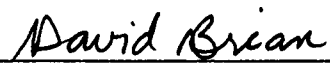


To the Graduate Council:

I am submitting herewith a thesis written by Aimee Elizabeth Cestra entitled "Amelioration of Experimental Allergic Encephalomyelitis Using an Agonist/Partial Agonist System in Immunoglobulin Constructs ". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.


Habib Zaghouani, Major Professor

We have read this thesis
and recommend its acceptance:




Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**AMELIORATION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS
USING AN AGONIST/PARTIAL AGONIST SYSTEM IN
IMMUNOGLOBULIN CONSTRUCTS**

A Thesis
Presented for the
Master of Science Degree

The University of Tennessee, Knoxville

Aimee Elizabeth Cestra
August 1998

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DEDICATION

This thesis is dedicated to my parents,
Mr. Carlo Cestra and Mrs. Diane George Cestra
who have given me the strength, courage and invaluable opportunities
to follow my dreams.

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ABSTRACT

In this study, a strategy to treat autoimmune disease was developed. In particular, a strategy was designed to ameliorate experimental allergic encephalomyelitis (EAE), a mouse model for the human disease multiple sclerosis.

Immunoglobulin constructs were designed to encode agonist and partial agonist peptides of the EAE system. Both agonist and partial agonist were efficiently presented and recognized by agonist specific T cells when in the context of immunoglobulin. Recognition of the agonist in vitro lead to the production of inflammatory cytokines. Conversely, recognition of the partial agonist yielded production of non-inflammatory cytokines. A series of in vivo experiments were designed based on this information. Evidence suggested that in mice co-immunized with agonist and partial agonist immunoglobulins, the two subsets of T cells generated downregulated one another to give a null response. These findings were used in a series of experiments designed to demonstrate the partial agonist's ability to reverse EAE in mice committed to disease. It was revealed that the partial agonist in the context of immunoglobulin was in fact able to significantly reduce the severity of EAE in disease mice.

TABLE OF CONTENTS

Part I: General Introduction and Overview.....	1
1. Survey of the Literature.....	2
T Cell Recognition of Antigen.....	2
Autoimmunity	11
Multiple Sclerosis	11
Experimental Allergic Encephalomyelitis.....	13
Treatment of EAE with a Peptide Analogue of Myelin Basic Protein	14
2. Objectives and Rationale	15
 Part II: Generation and Production of Chimeric Immunoglobulin	
Constructs, Ig-MBP3 and Ig-MBP3A.....	18
1. Introduction	19
2. Materials and methods	19
3. Results	27
4. Discussion	37
 Part III: Generation and Analyses of T Cell Hybridomas Specific for	
MBP3 Peptide	41
1. Introduction	42
2. Materials and Methods.....	43
3. Results.....	47
4. Discussion.....	53
 Part IV: T Cell Activation In Vivo	55
1. Introduction	56
2. Materials and Methods	56

3. Results	59
4. Discussion	79
Part V: Induction of EAE and Possible Treatment.....	90
1. Introduction	91
2. Materials and Methods	91
3. Results	93
4. Discussion	101
Part VI: Summary	106
References Cited	109
Vita	112

LIST OF FIGURES

FIGURE		PAGE
Part I		
1.	Recognition of antigens by T cells.....	3
2.	Costimulatory molecules generating signal 2.....	5
3.	Representation of TCR contacting residues.....	7
4.	Three possible outcomes of TCR interaction with a peptide-MHC complex	9
5.	Schematic representation of peptide delivery to APCs by immunoglobulins versus free peptide	16
 Part II		
6.	Insertion of MBP3 or MBP3A sequences in place of the D segment within the 91A3 H chain	20
7.	Schematic representation of the VDJ region of the anti-arsonate immunoglobulin, 91A3	28
8.	Schematic representation of the proposed D region replacement with MBP3 and MBP3A sequences.....	29
9.	PSV2 expression vector encoding the complete 91A3 heavy chain	30
10.	PSV2-gpt-91A3-MBP3 and PSV2-gpt-91A3-MBP3A.....	31
11.	Comparison of the chimeric heavy (H) chain gene sequences with that of the wild type	34
12.	PSV2-neo-91A3L	36
13.	Transfectoma secretion of Ig-MBP3 and Ig-MBP3A.....	38
14.	Schematic representation of Ig-MBP3 and Ig-MBP3A.....	39

FIGURE	PAGE
Part III	
15. Schematic representation of the generation of T cell hybridomas specific for MBP3	48
16. Efficient presentation of Ig-MBP3 and Ig-MBP3A, and cytokine profiles of TCH-MBP3 clones.....	50
Part IV	
17. Proliferative lymph node and spleen cell responses in mice immunized with Ig-MBP3.....	60
18. Proliferative lymph node and spleen cell responses in mice immunized with Ig-MBP3A.....	62
19. Proliferative lymph node and spleen cell responses in mice immunized with Ig-W.....	64
20. Cytokine profile of the spleen cells in mice immunized with Ig-MBP3.....	67
21. Cytokine profile of the spleen cells in mice immunized with Ig-MBP3A.....	69
22. Cytokine profile of the spleen cells in mice immunized with Ig-W.....	71
23. Cytokine profile of the lymph node cells in mice immunized with Ig-MBP3.....	73
24. Cytokine profile of the lymph node cells in mice immunized with Ig-MBP3A.....	75
25. Cytokine profile of the lymph node cells in mice immunized with Ig-W.....	77
26. Proliferative spleen cell response in mice co-immunized with Ig-MBP3 and Ig-MBP3A.....	80

FIGURE	PAGE
27. Proliferative spleen cell response in mice co-immunized with Ig-MBP3 and Ig-W	82
28. Cytokine profile of the spleen cells in mice co-immunized with Ig-MBP3 and Ig-MBP3A.....	84
29. Cytokine profile of the spleen cells in mice co-immunized with Ig-MBP3 and Ig-W	86

Part V

30. Induction of disease with MBP3, and treatment with Ig-MBP3A.....	94
31. Treatment of disease with Ig-W	97
32. Induction of multiple epitope disease with MBP3 and PLP1, and treatment with Ig-MBP3A.....	99
33. Treatment of multiple epitope disease with Ig-W	102

Part I: General Introduction and Overview

1. Survey of the Literature

T Cell Recognition of Antigen

Generally, T cells recognize and respond to antigens in the context of major histocompatibility complexes (MHCs) displayed by antigen presenting cells (APCs). The receptor found on T cells responsible for the recognition of antigens is known as the T cell receptor (TCR). The TCR will not bind to native antigen. Instead, an antigen must be internalized by an APC, processed into small fragments or peptides by proteolytic enzymes, loaded onto MHC class II molecules, and translocated to the surface for presentation to T cells (Figure 1). Antigens gain entry into the APCs by phagocytosis, fluid phase pinocytosis, or by receptor mediated endocytosis. Once internalized, antigens enter acidic vacuoles or endosomes. The antigens are then proteolysed to generate peptides, which access and associate with MHC class II molecules. In the case of T cells of the CD4 subset, recognition of the peptide is through association with MHC class II molecules. The process by which antigens are processed for display on MHC class II and recognition by CD4 T cells is known as the exogenous or endocytic pathway. The antigen can also be created by the APCs themselves, as in the case of viral infection, intracellular bacteria infection, or tumor growth. In this case, the antigen is proteolysed within the cytosol generating peptides that are transported to the endoplasmic reticulum.

The peptides associate with MHC class I molecules and the entire complex is translocated to the cell surface and displayed for recognition by T cells of the CD8 subset. The pathway leading to peptide-MHC class I complex display is called the endogenous pathway. These studies focus on the exogenous or endocytic pathway for antigen display, therefore, further information on the endogenous pathway will not be included.

Once on display at the APC surface, the peptide antigen-MHC complexes are accessible for recognition by T cells through their TCR. When the TCR binds to the peptide-MHC complex, it transduces a signal that contributes to the activation of the T cell. This signal, known as signal one, is not sufficient to activate the T cell by itself. An accompanying signal, known as signal two, is generated by other receptors expressed on T cells called costimulatory factors (Figure 2). During engagement of the TCR to the MHC-peptide complex, these molecules engage complementary costimulatory molecules on the surface of the APC. Signals one and two are required for activation of a T cell. T cell costimulatory factors that have been extensively studied are CD28/CTLA-4, LFA-1 and LFA-2. Complementary receptors for these molecules expressed on APCs are B7-1/B7-2, ICAM-1 and LFA-3, respectively.

A TCR engaged to its ligand without activation of signal two results in T cell anergy (1,2). Anergy has been defined as a state of unresponsiveness characterized by the inability of the T cell to proliferate in response to

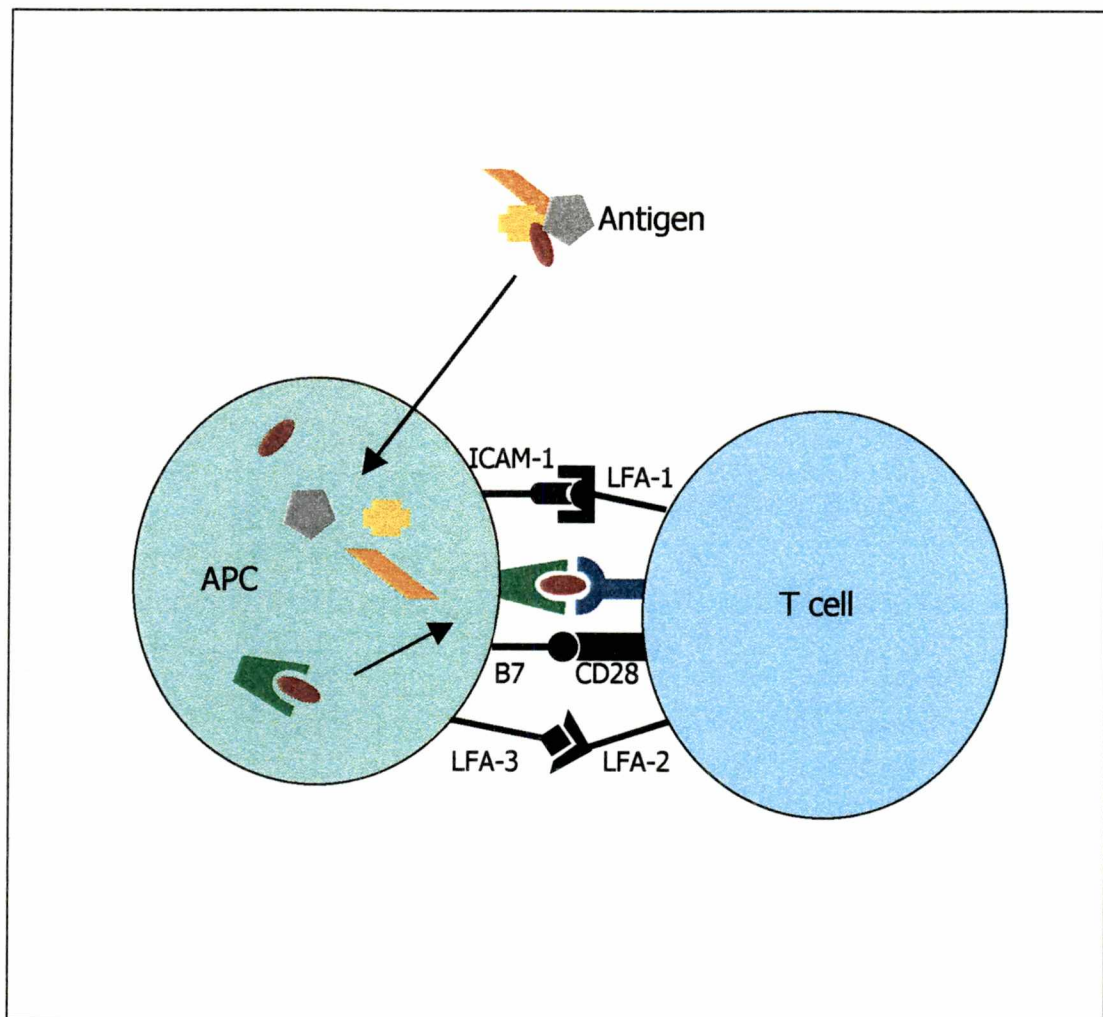


Figure 2: Costimulatory molecules generating signal 2

When both signal 1 and signal 2 are transduced to the T cell, the resulting intracellular biochemical events will drive the activation of the T cell.

stimulation with the peptide in which it is specific (3). For example, blockade of costimulatory molecules by monoclonal antibody (mAb) during TCR engagement leads to the inhibition of signal two and anergy of the T cell (4,5). This strategy seems attractive for the down regulation of autoreactive T cells and treatment of autoimmune diseases. However, this is not the case in that administration of anti-costimulatory antibodies would not be specific to autoreactive T cells and lead to a generalized suppression of the immune system.

Recent developments indicate that antigenic peptides encompass amino acid residues that are critical in the binding to MHC molecules, and others important in the binding to TCR. Those important in the binding of antigen to TCR are called TCR contacting residues (Figure 3). It has been shown that replacement of the major TCR contacting residue(s) with specific amino acids generates what is termed an altered peptide. This altered peptide retains its ability to bind MHC molecules in a manner equivalent to wild type peptide, and is able to engage the TCR. The response a T cell generates to the altered peptide is however, different than that achieved by the wild type. These results lead to the realization that a T cell's response to a peptide is variable and dependant on the TCR contact residues.

Avidity can be defined as the sum of all interaction affinities between the T cell and the APC. Affinity refers to the interaction strength between one

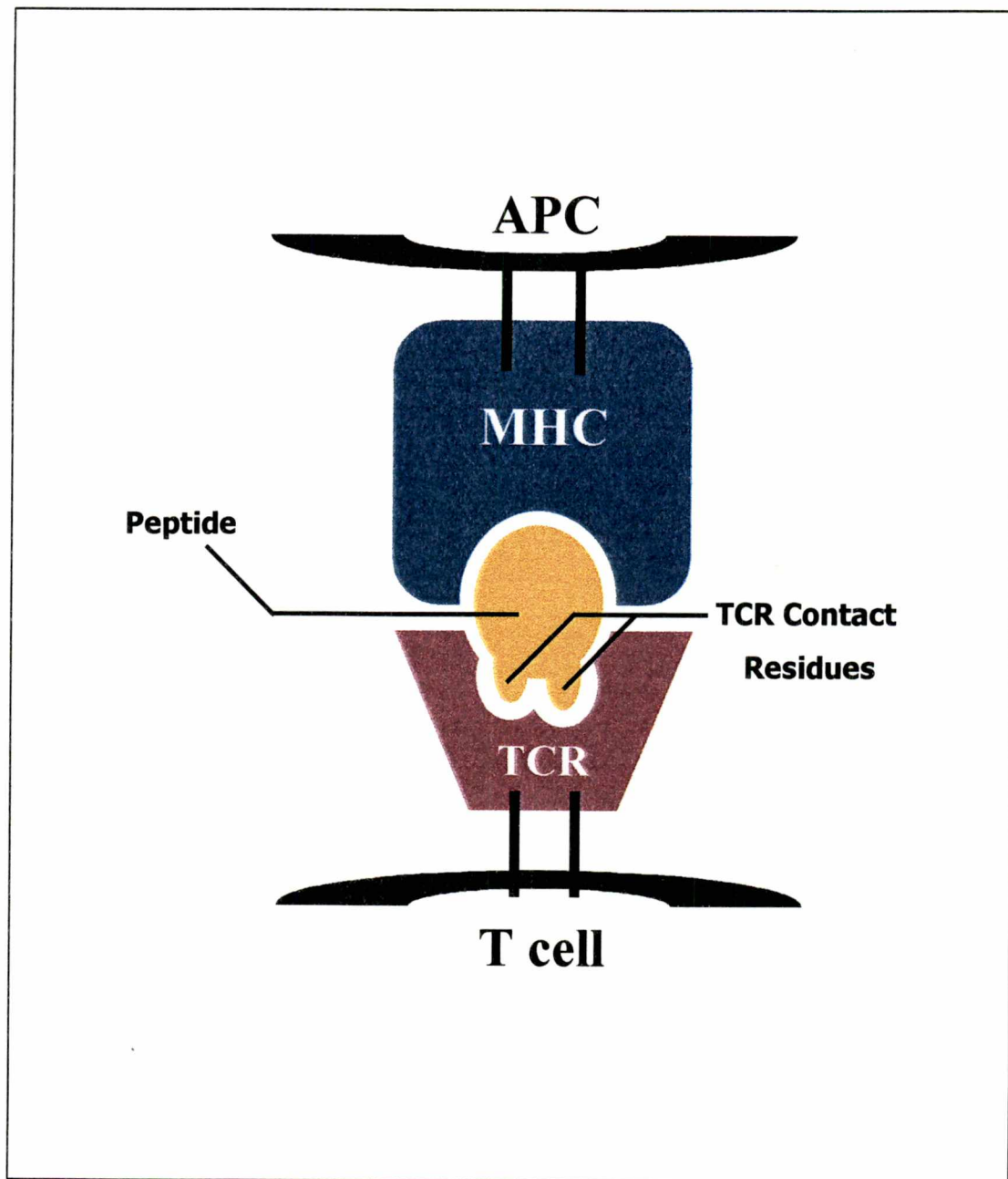


Figure 3: Representation of TCR contacting residues

Antigenic peptides encompass amino acid residues that are critical in the binding and activity of the T cell.

