-426.

17. Nemazee, D. A., and K. Bürki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* 337:562-566.

18. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, R. J. Trent, and A. Basten. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 334:676-682.

19. Crispe, I. N., R. P. Shimonkevitz, L. A. Husmann, J. Kimura, and J. P. Allison. 1987. Expression of T cell antigen receptor β-chains on subsets of mouse thymocytes. Analysis by three-color flow cytometry. *J. Immunol.* 139:3585-3589.

20. Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu, and K. Shortman. 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. *Immunol. Revs.* 104:81-120.

21. Petrie, H. T., P. Hugo, R. Scollay, and K. Shortman. 1990. Lineage relationships and developmental kinetics of immature thymocytes: CD3, CD4, and CD8 acquisition in vivo and in vitro. *J. Exp. Med.* 172:1583-1588.

22. Bevan, M. J. 1977. In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature* 269:417-418.

23. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, P. A. Klein, and J. Klein. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J. Exp. Med.* 147:882-896.

24. Kisielow, P., H. S. Teh, H. Blüthmann, and H. von Boehmer. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 335:730-733.

25. Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature* 336:73-76.

26. Berg, L. J., A. M. Pullen, B. Fazekas de St. Groth, D. Mathis, C. Benoist, and M. M. Davis. 1989. Antigen/MHC-specific T cells are preferentially

exported from the thymus in the presence of their MHC ligand. *Cell* 58:1035-1046.

27. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273-280.

28. Kisielow, P., H. Bluthmann, U. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T cell receptor transgenic mice involves deletion of nonmature $CD4^+CD8^+$ thymocytes. *Nature* 333:742-726.

29. Murphy, K. M., A. B. Meimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+$ $CD8^+$ TCR^{lo} thymocytes in vivo. *Science* 250:1720-1723.

30. Sprent, J., D. Lo, E.-K. Gao, and Y. Ron. 1988. T cell selection in the thymus. *Immunol. Revs.* 101:173-190.

31. Marrack, P., L. Ignatowicz, J. W. Kappler, J. Boymel, and J. H. Freed. 1993. Comparison of peptides bound to spleen and thymus class II. *J. Exp. Med.* 178:2173-2183.

32. Pawlowski, T., J. D. Elliot, D. Y. Loh, and U. D. Staerz. 1993. Positive selection of T lymphocytes on fibroblasts. *Nature* 364:642-645.

33. Bix, M., and D. Raulet. 1992. Inefficient positive selection of T cells directed by haematopoietic cells. *Nature* 359:330-333.

34. Ashton-Rickardt, P. G., A. Bandiera, J. R. Delaney, L. Van Kaer, H.-P. Pircher, R. M. Zinkernagel, and S. Tonegawa. 1994. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76:651-663.

35. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.

36. Schild, H., O. Rötzsche, H. Kalbacher, and H.-G. Rammensee. 1990. Limit of T cell tolerance to self proteins and peptide presentation. *Science* 247:1587-1589.

37. Benichou, G., P. A. Takizawa, P. T. Ho, C. C. Killion, C. A. Olson, M. McMillan, and E. E. Sercarz. 1990. Immunogenicity and tolerogenicity of self-major histocompatibility complex peptides. *J. Exp. Med.* 172:1341-1346.

38. Janeway, C. A., Jr., and P. Travers. 1994. The humoral immune response. *In* Immunobiology. The immune system in health and disease. M. Robertson. Current Biology Ltd., New York. 8:1-8:58.

39. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* 8:773-793.

40. Parker, D. C. 1993. T cell-dependent B cell activation. *Annu. Rev. Immunol.* 11:331-360.

41. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169:1042-1049.

42. Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symp. Quant. Biol.* 54:1-13.

43. Weaver, C. T., and E. R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today* 11:49-55.

44. Allison, J. P., and M. F. Krummel. 1995. The Yin and Yang of T cell costimulation. *Science* 270:932-933.

45. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248:1349-1356.

46. Lenschow, D. J., G. H.-T. Su, L. A. Zuckerman, N. Nabvi, C. L. Jellis, G. S. Gray, J. Miller, and J. A. Bluestone. 1993. Expression and functional significance of an additional ligand for CTLA-4. *Proc. Natl. Acad. Sci. USA* 90:11054-11058.