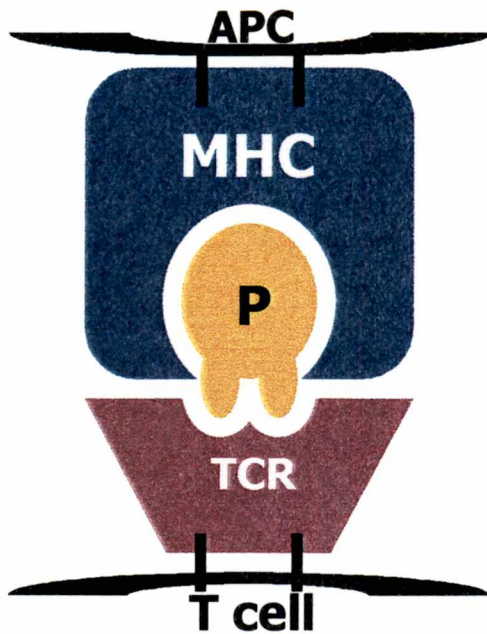
specific receptor and its ligand. The peptide can affect the interaction avidity in which the T cell response is dependent. Previous works that demonstrated a TCR's resemblance to an on/off switch are no longer applicable (2). Instead, a T cell can possess a variety of different activities depending on the affinity of the TCR-peptide interaction. These activities include super, normal, or partial activation, deviation, or antagonism (unresponsiveness). The term agonist refers to an antigen peptide that yields an optimal fit of the T cell contacting residues with the TCR. The TCR has a strong affinity for the contact residues and contact leads to full activation of the T cell (Figure 4) (3). Partial agonists or antagonists have one or more of their TCR contacting residues replaced so the TCR does not fit the antigen peptide optimally. The TCR does not have the strong affinity for the residues, as described previously, and the response to the peptide is altered (3). Another antigenic peptide, termed the null ligand, does not possess the correct TCR contacting residues and does not lead to activation (3).

Of importance to this work is the T cell activity called deviation. Deviation is defined as the production of cytokines other than those produced upon normal stimulation. Because altered peptides have the ability to change the avidity of the interaction and push T cells to deviate or become antagonized, they provide a possible strategy to down-regulate or deviate autoreactive T cells. Hence, autoimmunity could be ameliorated in a specific fashion. There have been several reports documenting the use of altered

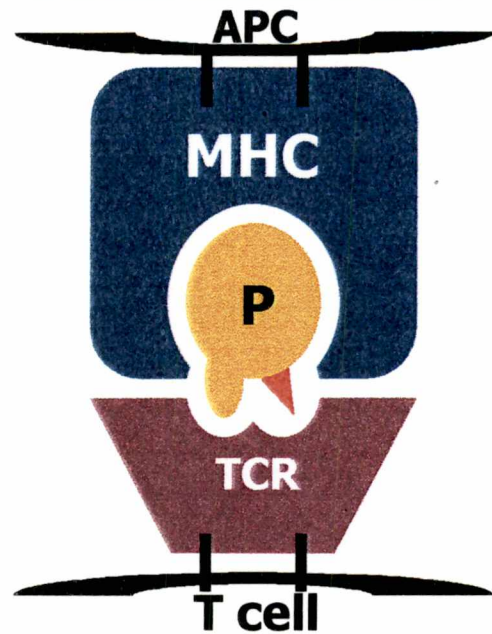
Figure 4: Three possible outcomes of TCR interaction with a peptide-MHC complex.

Full activation is achieved when the TCR recognizes an agonist peptide (P). The majority of altered ligands are created by making a single amino acid substitution, and are therefore called altered peptide ligands. This category of ligand includes partial agonists and antagonists. The final class of ligands are those that induce no response from the T cell. These are termed null ligands.

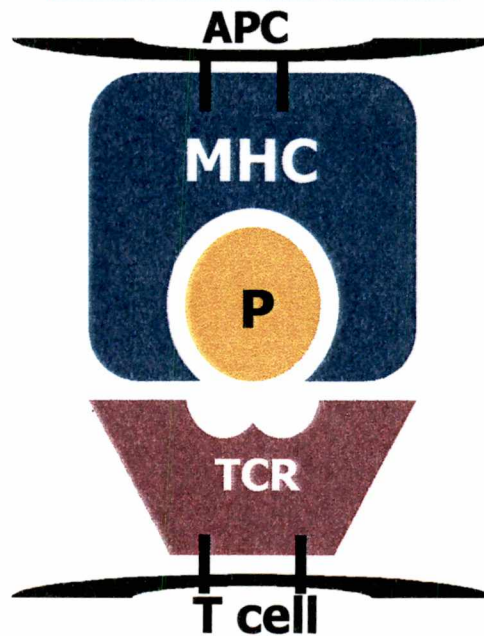
Agonist
(full activation)



Altered ligand
(partial activation)



Null ligands
(no activation)



peptide ligands (APLs) to inhibit mouse models for autoimmune diseases such as experimental allergic encephalomyelitis (6,7,8,9). More specifically, autoimmune disease induced in animals with free peptides was reversed upon treatment with free antagonist peptides (10,11).

Autoimmunity

Autoimmunity is an important cause of several human diseases. It results from a breakdown in the mechanisms normally responsible for the maintenance of self-tolerance. Autoimmunity is usually prevented by a selection process that ensures the development of self tolerant lymphocytes. Loss of self-tolerance may be the result of several phenomenas. Primarily, there may be selection of autoreactive clones. Second, abnormal stimulation of lymphocytes may cause them to become autoreactive. Lastly, autoimmunity could develop from the release of self antigens that are normally sequestered during the development of mature lymphocytes (12). There are several factors that may contribute to the conductance of autoimmunity. These factors can be genetic, environmental, and immunologic abnormalities (13).

Multiple Sclerosis

Multiple sclerosis (MS) is the major human demyelinating autoimmune disease (12). Although it has been studied for quite some time, the routes of pathogenesis and etiology of the disease have not yet been established. It is

currently believed that immunological mechanisms are involved in the course of the disease, with both genetic and environmental factors playing roles as well. MS is a chronic inflammatory disease confined to the central nervous system. The disease is quite variable depending on the individual with the common occurrence of progressive relapsing neurological deficits (13, 14).

Among other immunological aspects, inflammation of the central nervous system (CNS) in MS patients is associated with the infiltration of CD4 T cells as well as activated macrophages (14). More specifically, the CD4 T helper cells found within the sites of inflammation are of the Th1 type (15, 16). Accompanying the infiltration of these inflammatory Th1 cells is the synthesis of inflammatory cytokines such as Tumor Necrosis Factor (TNF), and Gamma-Interferon (IFN- γ). Both inflammatory cytokines are detrimental if expressed constantly by acting to recruit and activate mononuclear phagocytes and trigger T cells, as well as B cells, to differentiate and respond to self antigens (13, 14).

Although it has been understood for quite some time that the immune system plays a major role in this demyelinating disease, the cause for the inflammation remains to be discovered. A well accepted hypothesis reveals multiple sclerosis as a result of an autoimmune process mediated by autoreactive T cells specific for myelin antigens (13, 14). It is this hypothesis on which the forthcoming studies are based.

Experimental Allergic Encephalomyelitis

In order to study the pathogenesis and treatment of human disorders, animal models mimicking these diseases have been created. Because the pathology of multiple sclerosis is confined to the inaccessible CNS, animal models are the most valuable tools in researching this particular disease and its immunopathological features. Experimental allergic encephalomyelitis (EAE) is the well-characterized animal model for multiple sclerosis. It is well accepted that EAE is a T cell mediated autoimmune disease characterized by areas of inflammation and demyelination of the CNS (13). EAE shares a close resemblance to MS in both clinical and histological features. Chronic relapsing models of EAE have been developed and provide the basis for the study of in vivo regulation of autoreactive T cells and therapeutic manipulation (13). One characteristic of MS that EAE does not share is its spontaneous occurrence. Instead, EAE must be induced in genetically susceptible strains of mice and rats through immunization with myelin derived proteins, the major neural antigens (14). Adoptively transferring immune cells from diseased animals confers disease to non-sick animals, therefore, EAE is has been termed a cell mediated disorder (17). Further experimentation revealed EAE is transferred and mediated by CD4+ T cells (18). Success in the treatment of EAE has been achieved through the application of several methodologies. Antibodies directed against MHC class II molecules (19), administration of anti-TCR immunoglobulins (Ig) (20, 21), oral tolerance through CD8+ T cell TGF- β

production (22, 23), and vaccination with inactivated encephalogenic CD4+ T cells have all shown promise in the amelioration of EAE (24).

Treatment of EAE with a peptide analogue of Myelin Basic Protein

Brocke and colleagues conducted several experiments in an attempt to define therapeutic strategies for the reversal of EAE (25). Active disease was induced in susceptible mice by the transfer of a T cell clone specific for a myelin basic protein (MBP) epitope. The disease inducing epitope corresponds with MBP amino acid residues 87-99 (VHFFKNIVTPRTP). Interestingly, when the T cell clone was tolerized in vivo with a peptide analogue of the MBP epitope, the inflammatory infiltrates regressed, and the T cell infiltrates disappeared from the brain (25). The peptide analogue was created by a mutation at the 96th position in which a proline was substituted by an alanine. It was determined that mutation at this position caused the reversal of EAE because this particular amino acid is a major TCR contact residue and therefore, had an effect on the response the T cell generates to this peptide antigen. Also discovered was the reversal of this tolerance upon introduction of antibody against interleukin-4 (IL-4), displaying the role IL-4 has in the amelioration of the disease. It is therefore speculated that the production of IL-4 is from T cells that have deviated as a result of their interaction with the altered peptide ligand. This deviation in cytokine profile causing the production of IL-4 aids in the reversal of EAE by inhibiting TNF- α , an inflammatory

cytokine (25). Treatment with the altered peptide ligand selectively silenced pathogenic T cells and actively signaled for the efflux of other T cells as a result of the production of IL-4 (25).

2. Objectives and Rationale

The objective of this study is to develop a strategy for the prevention and treatment of autoimmune diseases. More specifically, research was conducted to control the activation of autoreactive T cells in EAE. It has been previously demonstrated that the variable regions of immunoglobulins are permissive to molecular grafting of MHC class II restricted T cell epitopes. In addition, it has been shown that peptides grafted into immunoglobulins are delivered to T cells in a manner that is 100-1000 fold more effective than by adding exogenous peptide (26). The rationale behind how this process works is attributed to a few theories. First, immunoglobulins have a half-life that is substantially greater than that of a peptide. Because of the globular nature of the immunoglobulin, they are resistant to extracellular degradation, and are therefore able to persist for long periods of time. The second reason behind the efficiency of the Ig constructs in presenting peptide epitopes is their ability to access APCs through an $Fc\gamma/Fc\gamma$ receptor interaction (Figure 5) (27). Internalization and eventual degradation of the chimeric Ig provides the epitope access to newly synthesized MHC molecules. Conversely, exogenous peptide must first displace the self-peptides already bound to the MHC

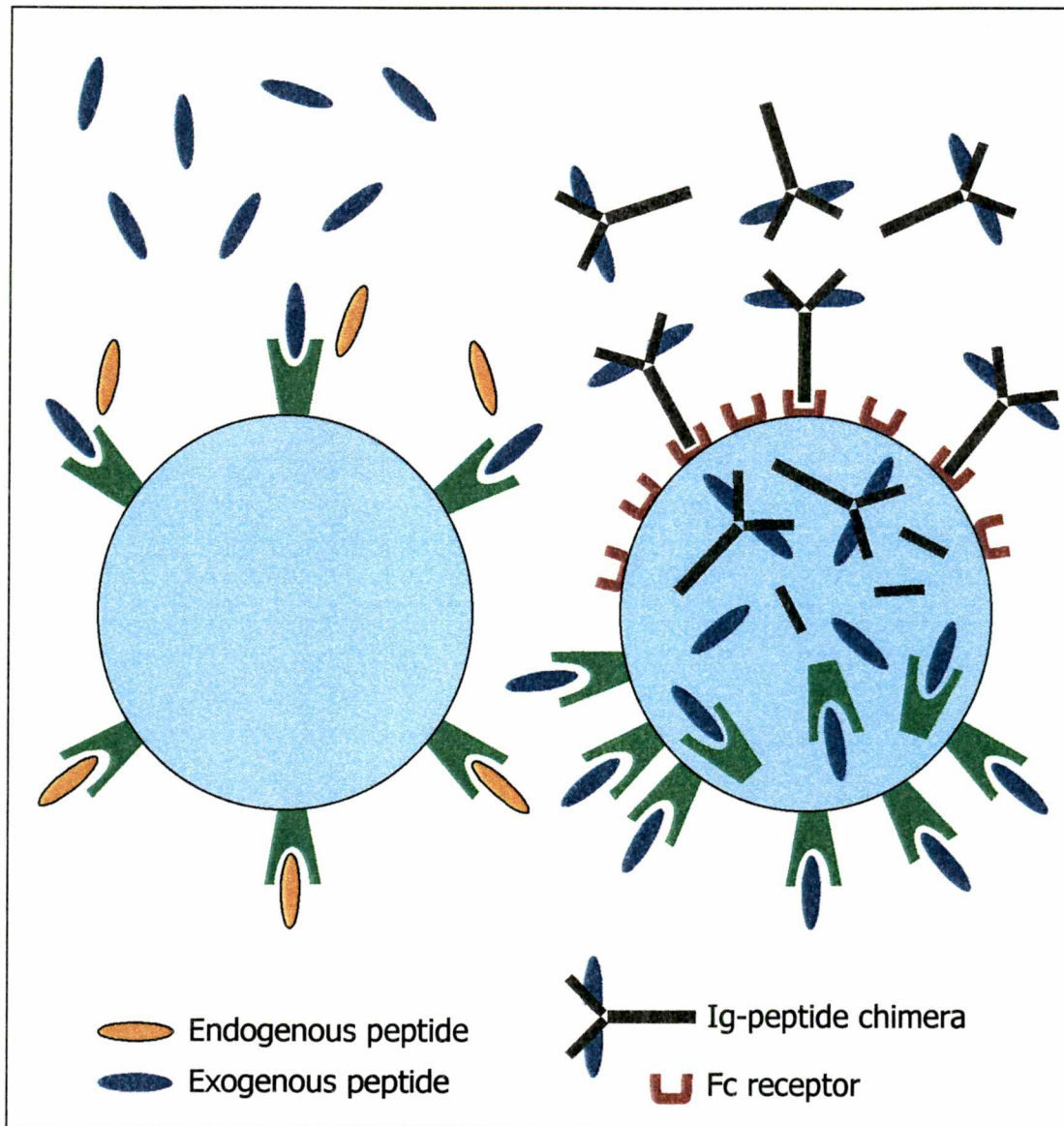


Figure 5: Schematic representation of peptide delivery to APCs by immunoglobulins versus free peptide

Peptides inserted into Igs are displayed to T cells in an effective manner because they are able to gain entry into the APC via Fc receptor mediated endocytosis. In addition, they are capable of accessing newly synthesized MHC molecules. Conversely, exogenous peptides must first displace endogenous peptides for their display.

molecule for their eventual presentation. In addition, MHC molecules expressed on the surface are subjected to physiological turnover. Therefore, the complexes formed with exogenous peptides after endogenous peptide replacement would remain at the surface for a shorter period of time in that these MHC molecules have been at the surface for some time already. Consequently, there are limitations to using synthetic peptides in the treatment of autoimmune diseases.

Grafting of antagonist peptides into chimeric immunoglobulin constructs proved to be a successful way to efficiently downregulate autoreactive T cells (27). Autoreactive T cells were antagonized through the recognition of an altered peptide delivered by an immunoglobulin (27). Because the immunoglobulin constructs were previously successful in the downregulation of autoreactive T cells specific for proteolipid protein (PLP), another target antigen in EAE, the strategy used in this study is the efficient delivery of altered peptide ligands grafted into immunoglobulins.

The specific aim of these experiments is to generate immunoglobulin constructs encoding the altered peptide ligand of MBP (87-99) and utilize this altered peptide ligand in the context of Ig to efficiently deliver the peptide to encephalogenic T cells and lead to their deviation and the reversal of EAE.

**Part II: Generation and Production of Chimeric
Immunoglobulin Constructs, Ig-MBP3 and Ig-MBP3A**

1. Introduction

Work performed by Brocke, et al on the EAE system provided insight leading to the development of new immunoglobulin constructs. Immunoglobulin constructs containing both the agonist (MBP 87-99), herein referred to as MBP3, as well as the partial agonist (96P→96A), MBP3A, will provide a more efficient means of peptide delivery. More importantly, delivery of the altered peptide ligand, MBP3A, through immunoglobulin constructs may lead to possible therapeutic ideas by deviating the cytokine response of encephalogenic T cells.

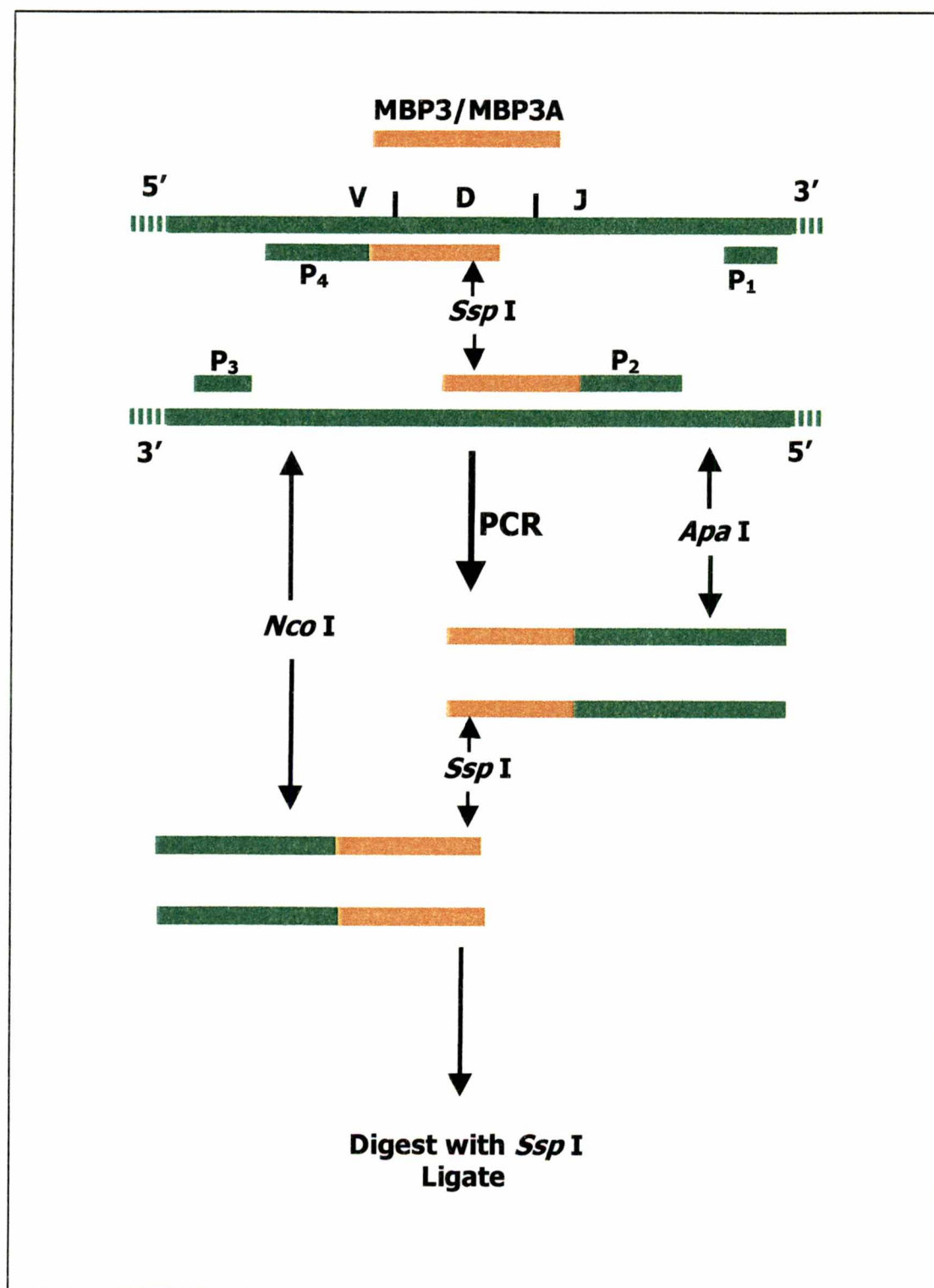
2. Materials and Methods

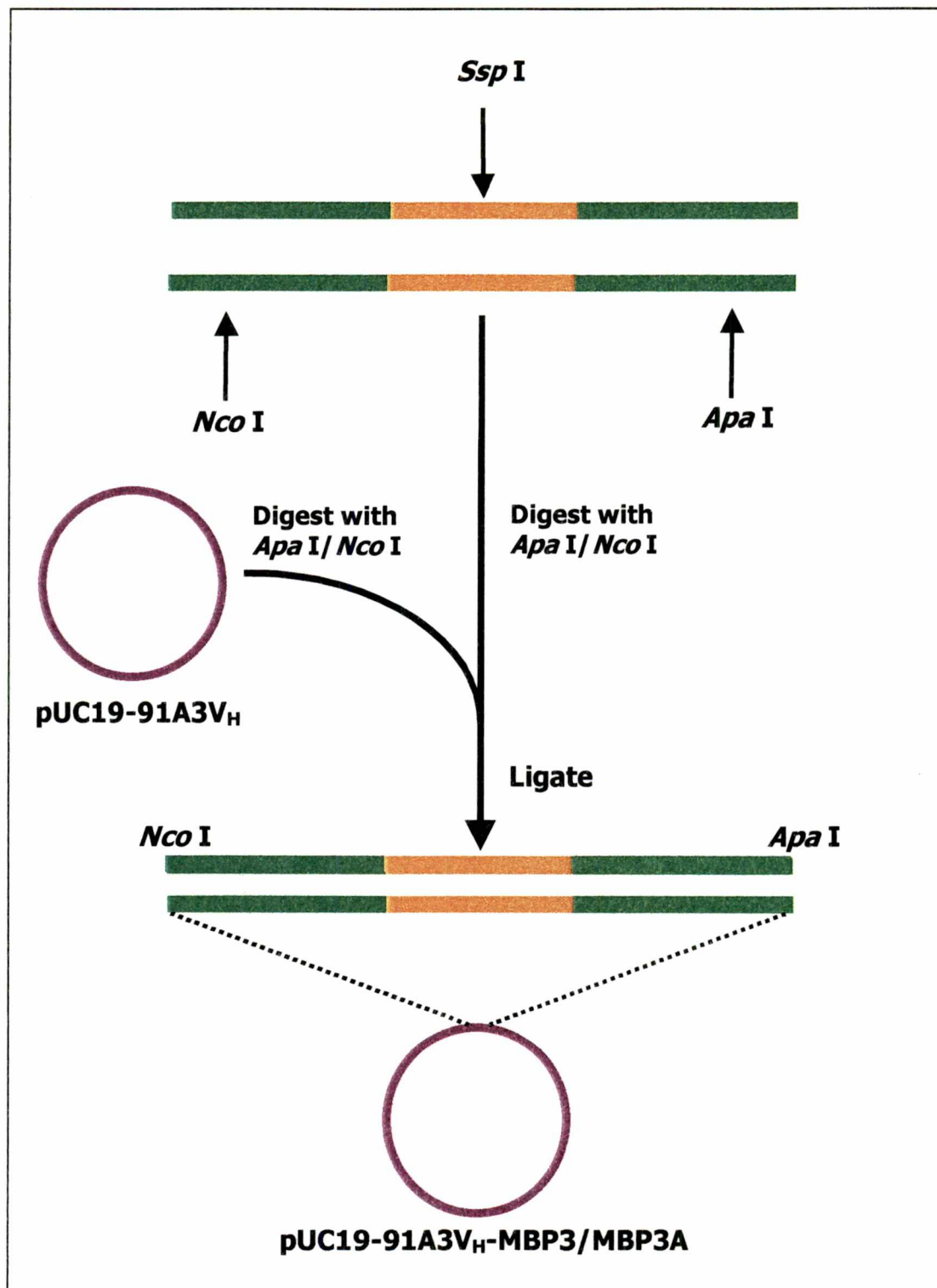
Deletion of Heavy Chain D region

Standard PCR, using 100ng of template DNA (pUC19-91A3 VDJ) and 500ng of designed primers (Figure 6), was carried out to delete the pre-existing diversity (D) segment from the 91A3 VDJ gene and replace it with the 39 nucleotides corresponding to the peptide sequences MBP3 and MBP3A. Procedures are based on those used to construct the Ig-NP chimera (28). Briefly, the 5.5-kb 91A3 fragment (29) was subcloned into the *EcoRI* site of the pUC19 plasmid and used as a template for two sets of PCR. The sequences of the primers for the generation of the MBP3 insertion are as follows: Primer 1 (5'-AGCCAGTGAGGACAAAGAAGATAGCA-3'), Primer 2 (5'-TTCAAGAATATTGTGACCCCGAGGACGCCGTTTGACTACTGGGGCCAAGGCTCC ACTCTC-3'),

Figure 6: Insertion of MBP3 or MBP3A sequences in place of the D segment within the 91A3 H chain

Schematic representation of the strategy used to delete the D segment and insert the peptides' nucleotide sequences. P_1 is Primer 1, P_2 is Primer 2, etc. Primer 1 is complementary to sequences within the intron region downstream of the *Apa* I site while Primer 3 is complementary to noncoding sequences upstream of the *Nco* I site. Primer 2 contains 3'-sequences starting at the first base upstream of the D region and 5'-sequences corresponding to a portion of the desired sequences (orange area) and Primer 4. Primer 4 contains 3'-nucleotides complementary to sequences starting at the first base upstream of the D segment and 5'-unmatched sequences corresponding to part of the MBP3 or MBP3A sequence (orange areas). Primers 3 and 4 contain overlapping sequences, so when joined at an internal *Ssp* I site, a complete MBP3 or MBP3A sequence is formed. Recombinant V-MBP3/MBP3A-J regions are subcloned into pUC19-91A3H.





Primer 3 (5'-CAAATCACCCAAGTGTATGGCA-3'), and Primer 4 (5'-CGTCCTCGGGGTCACAATATTCTTGAAGAAGTGTACTCTTGACAGAAATAGACCGCAGA-3'). Primers 1 and 3 are also used for the generation of the altered peptide, MBP3A. Primers 2 and 4 for the MBP3A construct are as follows: Primer 2 (5'-TTTAAAAATATTGTAACGGCGAGGACGCCGTTTGACTACTGGGGCCAAGGCTCCACTCTC-3') and Primer 4 (5'-GCCGTTACAATATTTTTAAAGAAGTGCACTCTTGACAGAAATAGACCGCAGAGTC-3'). Primers were purchased from Life Technologies (Grand Island, NY). PCR mutagenesis was conducted using the DNA Thermocycler 480 (Perkin Elmer, Branchburg, NJ). Primers 1 and 2 (Figure 6) generated a fragment spanning the MBP3 or MBP3A peptide sequences, the J region, and the intron sequences. Primers 3 and 4 were used in a second PCR reaction to generate a fragment containing the MBP3 or MBP3A sequence, the entire V region, as well as nontranscribed regions.

All molecular cloning techniques were done according to standard protocols (30). Briefly, recombinant fragments were digested and then ligated through a designed internal *Ssp* I site. This ligation yielded a complete MBP or MBP3A peptide sequence in the place of the D region. Complete fragments were digested with *Apa* I and *Nco* I and then subcloned into a cloning vector, pUC19-91A3VDJ that had the *Nco*I-VDJ-*APAI* site excised. The cloning vector was grown large scale and the recombinant sequences were subcloned into the *Eco*RI site of the pSV2-gpt-91A3H expression vector (31). Resultant vectors containing MBP3 and MBP3A were called pSV2-gpt-91A3-MBP3, and pSV2-gpt-

91A3H-MBP3A, respectively. Immunoglobulin chimeras were sequenced as per the Sanger method as stated in the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

Transfection

Transfection of SP2/0 cells (America Type Culture Collection, Rockville, MD) was accomplished through electroporation. 10 to 20 μ g of *Pvu* I linearized recombinant vectors were cotransfected separately with 20 μ g of an expression vector encoding a parental light chain, pSV2-neo-91A3L. Briefly, SP2/0 cells were grown to obtain the desired number in Dulbecco's Modification of Eagle's Medium 1X (DMEM) (Mediatech) supplemented with Fetal Calf Serum (FCS) (Intergen, Purchase, NY). Cells were harvested and spun down at 1600 revolutions per minute (RPM) for 10 minutes (min). Cells were resuspended in equal volume of ice cold D-PBS (Gibco-BRL, Grand Island, NY) and counted. Cells were then spun down and resuspended in D-PBS at 5×10^6 cells/ml. 0.9ml of cells were transferred to sterile culture tubes. Linearized plasmids were added to the culture tubes containing the cells and incubated on ice for 10min. 0.8ml of the mixture was transferred into a 0.4cm cuvette (BioRad Laboratories, Hercules, CA) and pulsed at 220 volts and 960 μ F using the BioRad Gene Pulser. Cells were then incubated on ice for 10 min. 10ml DMEM was added to the cells, and the cells were plated in a 24 well plate (Corning Costar, Cambridge, MA) at 0.5ml/well. Cells were incubated at 37°C, 7% CO₂.

After 48 hrs, 0.5ml selection media was added to each well. Selection media contains mycophenolic acid (MA) (Life Technologies, Gaithersburg, MD) and geneticin (G418) (Life Technologies). After an additional 48hrs, media was removed from the wells and replaced with 1ml of the G418-MA selection media. When the cells were grown to confluency, the cell culture supernatant was screened for antibody production.