47. Hathcock, K. S., G. Laszlo, C. Pucillo, P. Linsley, and R. J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: Expression and function. *J. Exp. Med.* 180:631-640.

48. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeman, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609-612.

49. Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson. 1994. Absence of B7-dependent responses in CD28 deficient mice. *Immunity* 1:501-508.

50. Gribben, J. G., G. J. Freeman, V. A. Boussiotis, P. Rennert, C. L. Jellis, E. Greenfield, M. Barber, V. A. Restivo, Jr., X. Ke, G. S. Gray, and L. M. Nadler. 1995. CTLA-4 mediates antigen-specific apoptosis of human T cells. *Proc. Natl. Acad. Sci. USA* 92:811-815.

51. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541-547.

52. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science* 270:985-988.

53. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271-296.

54. Rattis, F.-M., J. Péguet-Navarro, M.-J. Staquet, C. Dezutter-Dambuyant, P. Courtellemont, G. Redziniak, and D. Schmitt. 1996. Expression and function of B7-1 (CD80) and B7-2 (CD86) on human epidermal Langerhans cells. *Eur. J. Immunol.* 26:449-453.

55. Janeway, C. A., Jr., and P. Travers. 1994. Host defense against infection. *In* Immunobiology. The immune system in health and disease. M. Robertson. Current Biology Ltd., New York. 9:1-9:52.

56. Liu, Y., and J. Janeway, C.A. 1991. Microbial induction of co-stimulatory activity for CD4 T-cell growth. *Int. Immunol.* 3:323-332.

57. Tony, H.-P., and D. C. Parker. 1985. Major histocompatibility complex-restricted, polyclonal B cell responses resulting from helper T cell recognition of anti-immunoglobulin presented by small B lymphocytes. *J. Exp. Med.* 161:223-241.

58. Lenschow, D. J., A. I. Sperling, M. P. Cooke, G. Freeman, L. Rhee, D. C. Decker, G. Gray, L. M. Nadler, C. C. Goodnow, and J. A. Bluestone. 1994. Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen. *J. Immunol.* 153:1990-1997.

59. Smith, K. A. 1984. Interleukin 2. *Annu. Rev. Immunol.* 2:319-333.

60. Linsley, P. S., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721-730.

61. Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss. 1991. Regulation of interleukin 2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 251:313-316.

62. O'Garra, A., and K. Murphy. 1994. Role of cytokines in determining T-lymphocyte function. *Curr. Opinion Immunol.* 6:458-466.

63. Mosmann, T. R., and K. W. Moore. 1991. The role of IL-10 in crossregulation of Th1 and Th2 responses. *Immunol. Today* 12:A49-A53.

64. Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* 254:279-282.

65. Ben-Nun, A., H. Wekerle, and I. R. Cohen. 1981. Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. *Nature* 292:60-61.

66. Stockinger, B., and B. Hausmann. 1988. Induction of an immune response to a self antigen. *Eur. J. Immunol.* 18:249-253.

67. Russell, D. M., Z. Dembic, G. Monahan, J. F. A. P. Miller, K. Burki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. *Nature* 354:308-311.

68. Goodnow, C. C., J. Crosbie, H. Jorgensen, R. A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature* 342:385-391.

69. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302-319.

70. Quill, H., and R. H. Schwartz. 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.* 138:3704-3712.

71. Gaspari, A. A., M. K. Jenkins, and S. Katz. 1988. Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific Th1 clones. *J. Immunol.* 141:2216-2220.

72. Burkly, L. C., D. Lo, and R. A. Flavell. 1990. Tolerance in transgenic mice expressing major histocompatibility molecules extrathymically on pancreatic cells. *Science* 248:1364-1368.

73. Rammensee, H.-G., R. Kroschewski, and B. Frangoulis. 1989. Clonal anergy induced in mature V$\beta 6^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. *Nature* 339:541-544.

74. Fields, P. E., T. F. Gajewski, and F. W. Fitch. 1996. Blocked Ras activation in anergic $CD4^+$ T cells. *Science* 271:1276-1278.

75. Li, W., C. D. Whaley, A. Mondino, and D. L. Mueller. 1996. Blocked signal transduction to the ERK and JNK protein kinases in anergic $CD4^+$ T cells. *Science* 271:1272-1276.

76. Ohashi, P. S., S. Oehen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65:305-317.

77. Kumar, V., and E. E. Sercarz. 1993. The involvement of T cell receptor peptide-specific regulatory $CD4^+$ T cells in recovery from antigen-induced autoimmune disease. *J. Exp. Med.* 178:909-916.

78. Vandenbark, A. A., G. Hashim, and H. Offner. 1989. Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. *Nature* 341:541-544.

79. Shinohara, N., M. Watanabe, D. H. Sachs, and N. Hozumi. 1988. Killing of antigen-reactive B cells by class II-restricted, soluble antigen-specific $CD8^+$ cytolytic T lymphocytes. *Nature* 336:481-484.

80. Powrie, F., R. Correa-Oliveira, S. Mauze, and R. L. Coffman. 1994. Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J. Exp. Med.* 179:589-600.

81. Mitchison, N. A. 1964. Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. Ser. Biol. Sci.* 161:275-292.

82. Dietrich, F. M., and W. O. Weigle. 1964. Immunological unresponsiveness to heterologous serum proteins induced in adult mice and transfer of the unresponsive state. *J. Immunol.* 92:167-172.

83. Dresser, D. W. 1962. Specific inhibition of antibody production II. Paralysis induced in adult mice by small quantities of protein antigen. *Immunol.* 5:378-388.

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

85. Shellam, G. R., and G. J. V. Nossal. 1968. Mechanisms of induction of immunological tolerance IV. The effects of ultra-low doses of flagellin. *Immunol.* 14:273-284.

86. Parish, C. R., and F. Y. Liew. 1972. Immune response to chemically modified flagellin III. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *J. Exp. Med.* 135:298-311.

87. Eynon, E. E., and D. C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* 175:131-138.

88. Yuschenkoff, V. N., and D. C. Parker. 1996. Coexpression of B7-1 blocks tolerance induction to antigen presented by resting B cells. *Submitted.*

89. Parker, D. C., and E. E. Eynon. 1991. Antigen presentation in acquired immunological tolerance. *The FASEB Journal.* 5:2777-2784.

90. Fuchs, E. J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science* 258:1156-1159.

91. Eynon, E. E., and D. C. Parker. 1993. Parameters of tolerance induction by antigen targeted to B lymphocytes. *J. Immunol.* 151:2958-2964.

92. Morris, S. C., A. Lees, J. M. Holmes, R. D. Jeffries, and F. D. Finkelman. 1994. Induction of B cell and T cell tolerance in vivo by anti-CD23 mAb. *J. Immunol.* 152:3768-3776.

93. Parker, D. C., D. L. Greiner, N. E. Phillips, M. C. Appel, A. W. Steele, F. H. Durie, R. J. Noelle, J. P. Mordes, and A. A. Rossini. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc. Natl. Acad. Sci. USA* 92:9560-9564.

94. Adelstein, S., H. Pritchard-Briscoe, T. A. Anderson, J. Crosbie, G. Gammon, R. H. Loblay, A. Basten, and C. C. Goodnow. 1991. Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen. *Science* 251:1223-1225.

95. Chen, S., and G. A. Evans. 1990. A simple screening method for transgenic mice using the polymerase chain reaction. *Biotechniq*. 8:32-33.

96. Wysocki, L. J., and V. L. Sato. 1978. Panning for lymphocytes: A method for cell selection. *Proc. Natl. Acad. Sci. USA* 75:2844-2848.

97. Tony, H.-P., N. E. Phillips, and D. C. Parker. 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated, major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med.* 162:1695-1708.

98. Phillips, N. E., and D. C. Parker. 1985. Subclass specificity of Fc gamma receptor-mediated inhibition of mouse B cell activation. *J. Immunol.* 134:2835-2838.

99. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5 using modified cDNA expression vectors. *Eur. J. Immunol.* 18:98-104.

100. Hu-Li, J., J. Ohara, C. Watson, W. Tsang, and W. E. Paul. 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). *J. Immunol.* 142:800-807.

101. Epstein, M. M., F. Di Rosa, D. Jankovic, A. Sher, and P. Matzinger. 1995. Successful T cell priming in B cell deficient mice. *J. Exp. Med.* 182:915-922.