Radioimmunoassay(RIA)

The detection of antibodies in cell culture supernatant was performed as follows. Microtiter 96 well plates (Corning Costar, Cambridge, MA) were coated with rat anti-mouse Fc IgG2b antibodies (Zymed Laboratories Inc., South San Francisco, CA) at 2 μ g/ml and incubated overnight at 4°C. Plates were washed three times with phosphate buffered saline (PBS) and then saturated with 2% bovine serum albumin (BSA) (Boehringer Mannheim, Indianapolis, IN) in PBS for 1 hour at room temperature (RT). Plates were washed with PBS three times and then incubated with transfectoma supernatant for 2 hours at RT. Ig-W, a wildtype Ig with non-replaced D region, and G418-MA media were used in the incubation as positive and negative controls, respectively. Subsequent to supernatant incubation, plates were washed three times with PBS. Bound chimeric antibodies were revealed by incubation with 100 x 10³ ¹²⁵I-labeled rat anti-mouse κ L chain mAb for two hours at 37°C. Plates were washed 5 times

with PBS using the Nunc Immunowash 12. Results were obtained using an LKB gamma counter (Wallace Oy, Finland).

Limiting Dilution

Positive transfectomas were cloned by limiting dilution. Briefly, transfectomas were counted and then plated onto 96 well microtiter plates (Corning Costar, Cambridge, MA) at 1 cell per well in G418-MA media. After clones were grown to confluency, they were tested by RIA to detect clones most successful in the secretion of chimeric immunoglobulin.

Purification and Concentration

Antibodies were purified from cell culture supernatant using the technique affinity chromatography. Briefly, supernatant was passed over columns (BioRad Laboratories, Hercules, CA) containing rat anti-mouse κ Light chain mAb coupled to CNBr activated sepharose beads 4B (Pharmacia Biotech, Piscataway, NJ). Bound antibody was eluted from the columns using 0.1M Na Citrate, pH 2.6. Fractions were read at an optical density (OD) of 280. Positive fractions were neutralized with 1M K_2HPO_4 , pH 8.0 and dialyzed against PBS overnight. To avoid cross contamination, separate columns were used to purify each chimera. Purified antibodies were concentrated using collodium membranes (Schleicher & Schuell, Keene, NH). Concentration was performed as per manufacturer's specifications.

3. Results

Immunoglobulin molecules were engineered to carry the epitopes, MBP3 and MBP3A, by replacing the diversity (D) gene segment of the heavy chain variable region of the anti-arsonate antibody, 91A3 (Figures 7 & 8) (29). The immunoglobulin D segment was chosen as an appropriate deletion sequence for several reasons. The first being that this region can accommodate peptides that are very diverse in amino acid length without altering the structure of the heavy chain. The second reason is that folding in this region due to amino acid interactions does not affect its pairing with the light chain, and the generation of a functional immunoglobulin.

Insertion of MBP3 and MBP3A sequences in place of the D region within the 91A3H chain was performed using the technique PCR mutagenesis. A pUC plasmid containing the 91A3-VDJ segment was used as a template for the PCR reactions (Figure 6) (29). Four primers were designed to be used in two sets of PCR reactions for the deletion of the diversity region and replacement with the selected peptide sequences. The complete recombinant sequences, V-MBP3 (or MBP3A) -J, were subcloned into a cloning vector, pUC19-91A3, for large-scale growth. The V-MBP3 (or MBP3A)-J regions were then subcloned into an expression vector, PSV2-gpt-91A3H (Figure 9) to generate PSV2-gpt-91A3-MBP3 (Figure 10a) and PSV2-gpt-91A3-MBP3A (Figure 10b). The pSV2 plasmid was chosen as an appropriate expression vector for several reasons. Reasons for using this particular expression vector are as follows: 1) it

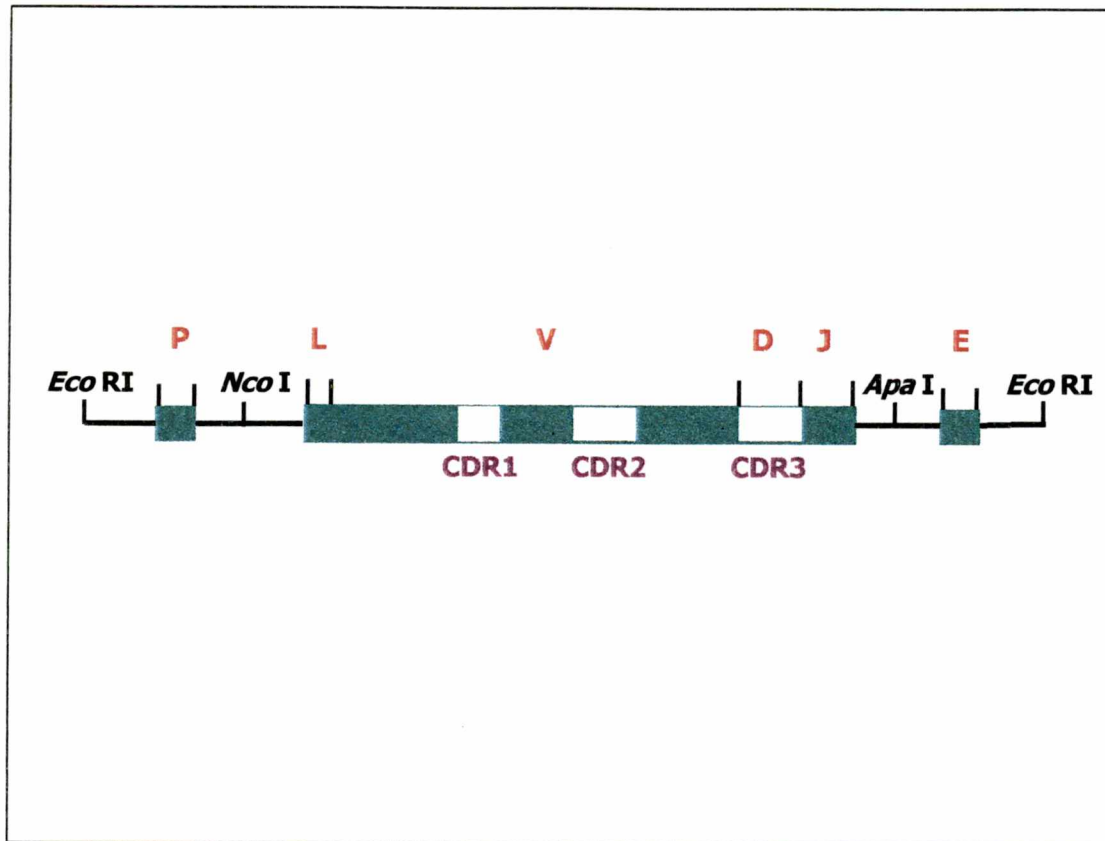


Figure 7: Schematic representation of the VDJ region of the anti-arsonate immunoglobulin, 91A3

Note promoter (P), leader (L) and enhancer (E) sequences.

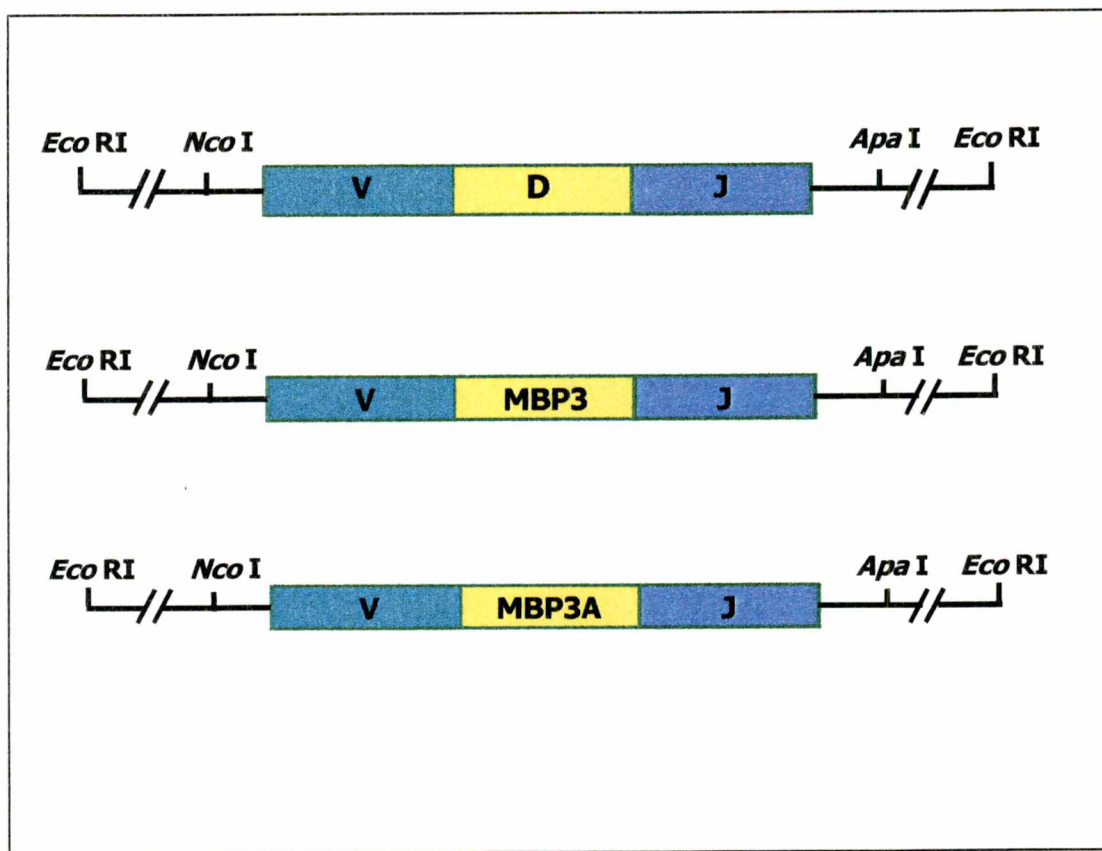


Figure 8: Schematic representation of the proposed D region replacement with MBP3 and MBP3A sequences

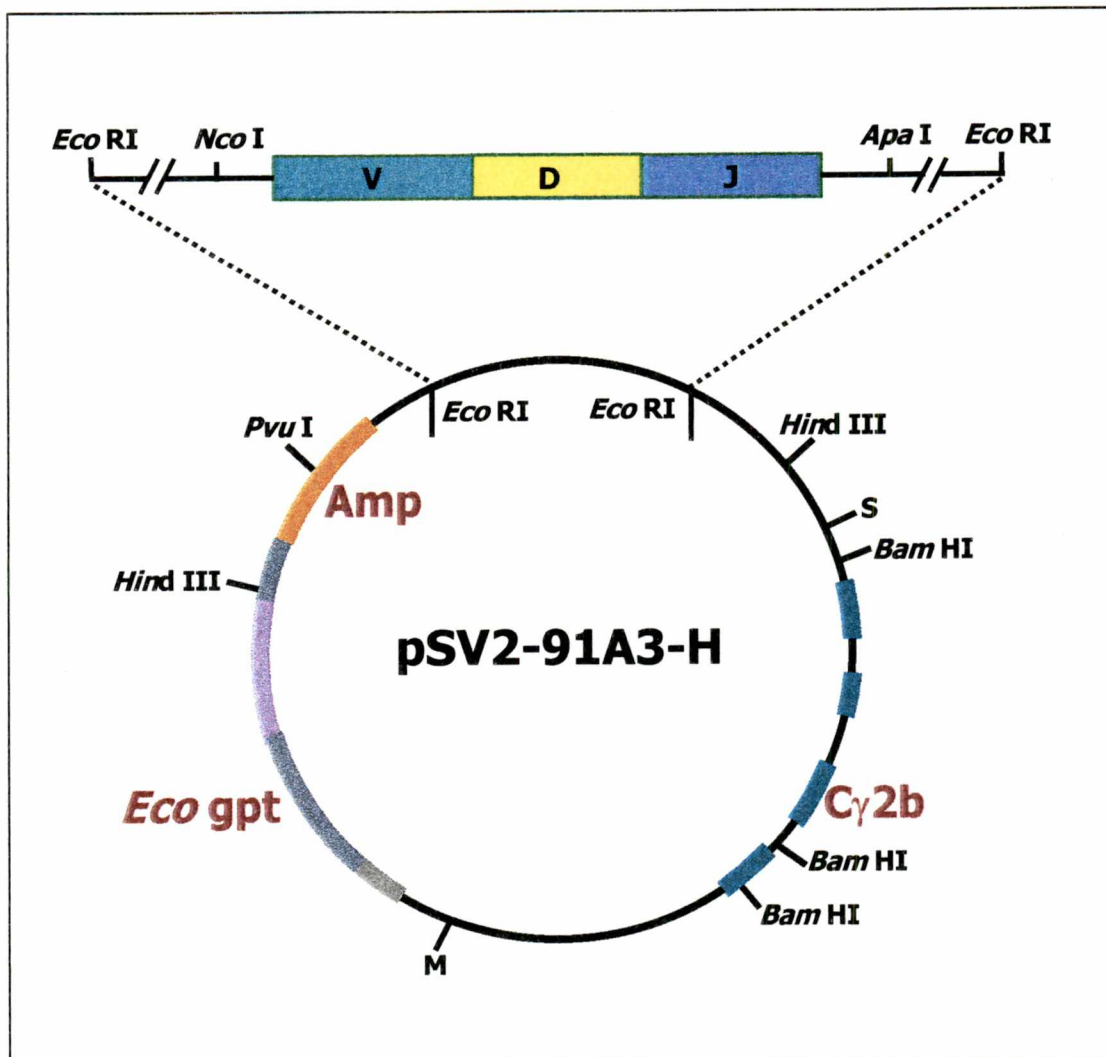
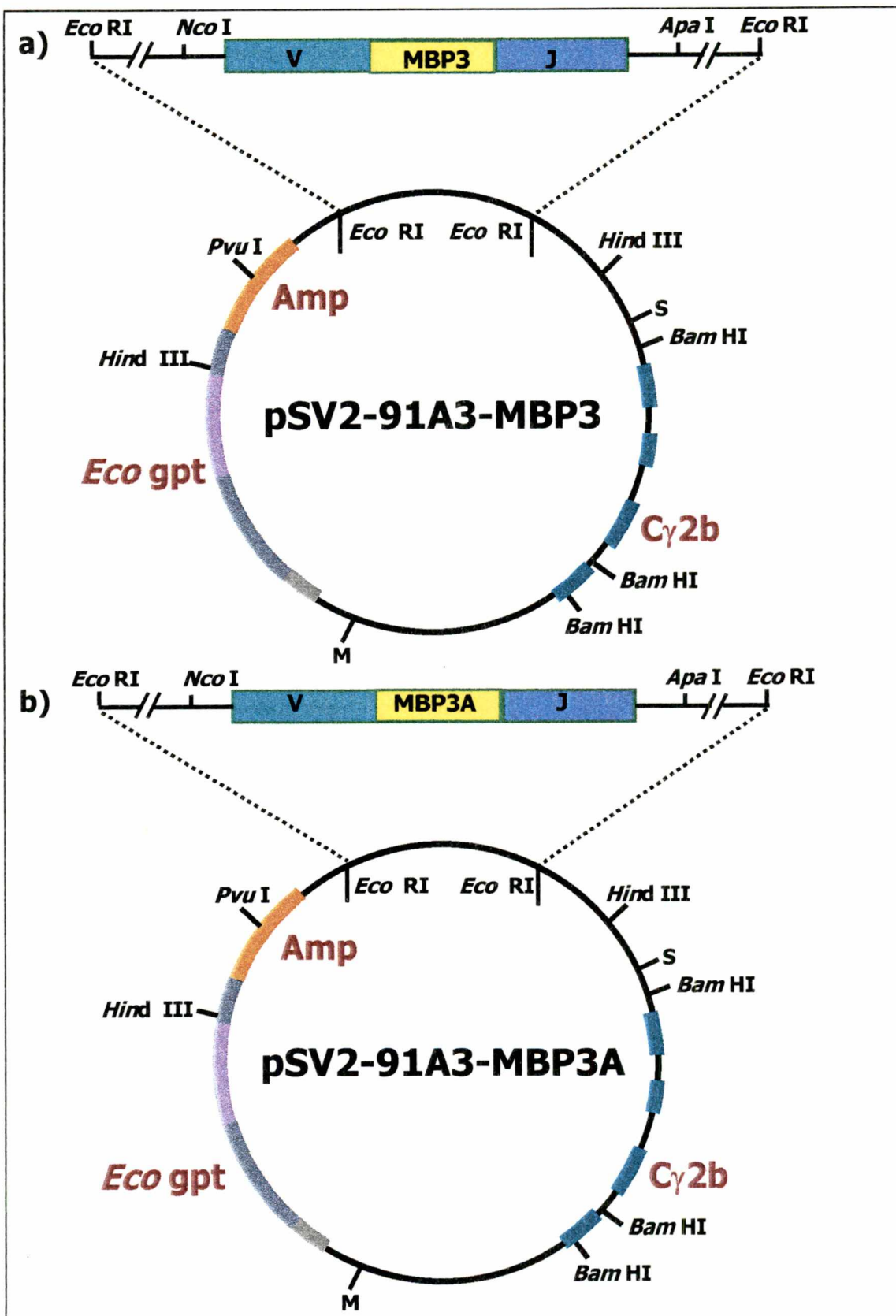


Figure 9: PSV2 expression vector encoding the complete 91A3 heavy chain

Note the Amp resistance gene, the guanine phosphoribosyl transferase gene (gpt), and exons coding for the constant region of a BALB/c γ 2b constant region.