102. Ron, Y., P. D. Baetselier, J. Gordon, M. Feldman, and S. Segal. 1981. Defective induction of antigen-reactive proliferating T cells in B cell-deprived mice. *Eur. J. Immunol.* 11:964-968.

103. Ron, Y., and J. Sprent. 1987. T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. *J. Immunol.* 138:2848-2856.

104. Janeway, C. A., Jr, J. Ron, and M. E. Katz. 1987. The B cell is the initiating antigen-presenting cell in peripheral lymph nodes. *J. Immunol.* 138:1051-1055.

105. Kurt-Jones, E. A., D. Liano, K. A. HayGlass, B. Benacerraf, M. S. Sy, and A. K. Abbas. 1988. The role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice. *J. Immunol.* 140:3773-3778.

106. Sunshine, G. H., B. L. Jimmo, C. Ianelli, and L. Jarvis. 1991. Strong priming of T cells adoptively transferred into *scid* mice. *J. Exp. Med.* 174:1653-1656.

107. Ronchese, F., and B. Hausmann. 1993. B lymphocytes in vivo fail to prime naive T cells but can stimulate antigen-experienced T lymphocytes. *J. Exp. Med.* 177:679-690.

108. Malynn, B. A., D. T. Romeo, and H. H. Wortis. 1985. Antigen-specific B cells efficiently present low doses of antigen for induction of T cell proliferation. *J. Immunol.* 135:980-988.

109. Constant, S., D. Sant'Angelo, T. Pasqualini, T. Taylor, D. Levin, R. Flavell, and K. Bottomly. 1995. Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of $CD4^+$ T cells. *J. Immunol.* 154:4915-4923.

110. Liu, Y., Y. Wu, L. Ramarathinam, Y. Guo, D. Huszar, M. Trounstine, and M. Zhao. 1995. Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response. *Int. Immunol.* 7:1353-1362.

111. Ho, W. Y., M. P. Cooke, C. C. Goodnow, and M. M. Davis. 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J. Exp. Med.* 179:1539-1549.

112. Snapper, C. M., and W. E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236:944-947.

113. Rizzo, L. V., R. H. DeKruyff, D. T. Umetsu, and R. R. Caspi. 1995. Regulation of the interaction between Th1 and Th2 T cell clones to provide help for antibody production *in vivo*. *Eur. J. Immunol.* 25:708-716.

114. McHeyzer-Williams, M. G., and M. M. Davis. 1995. Antigen-specific development of primary and memory T cells in vivo. *Science* 268:106-111.

115. Beverly, B., S.-M. Kang, M. J. Lenardo, and R. H. Schwartz. 1992. Reversal of *in vitro* T cell clonal anergy by IL-2 stimulation. *Int. Immunol.* 4:661-671.

116. Jacobs, M. J. M., A. E. M. Van Den Hoek, L. B. A. Van De Putte, and W. B. Van Den Berg. 1994. Anergy of antigen-specific T lymphocytes is a potent mechanism of intravenously induced tolerance. *Immunol.* 82:294-300.

117. Parks, D. E., M. V. Doyle, and W. O. Weigle. 1978. Induction and mode of action of suppressor cells generated against human gamma globulin. I. An immunologic unresponsive state devoid of demonstrable suppressor cells. *J. Exp. Med.* 148:625-638.

118. Grewal, I. S., K. D. Moudgil, and E. E. Sercarz. 1995. Hindrance of binding to class II major histocompatibility complex molecules by a single amino acid residue contiguous to a determinant leads to crypticity of the determinant as well as lack of response to the protein antigen. *Proc. Natl. Acad. Sci. USA* 92:1179-1783.

119. Gammon, G., N. Shastri, J. Cogswell, S. Wilbur, S. Sadegh-Nasseri, U. Krzych, A. Miller, and E. Sercarz. 1987. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Revs.* 98:53-73.

120. Gammon, G., H. M. Geysen, R. J. Apple, E. Pickett, M. Palmer, A. Ametani, and E. E. Sercarz. 1991. T cell determinant structure: cores and

determinant envelopes in three mouse major histocompatibility complex haplotypes. *J. Exp. Med.* 173:609-617.

121. Hill, S. W., D. E. Kipp, I. Melchers, J. A. Frelinger, and E. E. Sercarz. 1980. Multiple H-2 and non-H-2 genes controlling the anti-lysozyme response: alternative gene constellations can lead to responsiveness. *Eur. J. Immunol.* 10:384-391.

122. Shastri, N., D. J. Kawahara, A. Miller, and E. E. Sercarz. 1984. Antigen-specific T helper clones in a nonresponder strain require augmentation for expression of helper activity. Evidence for a possible antigen presentation defect in B cells. *J. Immunol.* 133:1215-1221.

123. Cabaniols, J. P., R. Cibotti, P. Kourilsky, K. Kosmatopoulos, and J. M. Kanellopoulos. 1994. Dose-dependent T cell tolerance to an immunodominant self peptide. *Eur. J. Immunol.* 24:1743-1749.

124. Cibotti, R., J. P. Cabaniols, C. Pannetier, C. Delarbre, I. Vergnon, J. M. Kanellopoulos, and P. Kourilsky. 1994. Public and private V beta T cell receptor repertoires against hen egg white lysozyme (HEL) in nontransgenic versus HEL transgenic mice. *J. Exp. Med.* 180:861-872.

125. Deng, H., R. Apple, M. Clare-Salzler, S. Trembleau, D. Mathis, L. Adorini, and E. Sercarz. 1993. Determinant capture as a possible mechanism of protection afforded by major histocompatibility complex class II molecules in autoimmune disease. *J. Exp. Med.* 178:1675-1680.

126. Pfeiffer, C., J. Stein, S. Southwood, H. Ketelaar, A. Sette, and K. Bottomly. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* 181:1569-1574.

127. Constant, S., N. Schweitzer, J. West, P. Ranney, and K. Bottomly. 1995. B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. *J. Immunol.* 155:3734-3741.

128. Mamula, M. J., and C. A. Janeway, Jr. 1993. Do B cells drive diversification of immune responses? *Immunol. Today* 14:151-152.

129. Croft, M., D. D. Duncan, and S. L. Swain. 1992. Response of naive antigen-specific CD4+ T cells in vitro: Characteristics and antigen-presenting cells requirements. *J. Exp. Med.* 176:1431-1437.

130. Cassell, D., and R. H. Schwartz. 1994. A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells

but are inferior to dendritic cells in providing costimulation. *J. Exp. Med.* 180:1829-1840.

131. Simitsek, P. D., D. G. Campbell, A. Lanzavecchia, N. Fairweather, and C. Watts. 1995. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J. Exp. Med.* 181:1957-1963.

132. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358:155-157.

133. Rock, K. L., B. Benacerraf, and A. K. Abbas. 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J. Exp. Med.* 160:1102-1113.

134. Lanzavecchia, A. 1985. Antigen specific interaction between T and B cells. *Nature* 314:537-539.

135. Gosselin, E. J., H.-P. Tony, and D. C. Parker. 1988. Characterization of antigen processing and presentation by resting B lymphocytes. *J. Immunol.* 140:1408-1413.

136. Krieger, J. I., S. F. Grammer, H. M. Grey, and R. W. Chestnut. 1985. Antigen presentation by splenic B cells: Resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol.* 135:2937-2945.

137. Metlay, J. P., E. Puré, and R. M. Steinman. 1989. Control of the immune response at the level of antigen-presenting cells: a comparison of the function of dendritic cells and B lymphocytes. *Adv. Immunol.* 47:45-116.

138. Jenkins, M. K., E. Burrell, and J. D. Ashwell. 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.* 144:1585-1590.

139. Miethke, T., C. Wahl, K. Heeg, and H. Wagner. 1995. Bacterial superantigens induce T cell unresponsiveness in B cell-deficient mice. *Eur. J. Immunol.* 25:3187-3190.

140. Vella, A. T., M. T. Scherer, L. Shultz, J. W. Kappler, and P. Marrack. 1996. B cells are not essential for peripheral T cell tolerance. *Proc. Natl. Acad. Sci. USA* 93:951-955.

141. Phillips, J. A., C. G. Romball, M. V. Hobbs, D. N. Ernst, L. Shultz, and W. O. Weigle. 1996. $CD4^+$ T cell activation and tolerance induction in B cell knockout mice. *J. Exp. Med.* 183:1339.