Figure 10: PSV2-gpt-91A3-MBP3 and PSV2-gpt-91A3-MBP3A

- a) PSV2-gpt-91A3-MBP3. Note the MBP3 sequence has replaced the D region.
- b) PSV2-gpt-91A3-MBP3A. Note the MBP3A sequence has replaced the D region.



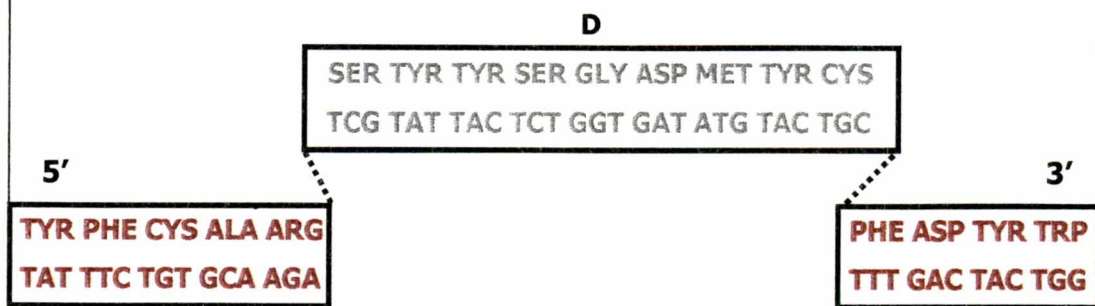
contains a gene for ampicillin resistance applicable in large scale production; 2) it encodes a guanine phosphoribosyl transferase (gpt) gene useful in selection; 3) it contains exons coding for the constant region of a BALB/c $\gamma 2b$ immunoglobulin; and 4) it has a SV40 replication site. Sequencing analyses of the recombinant expression vectors revealed that the D segment was deleted and replaced with complete MBP3 or MBP3A sequences in the correct reading frame (Figure 11).

Recombinant heavy chain expression vectors were cotransfected into SP2/0 cells, a non-immunoglobulin producing B myeloma cell, with an expression vector encoding the genetic material to make a complete light chain (Figure 12). Transfectomas producing the immunoglobulin chimeras were obtained in the presence of a selective medium containing geneticin and mycophenolic acid. Briefly, the guanine phosphoribosyl transferase gene encoded by the heavy chain construct provides resistance to mycophenolic acid (MA), and the neomycin resistance gene (neo) encoded by the light chain vector confers resistance to the antibiotic geneticin. MA blocks the *de novo* synthesis of guanosine monophosphate (GMP) from guanine. The presence of guanine phosphoribosyl transferase encoded by the heavy chain vector allows the synthesis of GMP from xanthine, a precursor metabolite added to the selective media. Geneticin is an antibiotic that inhibits protein synthesis, and the presence of the neo gene encoded by the light chain vector allows for its detoxification. Transfectomas that are actively manufacturing the heavy and

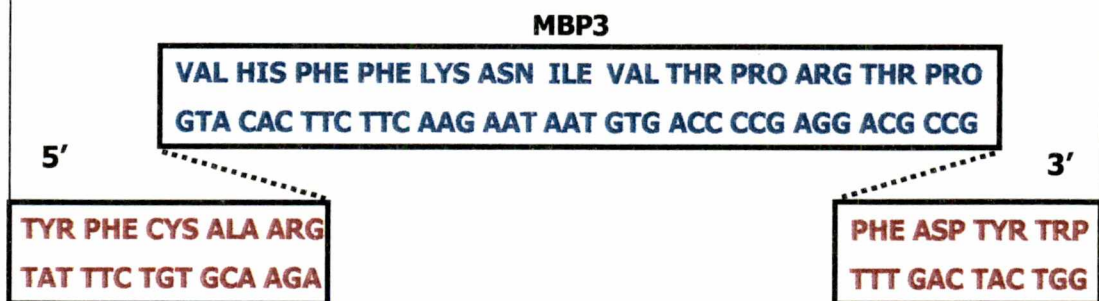
Figure 11: Comparison of the chimeric heavy (H) chain gene sequences with that of the wild type

Burgundy, amino acids and nucleotides remaining unchanged (flanking region); Blue, MBP3/MBP3A sequences; Red, alanine substituted for proline. All of the *Nco* I-*Apa* I fragments were sequenced, but only the MBP3 or MBP3A sequences and partial surrounding sequences are shown.

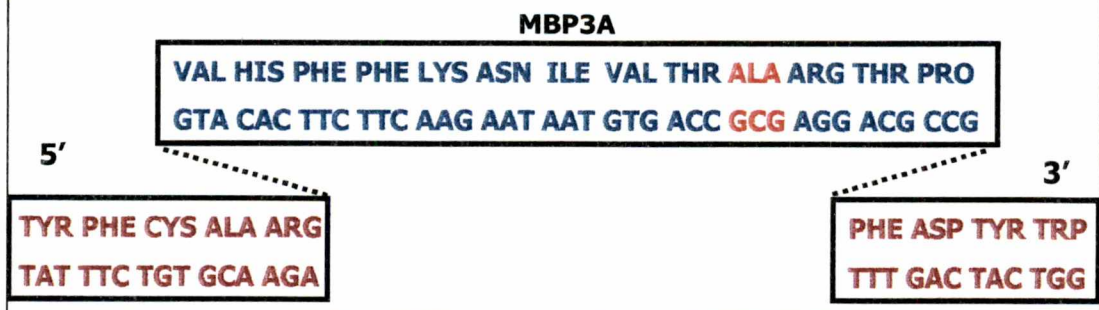
91A₃H



91A₃H-MBP3



91A₃H-MBP3A



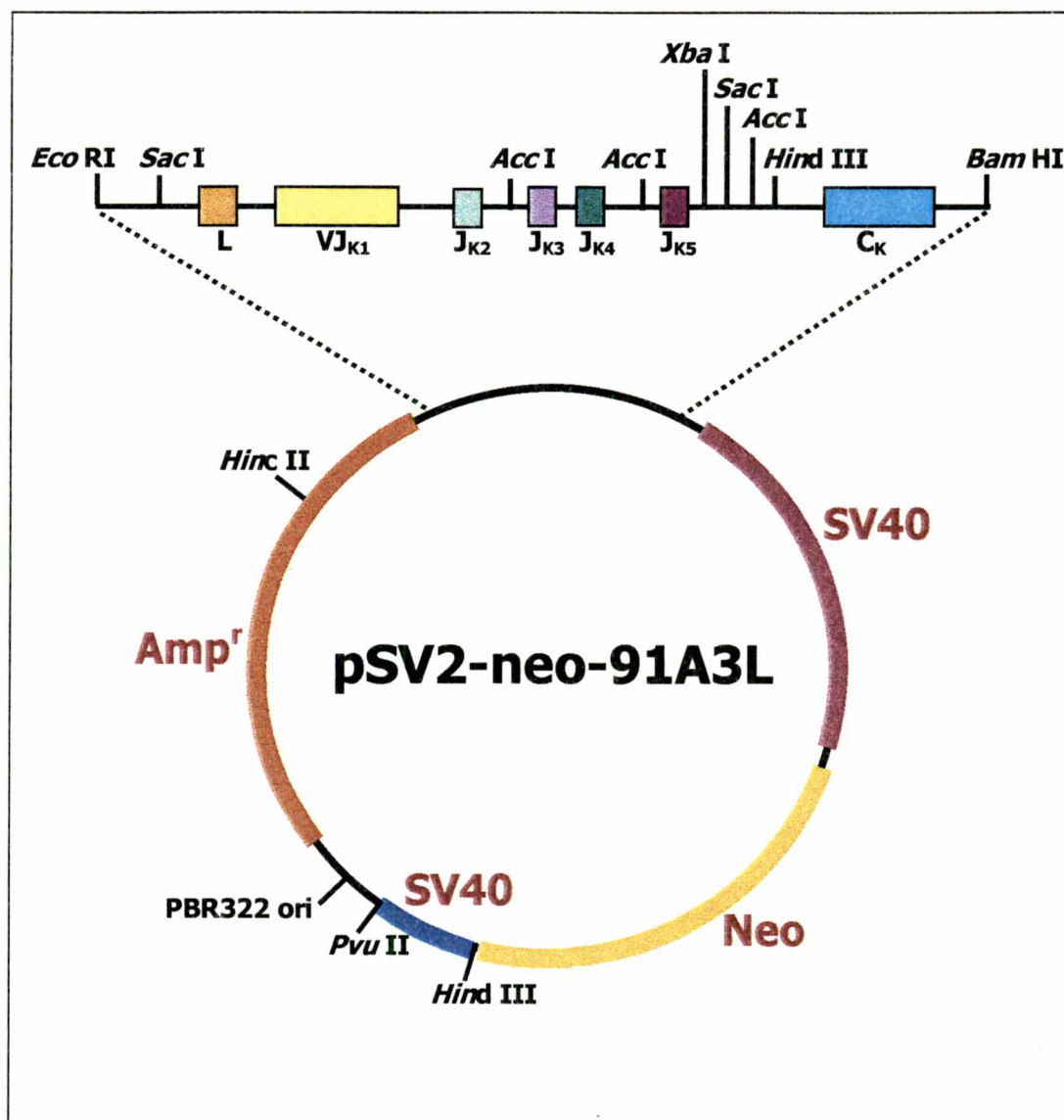


Figure 12: PSV2-neo-91A3L

PSV2 expression vector encoding the genetic material for the synthesis of a complete rearranged κ light chain. Notice the Amp resistance gene as well as the neo gene.

light chains, are able to survive in the selective media because of the resistance genes present. However, survival does not necessarily mean that the heavy and light chains are being paired successfully to form complete immunoglobulins, or that the immunoglobulins are being secreted in adequate quantities. Therefore, radioimmunoassays (RIA) were performed to detect the secretion of 1 to 2 $\mu\text{g/ml}$ of complete immunoglobulins. Positive RIA results were obtained demonstrating that immunoglobulins were being secreted at competitive levels with the positive control, Ig-W (Figure 13). The RIA also revealed that the Ig constructs were complete with both heavy and light chains because they are sandwiched between anti-heavy chain and anti-light chain immunoglobulins in the assay. Positive transfectomas were then grown for large-scale antibody production. The antibodies were purified from cell culture supernatant by affinity chromatography on rat anti-mouse kappa light chain mAb coupled to sepharose beads. Immunoglobulin constructs, Ig-MBP3 and Ig-MBP3A were then concentrated to working levels (2mg/ml). A schematic representation of the immunoglobulin constructs can be seen in Figure 14.

4. Discussion

Immunoglobulin constructs were designed to contain mutated diversity regions within the variable region of the heavy chain of an anti-arsenate antibody. These regions were altered to encode two peptides previously shown to be of importance in research proving the amelioration of EAE. Both

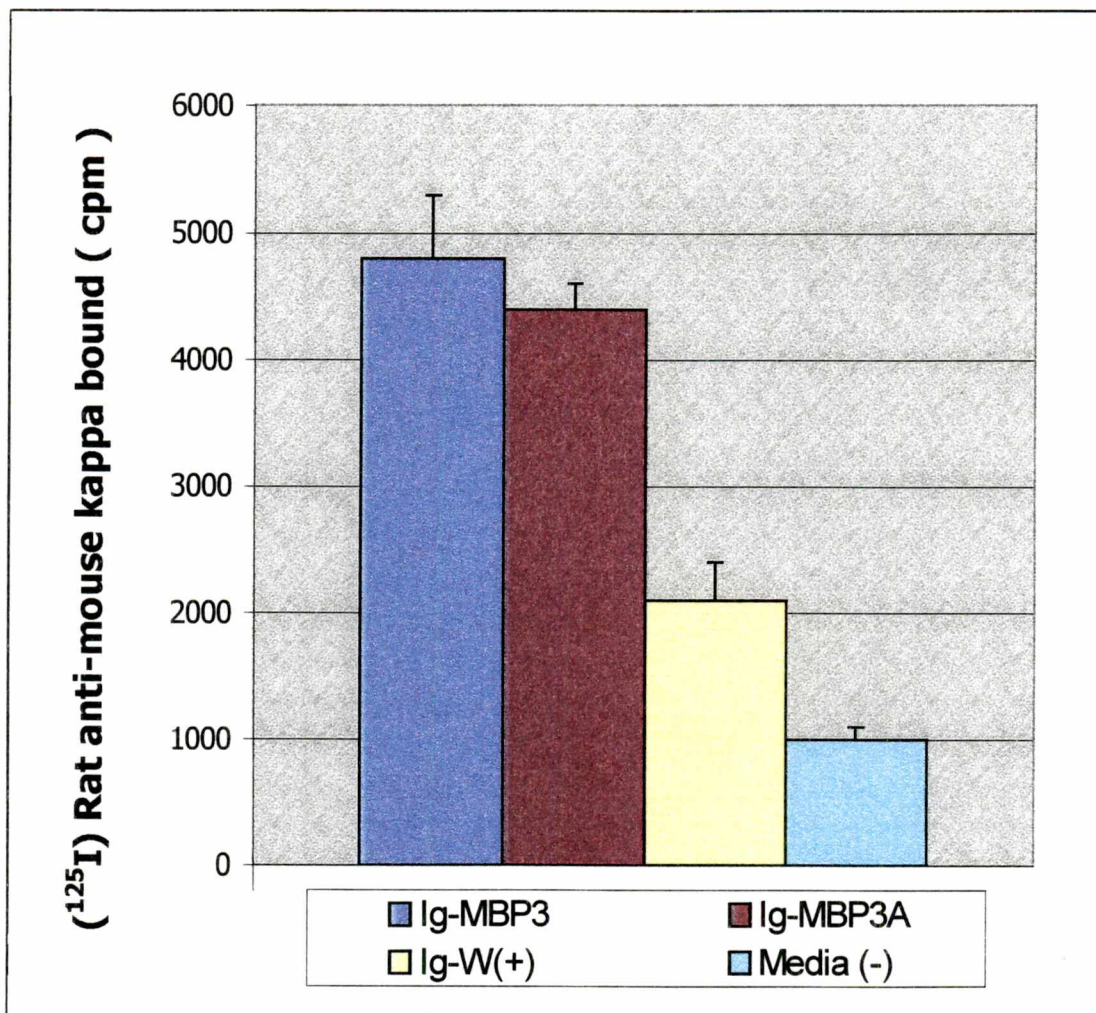


Figure 13: Transfectoma secretion of Ig-MBP3 and Ig-MBP3A

Microtiter plates were coated with anti- γ 2b, blocked with BSA, and incubated with transfectoma supernatant, negative control media, or positive control Ig-W. Captured Igs were revealed with ¹²⁵I-labeled rat anti-mouse κ light chain mAb. Shown is the mean $\pm$ standard deviation (SD) of triplicates.

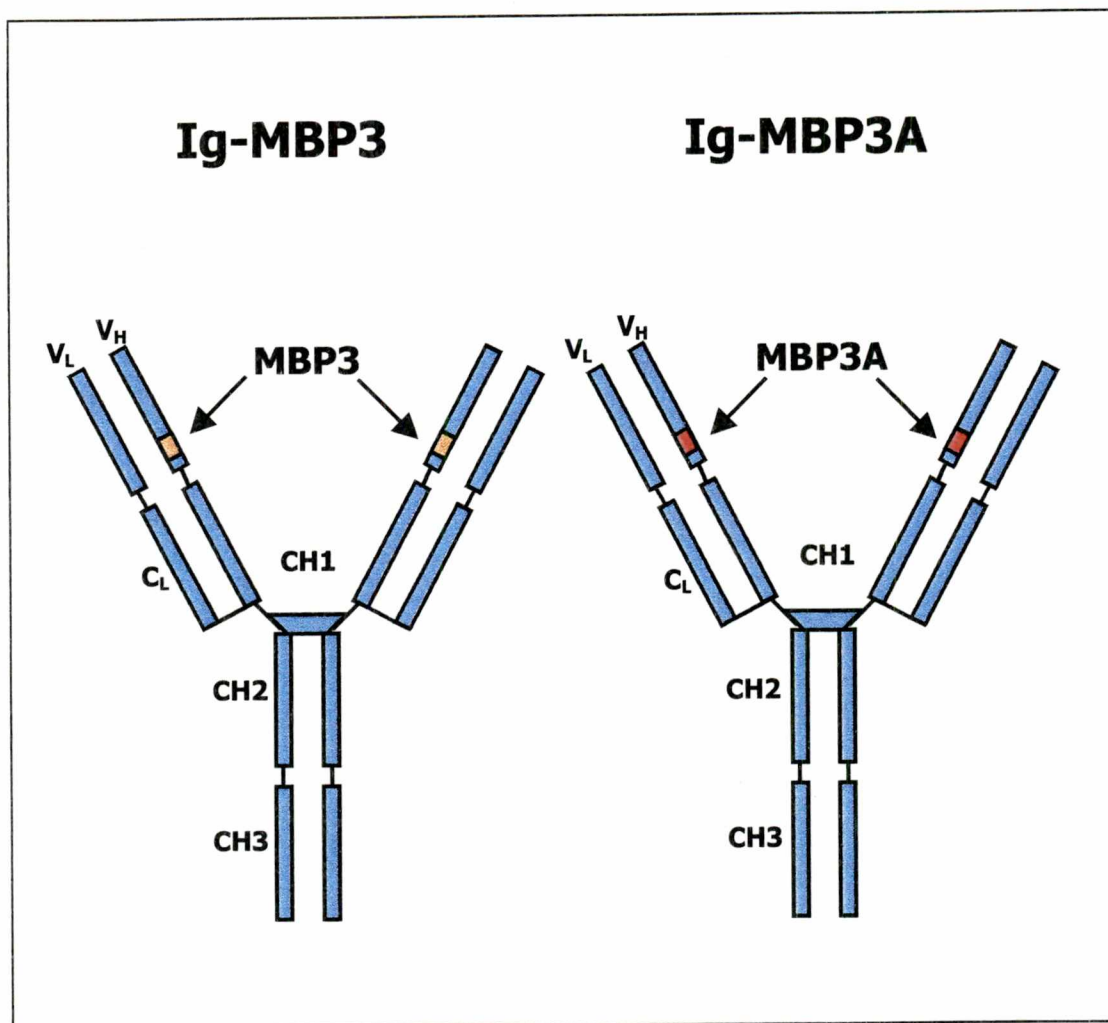


Figure 14: Schematic representation of Ig-MBP3 and Ig-MBP3A

The CDR3 loop (D region) of the heavy chain variable region of the anti-arsonate antibody, 91A3, was deleted and replaced with the nucleotide sequences that encode MBP3 and MBP3A peptides.

the agonist peptide, MBP3 as well as the altered peptide ligand, or partial agonist, MBP3A were grafted into immunoglobulins. These chimeric immunoglobulins were designated Ig-MBP3 and Ig-MBP3A, respectively. The immunoglobulins were designed to provide a more efficient and effective way to deliver peptides to autoreactive T cells. More specifically, the partial agonist, MBP3A, should be presented to autoreactive T cells and lead to their eventual downregulation through IL-4 production, as shown by Brocke, et al. This prospective downregulation would occur at a rate much quicker than that of exogenous peptide due to the antibody's ability to enter the APCs through Fc γ receptor mediated endocytosis as previously shown (26). Once inside the APC, the immunoglobulins are processed, liberating the peptides and allowing their access to newly synthesized MHC class II molecules. This process is much more efficient than the displacement of self-peptides already bound to MHC present on the surface of the APC, as performed by exogenous peptides. Also, immunoglobulin constructs should prove invaluable in the treatment of EAE in that a lower concentration than that of peptide is needed to reach the same effect because of extended half-life.

Part III: Generation and Analyses of T Cell Hybridomas
Specific for MBP3 Peptide

1. Introduction

After the construction of the immunoglobulin chimeras, Ig-MBP3 and Ig-MBP3A, T cell hybridomas (TCH) were generated against the MBP3 peptide. MBP3 specific hybridomas were constructed for several reasons. The first is one of a matter of convenience. The prospective cells are hybridomas, as a result of a fusion with cancer cells, therefore, they contain the genetic material to live indefinitely. Non-hybridoma T cell lines or clones require restimulation with specific peptide and APCs, followed by rest, in an everlasting cycle. This is a tedious cycle that requires a lot of media, antigen and APCs, all of which are quite pricey. Generation of hybridomas would eliminate these aforementioned issues. Included as the second reason for generating T cell hybridomas is the intent to prove the efficient delivery of antigens grafted into chimeric immunoglobulins. T cell hybridomas would be made in order to prove the internalization of chimeric immunoglobulins by APCs, the presentation of the peptides on MHC class II, and the recognition of these peptides by MBP3 specific CD4 T cells. Also of importance is the ability to demonstrate the lesser quantity of immunoglobulin needed to generate a response equivalent to that of exogenous peptide. The final reason for generating hybridomas is to conduct several analyses of cytokine profiles in response to various antigens, both peptide and immunoglobulin.

2. Materials and Methods

Animals

Six to eight week old SJL/J mice (H-2^s) were purchased from Harlan Sprague Dawley (Fredrick, MD) and maintained in the University of Tennessee animal facility for the duration of the experiments.

Peptides

All peptides used in these experiments were purchased from Research Genetics, Inc. (Huntsville, Alabama) and purified on an HPLC column to >90% purity. MBP3 peptide (VHFFKNIVTPRTP) encompasses an encephalogenic sequence corresponding to amino acid residues 87-99 of MBP (25). MBP3A (VHFFKNIVTARTP) is a mutant form of MBP3 in which Pro 96 was replaced with Ala. Both peptides bind to I-A^s class II molecules (25). PLP2 peptide (NTWTTCQSIAPFSK) encompasses a sequence corresponding to amino acid residues 178-191 of the proteolipid protein (PLP) and is used as a control peptide as it also binds to I-A^s class II molecules (32).

Immunoglobulin Chimeras

MBP3 and MBP3A peptides were expressed on Ig chimeras designated Ig-MBP3 and Ig-MBP3A, respectively. Procedures explaining the insertion of these sequences were described previously in this report. PLP2 peptide was

expressed on an Ig chimera and designated Ig-PLP2 (33). Ig-PLP2 was used as a control immunoglobulin.

Immunization

Five SJL/J mice were immunized sub-cutaneously in the footpads and at the base of the limbs with 400 μ g of MBP3 emulsified in a 200 μ l mixture 1:1 vol/vol PBS/Complete Freund's Adjuvant (CFA) (Difco Laboratories, Detroit, MI). Ten days later, the mice were terminated by cervical dislocation and the lymph nodes (axillary, inguinal, and popliteal) were removed and aseptically prepared in single cell suspension.

In Vitro Stimulation of Lines

Lymph node cells in single cell suspension were counted. Cells were resuspended at 5×10^5 cells/ml in HT-2 media containing 2×10^6 cells/ml irradiated APCs, 5% T-Stim (Collaborative Biomedical Products, Bedford, MA), and 15 μ g/ml of the stimulant, MBP3 peptide. HT-2 media is DMEM supplemented with FCS and 2-Mercaptoethanol (Fisher Scientific). Cultures were incubated at 37°C in 7% CO₂ for 7 days. This in vitro stimulation process was performed a total of two times. Resultant cells were defined as T cell lines specific for MBP3 peptide (TCL-MBP3).

Fusion

After TCL-MBP3 cells were stimulated 2 times with MBP3 peptide they reached a number sufficient to perform fusion for the generation of T cell hybridomas. The T cells and their fusion partner, BW1100 α - β -, a TCR deficient thymoma cell line, were collected and spun down 10 min at 1600 RPM, 4°C. Each cell type was resuspended in 30ml DMEM and counted. Cells were spun at 1600 RPM for 6 min at 4°C. Cells were combined in a 1:1 ratio in 30ml DMEM. Cells were spun at 1000 RPM for 10 min at 4°C. The pellet was aspirated. 1ml of pre-warmed DMEM containing PEG 4000 (Fisher Scientific, Pittsburgh, PA) was added to the pellet over a 1min period while rotating the tube. One ml of pre-warmed DMEM was added to the cells over a period of 30 seconds (sec). An additional 9ml of DMEM was added over a period of 30 sec. Cells were spun at 1600 RPM for 10 min at RT. 1ml of pre-warmed fusion media (DMEM supplemented with FCS, Non-essential amino acids (Sigma, St. Louis, MO), and 2-Mercaptoethanol) was added to break the pellet. An additional 10ml fusion media was added slowly. Cell suspension was brought to 30ml. Cells were plated in a microtiter 96 well flat bottom plate (Costar Corning) at 100 μ l/well. Hybridoma cells were incubated at 37°C in 7% CO₂. After 24 hours (hrs), 100 μ l/well of 2X HA media (fusion media supplemented with HA (Sigma)) was added. After an additional 24 hrs, 100 μ l/well media was removed and replaced with 1X HA fusion media. After an additional 36 hrs, 100 μ l/well media was removed and replaced with 1X HA fusion media.

After cells were grown to confluency, cells were screened by enzyme linked immunosorbent assay (ELISA).

Enzyme Linked Immunosorbent Assay (ELISA)

T cell hybridomas were incubated in 96 well round-bottom plates (Corning Costar) with 15 µg/ml MBP3 and PLP2 peptides, separately. 5×10^5 cells/well of irradiated APCs were added. Plates were incubated at 37°C in 7% CO₂ for 24 hrs. The cells were then pelleted by centrifugation and 100µl of supernatant was transferred to ELISA plates (Corning Costar). ELISAs were performed according to Pharmingen's cytokine ELISA protocol. Briefly, capture antibodies (rat anti-mouse IL-2, JES6-1A12; rat anti-mouse IFN-γ, R4-6A2; rat anti-mouse IL-4, 11B11) were incubated at 2µg/ml (50µl/well) overnight at 4°C. The plates were then washed with PBS-0.05% Tween 20 (Fisher) and blocked with PBS-3% BSA for 2 hours at RT. After subsequent washing, the culture supernatant was added and incubated overnight at 4°C. The plates were then washed and incubated with 100µl/well of a 1µg/ml solution of Pharmingen biotinylated anti-cytokine antibody (rat anti-mouse IL-2, JES6-5H4; rat anti-mouse IFN-γ, XMG1.2; rat anti-mouse IL-4, BVD6-24G2) for 45 minutes at RT. The plates were washed and incubated with 100µl/well (2.5µg/ml) of avidin-peroxidase (Sigma), washed 3 times with PBS-0.05% Tween, and developed with 100µl/well (300µg/ml) of 2,2'-Azido-bis(3-ethylbenzthiazoline-6-

sulfonic acid) (ABTS) (Sigma) in 0.03% H₂O₂ for ~20 min. The OD₄₀₅ was then measured on a spectraMAX 340 counter (Molecular Devices) using the SoftMAX PRO version 1.2.0 software. Graded amounts of recombinant mouse IL-2, IFN- γ , and IL-4 (Pharmingen) were included in all experiments to construct standard curves. The concentration of the cytokines in cell supernatant was estimated by extrapolation from the linear portion of each standard curve.

For the detection of complete cytokine profiles of the hybridomas the stimulators were used at the following concentrations: peptides at 0.1, 0.5, 1, 5, 10, and 50 μ M; immunoglobulins at 0.0001, 0.001, 0.01, 0.1, 0.5, and 1 μ M. The assay was performed in duplicate.

3. Results

Mice were immunized with MBP3 peptide for the generation of MBP3 specific T cells (schematic representation, Figure 15). T cell lines were harvested from the lymph nodes of MBP3 immunized mice. MBP3 specific T cell lines were restimulated with peptide in vitro a total of two times in order to raise the number of T cells to an optimal number for hybridoma generation. Once T cells reached an adequate number for fusion, the T cell line was fused with the BW1100 α - β - fusion partner. The BW1100 α - β - cell line is a TCR negative thymoma cell. Fusion was performed by using a viscous medium containing PEG 4000.