142. Munn, D. H., J. Pressey, A. C. Beall, R. Hudes, and M. R. Alderson. 1996. Selective activation-induced apoptosis of peripheral T cells imposed by macrophages. *J. Immunol.* 156:523-532.

143. Mitchison, N. A. 1968. The dosage requirements for immunological paralysis by soluble proteins. *Immunol.* 15:509-530.

144. Chiller, J. M., G. S. Habicht, and W. O. Weigle. 1971. Kinetic differences in unresponsiveness of thymus and bone marrow cells. *Science* 171:813-815.

145. Kündig, T. M., H. Schorle, M. F. Bachmann, H. Hengartner, R. M. Zinkernagel, and I. Horak. 1993. Immune responses in interleukin-2-deficient mice. *Science* 262:1059-1061.

146. Graham, M. B., D. K. Dalton, D. Giltinan, V. L. Braciale, T. A. Stewart, and T. J. Braciale. 1993. Response to influenza infection in mice with a targeted disruption in the interferon γ gene. *J. Exp. Med.* 178:1725-1732.

147. Guéry, J.-C., F. Galbiati, S. Smiroldo, and L. Adorini. 1996. Selective development of T helper (Th)2 cells induced by continuous administration of low dose soluble proteins to normal and β2-microglobulin-deficient Balb/c mice. *J. Exp. Med.* 183:485-497.

148. Burstein, H. J., C. M. Shea, and A. K. Abbas. 1992. Aqueous antigens induce in vivo tolerance selectively in IL-2-producing and IFN-γ-producing (Th1) cells. *J. Immunol.* 148:3687-3691.

149. Burstein, H. J., and A. K. Abbas. 1993. In vivo role of interleukin-4 in T cell tolerance induced by aqueous protein antigen. *J. Exp. Med.* 177:457-463.

150. De Wit, D., M. Van Mechelen, M. Ryelandt, A. C. Figueiredo, D. Abramowicz, M. Goldman, H. Bazin, J. Urbain, and O. Leo. 1992. The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes. *J. Exp. Med.* 175:9-14.

151. Vidard, L., L. J. Colarusso, and B. Benacerraf. 1995. Specific T-cell tolerance may reflect selective activation of lymphokine synthesis. *Proc. Natl. Acad. Sci. USA* 92:2259-2262.

152. Van Mechelen, M., D. De Wit, M. Ryelandt, S. Hjulström, M. Heynderickx, H. Bazin, J. Urbain, and O. Leo. 1995. Induction of Th2 responses to soluble proteins is independent of B cell tolerance status. *Internat. Immunol.* 7:199-205.

153. Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. of Immunol.* 12:635-73.

154. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor-αβ-transgenic model. *J. Exp. Med.* 182:1579-1584.

155. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med.* 182:1591-1596.

156. Romball, C. G., and W. O. Weigle. 1993. In vivo induction of tolerance in murine CD4+ cell subsets. *J. Exp. Med.* 178:1637-1644.

157. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T-cell immunity and peripheral tolerance in vivo. *Immunity* 1:327-339.

158. Doyle, M. V., D. Elliot Parks, and W. O. Weigle. 1976. Specific suppression of the immune response by HGG tolerant spleen cells. I. Parameters affecting the level of suppression. *J. Immunol.* 116:1640-1645.

159. Basten, A., J. F. A. P. Miller, R. Loblay, P. Johnson, J. Gamble, E. Chia, H. Pritchard-Briscoe, R. Callard, and I. F. C. McKenzie. 1978. T cell-dependent suppression of antibody production. I. Characteristics of suppressor T cells following tolerance induction. *Eur. J. Immunol.* 8:360-370.

160. Loblay, R., B. Fazekas de St. Groth, H. Pritchard-Briscoe, and A. Basten. 1983. Suppressor T cell memory. II. The role of memory suppressor T cells in tolerance to human gamma globulin. *J. Exp. Med.* 157:957-973.

161. Gahring, L. C., and W. O. Weigle. 1989. The induction of peripheral T cell unresponsiveness in adult mice by monomeric human γ-globulin. *J. Immunol.* 143:2094-2100.

162. Evavold, B. D., J. Sloan-Lancaster, and P. M. Allen. 1993. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today* 14:602-609.

163. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 363:156-159.

164. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1994. Th2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med.* 180:1195-205.