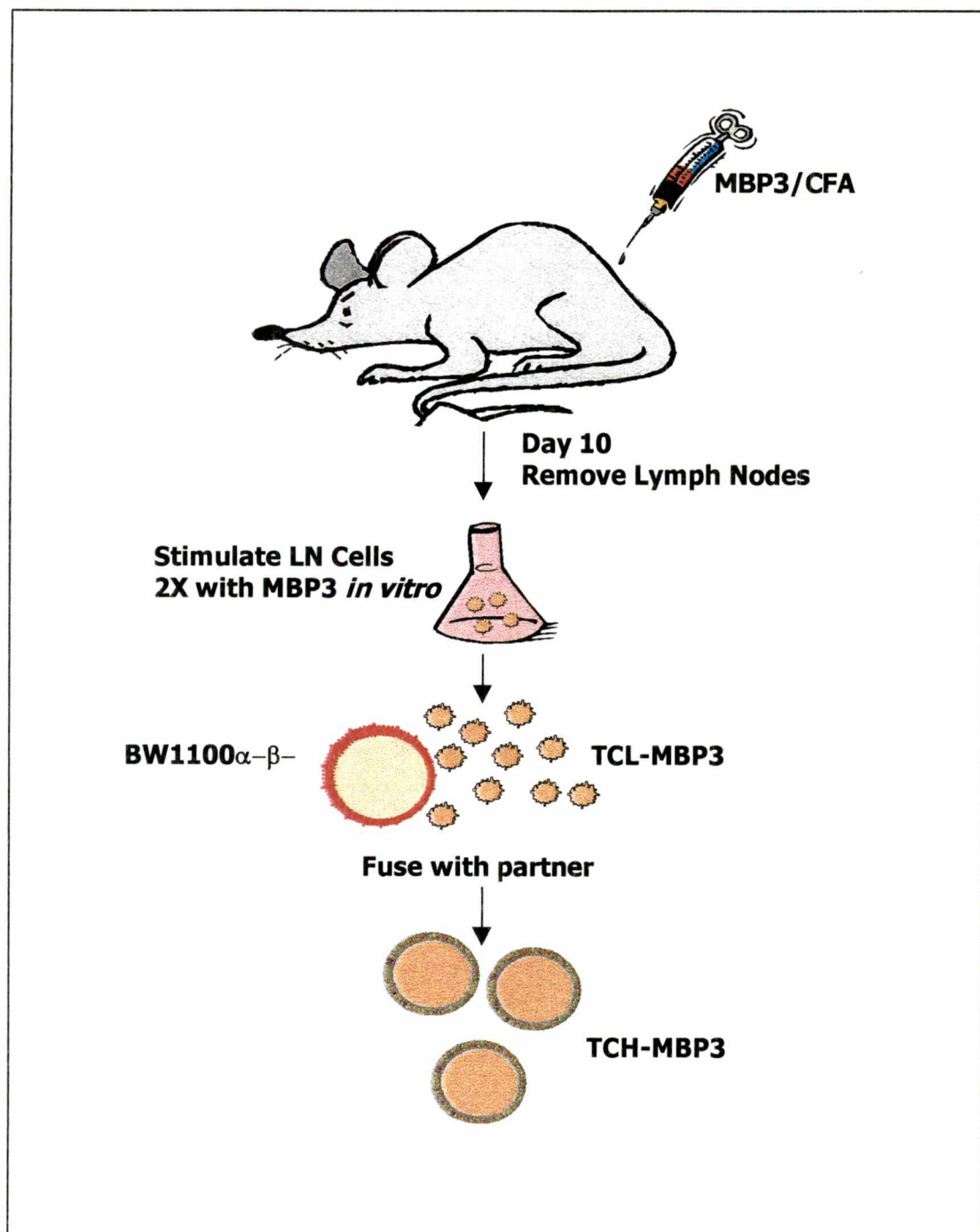


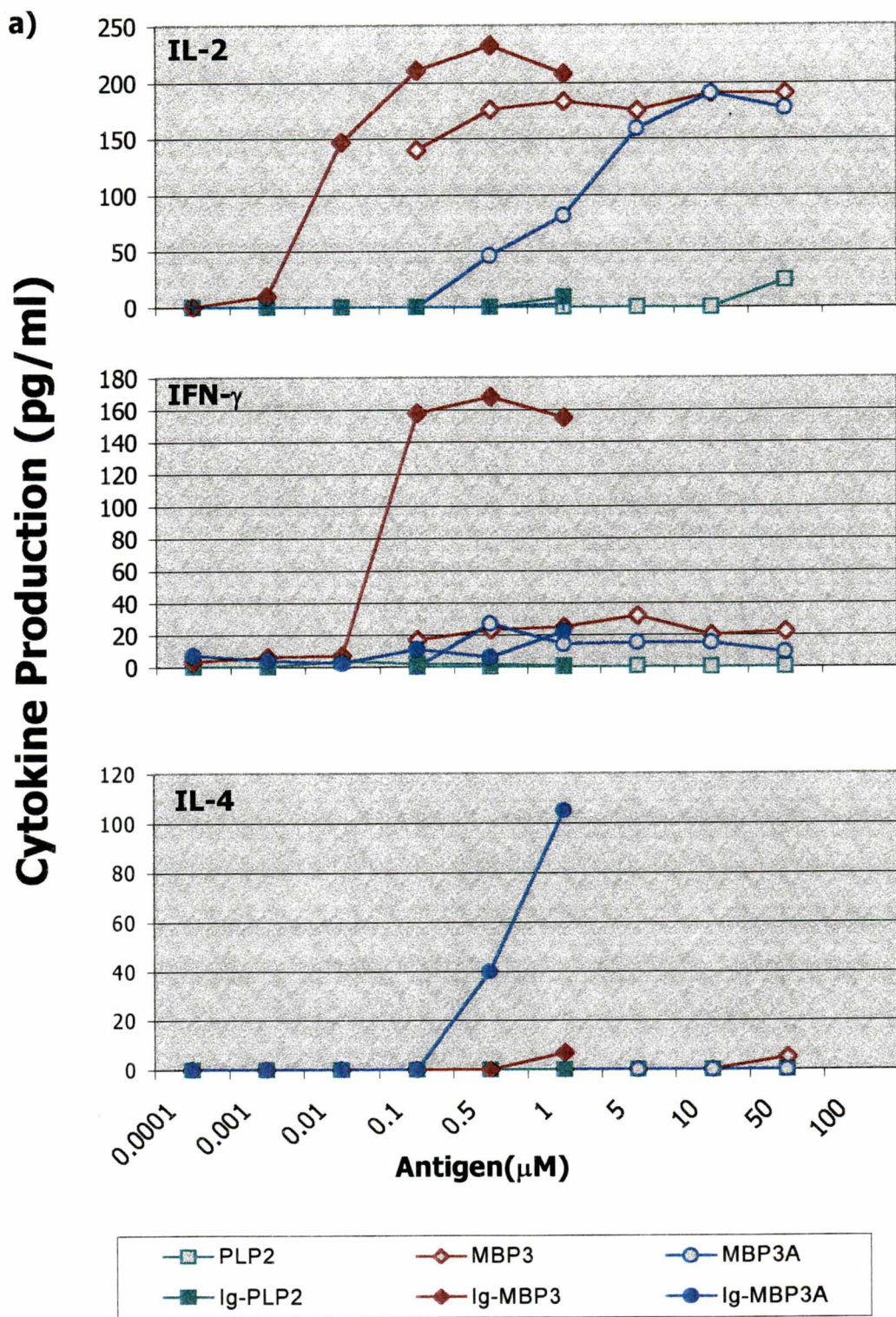
Figure 15: Schematic representation of the generation of T cell hybridomas specific for MBP3

T cell hybridomas were selected based on their specificity to MBP3 peptide, and non-recognition of PLP2, an irrelevant peptide. A series of enzyme linked immunosorbent assays (ELISAs) were used to detect hybridoma IL-2 and IFN- γ levels in response to the two antigens. T cells specific for MBP3 secreted significant levels of IL-2 and IFN- γ in response to the peptide antigen. After identifying several hybridomas positive for MBP3 specificity, clones were generated by limiting dilution. Clones specific for MBP3 peptide were detected by ELISA in a manner equivalent to that used in the initial hybridoma selection. Two resultant T cell hybridoma clones were selected to be used in a series of ELISAs to demonstrate the clones' cytokine profiles in response to various antigens. Antigens used included the peptides MBP3, MBP3A and PLP2, as well as the immunoglobulin constructs Ig-MBP3, Ig-MBP3A, and Ig-PLP2. Again, PLP2 and Ig-PLP2 were used as irrelevant antigens or negative controls. Antigens were tested at various concentrations depending on whether in peptide or immunoglobulin form. IL-2 and IFN- γ levels were detected and are displayed in figure 16.

Also observed were the IL-4 responses of these hybridomas in the presence of the same antigens. IL-4 levels detected are displayed in figure 16 as well.

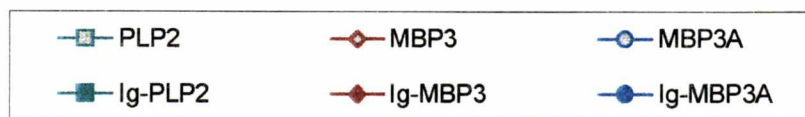
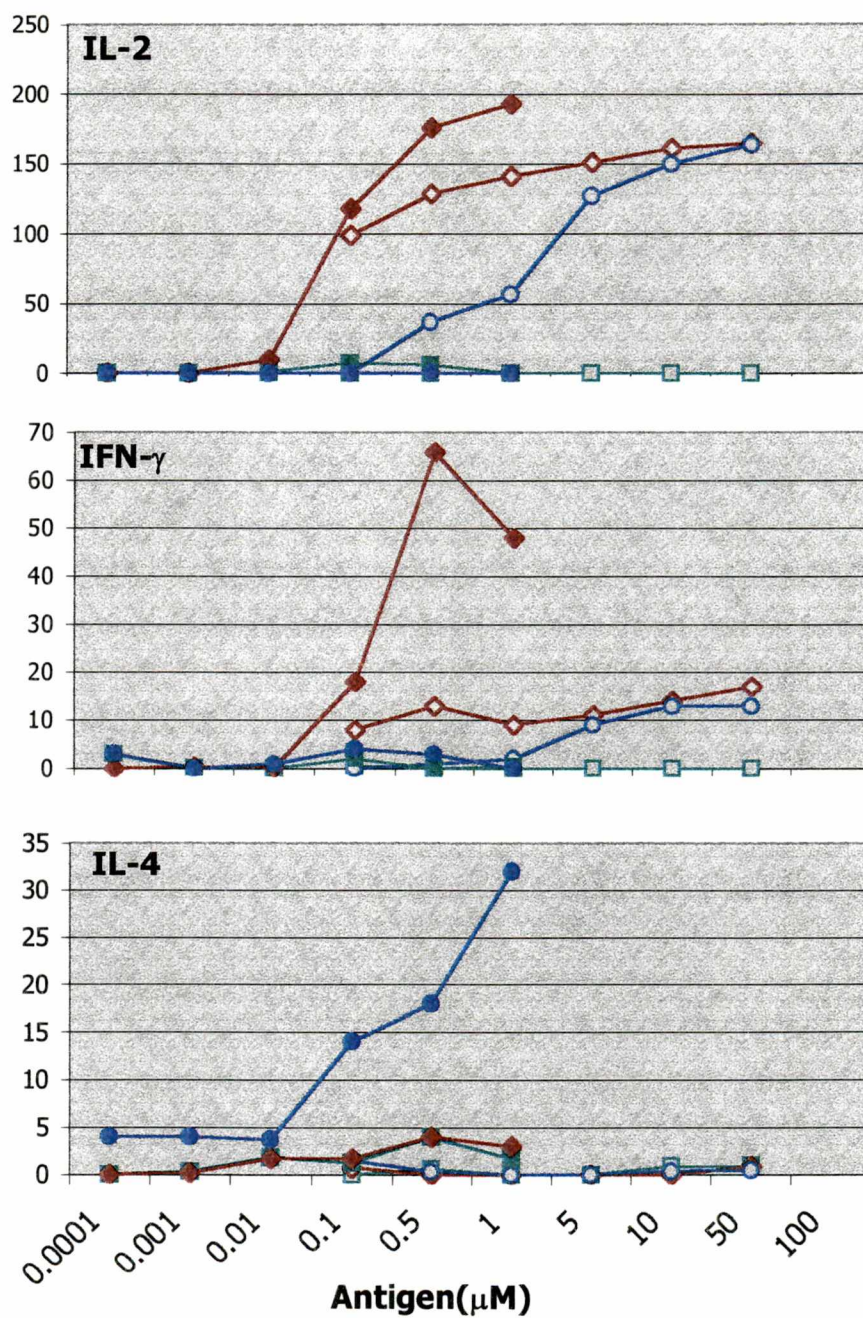
Figure 16: Efficient presentation of Ig-MBP3 and Ig-MBP3A, and cytokine profiles of TCH-MBP3 clones

IL-2, IFN- γ , and IL-4 responses were determined by ELISA. Graded amounts of each antigen were incubated with splenic SJL/J APCs and the MBP3 specific T cell hybridomas, (a) TCH-MBP3 A7 and (b) TCH-MBP3 E8. Each point represents the mean of duplicates. The SD did not exceed more than 10% of the mean value.



b)

Cytokine Production (pg/ml)



4. Discussion

Resultant T cell hybridoma cytokine profiles to the various antigens revealed several things. Primarily, data demonstrated that T cell hybridomas A7 (TCH-MBP3 A7) and E8 (TCH-MBP3 E8) were specific for MBP3 peptide due to the production of IL-2 in response to the peptide stimulus. Of most importance is the fact that these hybridomas were able to recognize the epitope, MBP3, when present as part of the immunoglobulin. Therefore, the immunoglobulin construct as a means for effective peptide presentation was a success with the MBP peptides. The results also revealed that the MBP3 epitope was better presented by Ig-MBP3 than by free MBP3 peptide. This is demonstrated by the lesser quantity of Ig construct needed to achieve maximum results in cytokine production. Therefore, efficacy in T cell activation is optimal with the immunoglobulin constructs. An additional observation concluded from the data is that stimulation with the immunoglobulin, Ig-MBP3, yields a Th1 response with the production of IL-2 and IFN- γ , inflammatory cytokines important in autoreactivity. The altered peptide ligand, MBP3A yields IL-2 production when the stimulant is MBP3A peptide, whereas peptide in the context of immunoglobulin yields the production of IL-4, a Th2 type anti-inflammatory response. Reasons for the differing effects are unknown. Perhaps a technical error occurred in the development of the assay. Amazingly, the altered peptide immunoglobulin, Ig-MBP3A, causes a deviation in MBP3 specific T cell responses in that cells which under normal situations

produce IL-2, where the stimulant is the agonist, now produce IL-4. The deviation the partial agonist causes may have important therapeutic possibilities in that inflammatory cells specific in the destruction of the myelin sheath may deviate after partial agonist contact to secrete non-inflammatory cytokines and aid in the reversal of the disease.

Part IV: T Cell Activation in Vivo

1. Introduction

After the effective in vitro presentation and recognition of Ig-MBP3 and Ig-MBP3A to T cell hybridomas specific for MBP3, and the enlightening information that Ig-MBP3A induces a Th2 type T cell response in the same MBP3 specific T cells, several in vivo analyses were performed using the two constructs. In vivo experiments were designed and carried out in an attempt to reveal the T cells' ability to recognize and respond to the immunoglobulins as previously demonstrated in vitro.

2. Materials and Methods

Animals

Six to eight week old SJL/J mice were purchased from Harlan Sprague Dawley (Fredrick, MD) and maintained in the University of Tennessee animal facility for the duration of the experiments.

Antigens

As previously discussed, all peptides were purchased from Research Genetics, Inc. (Huntsville, Alabama). Generation of immunoglobulin constructs was described previously in this report. Antigens used in the following experiments were; MBP3, MBP protein, HA110-120 (influenza hemagglutinin residues 110-120), Ig-MBP3, Ig-MBP3A, and Ig-W.

Single Immunizations

Three groups of 5 Mice were each injected sub-cutaneously in the foot pads and at the base of the limbs separately with 50 μ g of Ig-MBP3, Ig-MBP3A or Ig-W emulsified in a 200 μ l mixture 1:1 vol/vol PBS/CFA. After 10 days, the mice were sacrificed by cervical dislocation. Lymph nodes (axillary, inguinal, and popliteal) and spleens were removed from each mouse and prepared as single cell suspensions in DMEM. T cell responses were analyzed as described below.

Co-Immunizations

Individual mice from two groups (five mice per group) were injected sub-cutaneously as above with a 200 μ l mixture (PBS/CFA, 1:1 vol/vol) containing 50 μ g Ig-MBP3 and 150 μ g Ig-MBP3A; or 50 μ g Ig-MBP3 and 150 μ g Ig-W. Splenic and lymph node cell responses were analyzed 10 days post immunization.

T Cell Proliferation Assay

Assays for lymph node (LN) and spleen (SP) proliferative responses were conducted as follows. LN and SP cells were incubated in 96 well flat bottom plates at 4 and 10 $\times 10^5$ cells/100 μ l/well, respectively, with 100 μ l of stimulator for 3 days. Subsequent to incubation, 1 μ Ci [^{3} H]thymidine (ICN Pharmaceuticals Inc., Costa Mesa, CA) was added per well, and the culture

incubated for an additional 14.5 hours at 37°C, 7% CO₂. The cells were then harvested on glass fiber filters (Inotech Biosystems International Inc., Rockville, MD). Incorporated [³H]thymidine was counted using the trace 96 program and an Inotech β counter. Antigens were used at the following concentrations: MBP 5μM; HA 15μg/ml.

ELISA and Enzyme Linked Immunosorbent Spot (ELISPOT)

ELISAs were performed as stated previously. ELISPOT was performed as follows. Briefly, ELISPOT plates (Millipore) were coated with 100μl/well of capture anti-cytokine antibodies (as used in ELISA) at 2μg/ml in sterile 1M NaHCO₃. Plates were incubated overnight at 4°C. The plates were washed three times with sterile PBS then blocked for two hours at 37°C with complete HT-2 media. 50μl/well were then removed and replaced with 50μl/well of lymph node cells at 10 x 10⁶ cells/ml. 100μl of antigen, MBP protein or HA, was added at 50mM and 15μg/ml, respectively. Plates were incubated overnight at 37°C, 7% CO₂. Subsequent to incubation, the plates were washed three times with PBS-0.05% Tween. 100μl/well of 1μg/ml solutions of Pharmingen biotinylated anti-cytokine antibodies (as discussed for ELISA) were then added to the plates and incubated overnight at 4°C. After incubation, plates were washed three times with PBS-0.05% Tween. Avidin peroxidase (Sigma) was added in 100μl/well (2.5μg/ml) and incubated for 1 hr at 37°C.

Plates were washed again and developed with 200 μ l 3-amino-9-ethylcarbazole (AEC) (Sigma) dissolved in dimethylformamide (Fisher) and 50mM acetate buffer pH5.0 (74ml 0.2N acetic acid and 176 ml of 0.2M sodium acetate to 1L water), 0.03% H₂O₂ for 15 min. After developing, plates were washed with water and dried overnight. Spots were counted under a dissection microscope.

Antigens were used at concentrations described previously in the protocol for proliferation assay. The assay was performed in triplicate.

3. Results

Primarily, three sets of five individual mice were immunized with Ig-MBP3, Ig-MBP3A or Ig-W. A series of experiments were designed which included proliferation assays for specificity to antigen, as well as ELISAs and ELISPOTs for the construction of cytokine profiles in response to antigen. When mice were immunized with Ig-MBP3 in the footpads, they developed strong myelin basic protein (MBP) responses both in the lymph nodes and in the spleen (Figure 17). Mice immunized with altered peptide immunoglobulin, Ig-MBP3A displayed similar proliferative results to those immunized with the agonist, Ig-MBP3 (Figure 18). Mice immunized with the negative control, wild type immunoglobulin, Ig-W, did not show signs of proliferation to MBP protein (Figure 19). Responses to HA were considered a negative control.

Figure 17: Proliferative lymph node and spleen cell responses in mice immunized with Ig-MBP3

In vivo priming of MBP specific T cells by immunization with Ig-MBP3. Five mice were each immunized with 50 μ g of Ig-MBP3 in CFA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Each value represents the mean $\pm$ SD of triplicates after deduction of background (BG) counts per minute (cpms) obtained with no stimulator in the medium. LN, lymph node; SP, spleen.

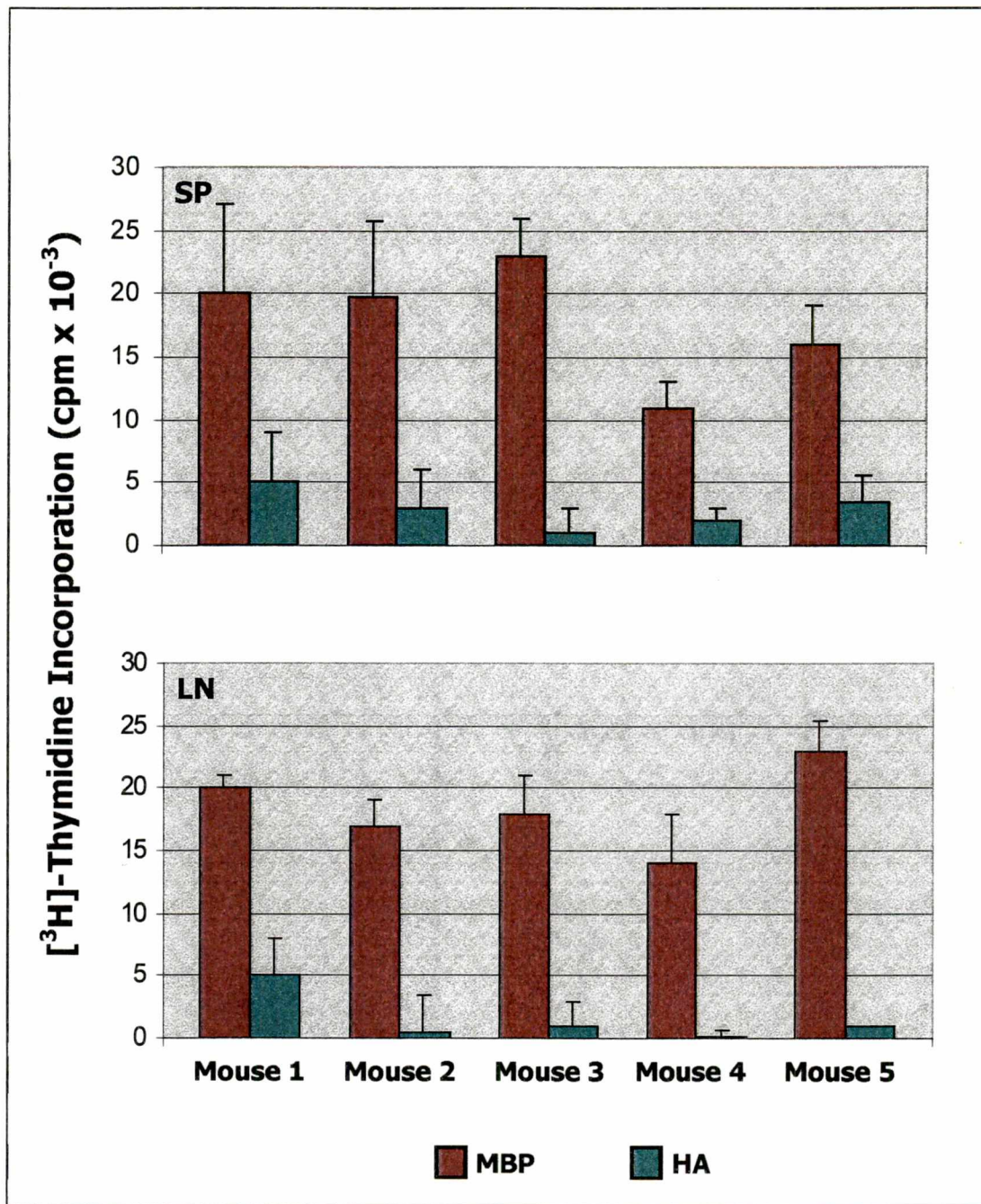


Figure 18: Proliferative lymph node and spleen cell responses in mice immunized with Ig-MBP3A

In vivo priming of MBP specific T cells by immunization with Ig-MBP3A. Five mice were each immunized with 50 μ g of Ig-MBP3A in CFA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Each value represents the mean $\pm$ SD of triplicates after deduction of BG cpms obtained with no stimulator in the medium. LN, lymph node; SP, spleen.

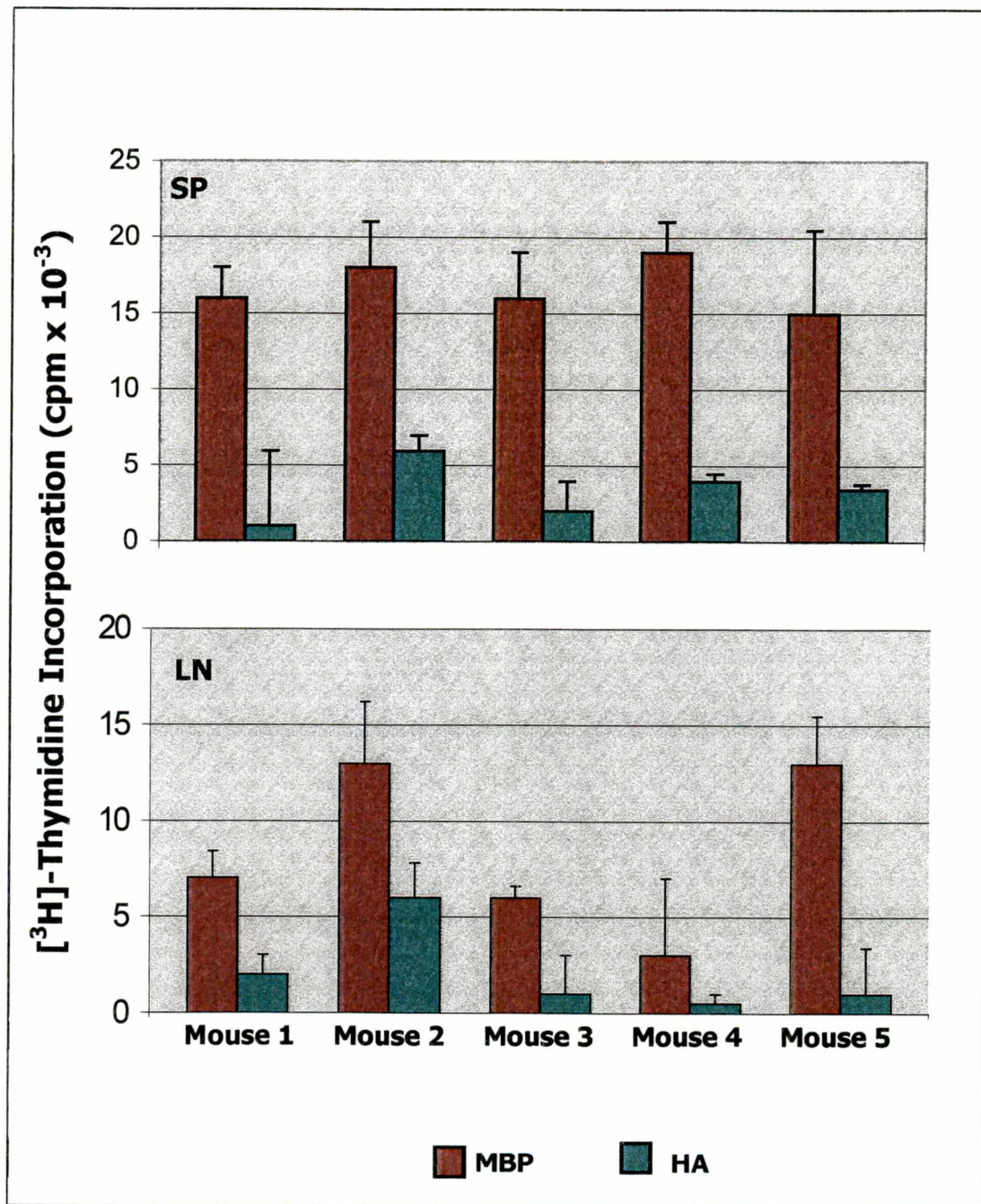
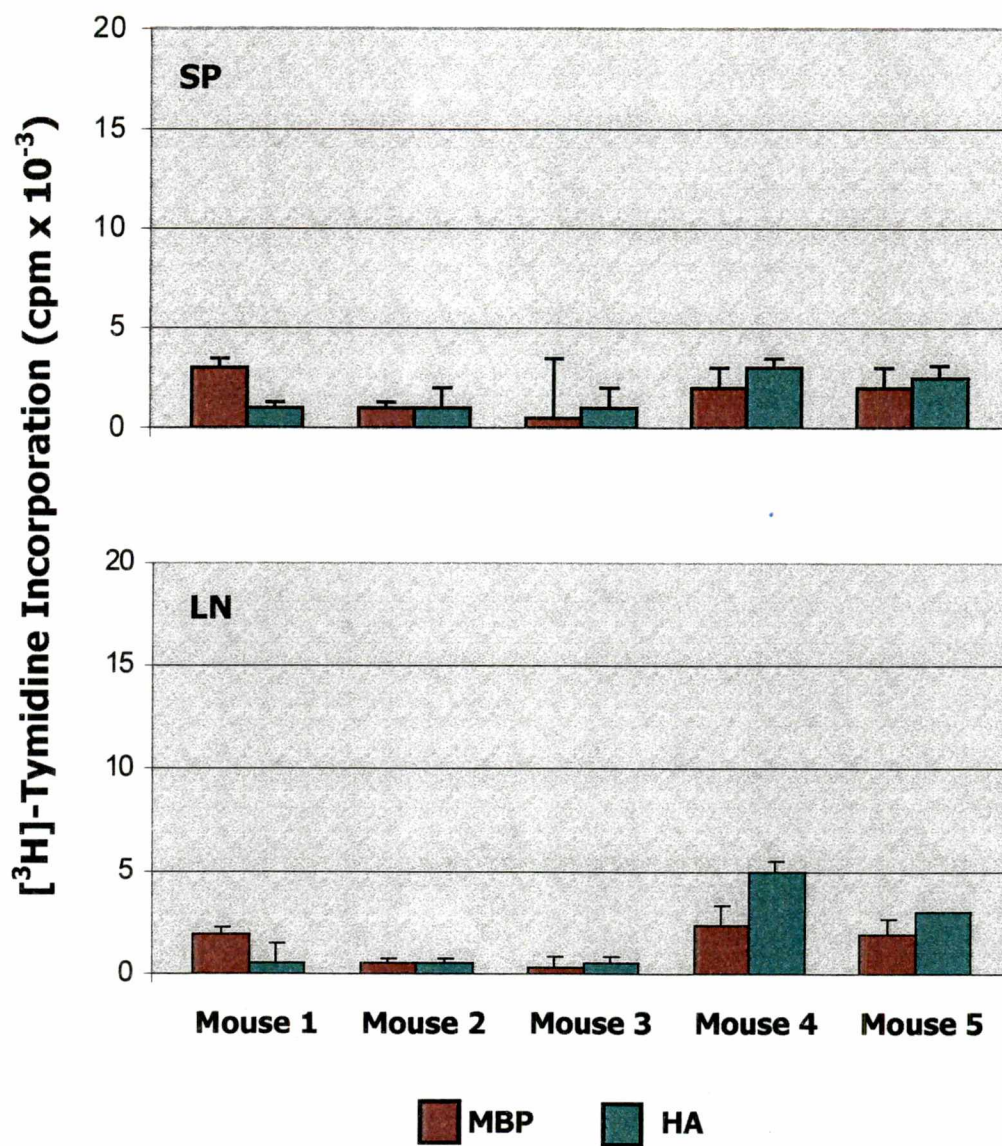


Figure 19: Proliferative lymph node and spleen cell responses in mice immunized with Ig-W

Five mice were each immunized with 50 μ g of Ig-W in CFA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Each value represents the mean $\pm$ SD of triplicates after deduction of BG cpms obtained with no stimulator in the medium. LN, lymph nodes; SP, spleen.



ELISAs were performed on the combined mouse spleen cells for each immunoglobulin set. The Ig-MBP3 immunized mice (Figure 20) showed a significant IL-2 response to MBP, as well as a strong IFN- γ response, both of which are cytokines characteristic of Th1 T cells promoting an inflammatory response. The Ig-MBP3A immunized mice (Figure 21) showed no significant IL-2 or IFN- γ response, but did show a significant IL-4 response, a cytokine produced by Th2 type non-inflammatory T cells. Negative control Ig-W immunized mice did not produce any cytokines in response to the protein (Figure 22). Again, the results to HA were used as a negative control.

ELISPOTs were performed on the combined mouse lymph node cells for each immunoglobulin set. The mouse set immunized with Ig-MBP3 showed a slightly elevated level of IL-2 only (Figure 23). The Ig-MBP3A immunized group did not show detectable levels for any of the cytokines, as did the Ig-W group (Figures 24 & 25).

Immunizations with Ig-MBP3 yielded a Th1 type of cytokine response to MBP, and Ig-MBP3A, resulted in a Th2 type response to the same in vitro stimulation. Therefore, a series of co-immunizations using both Ig constructs were designed and carried out. Co-immunizations were designed to demonstrate the ability of the APL, in the form of the immunoglobulin, to deviate T cells specific for protein in vivo. One set of mice was co-immunized with the agonist and partial agonist, Ig-MBP3 and Ig-MBP3A. Another set was immunized with Ig-MBP3 and Ig-W as a negative control. Once again, T cell

Figure 20: Cytokine profile of the spleen cells in mice immunized with Ig-MBP3

IL-2, IFN- γ , and IL-4 responses were determined by ELISA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Duplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice.

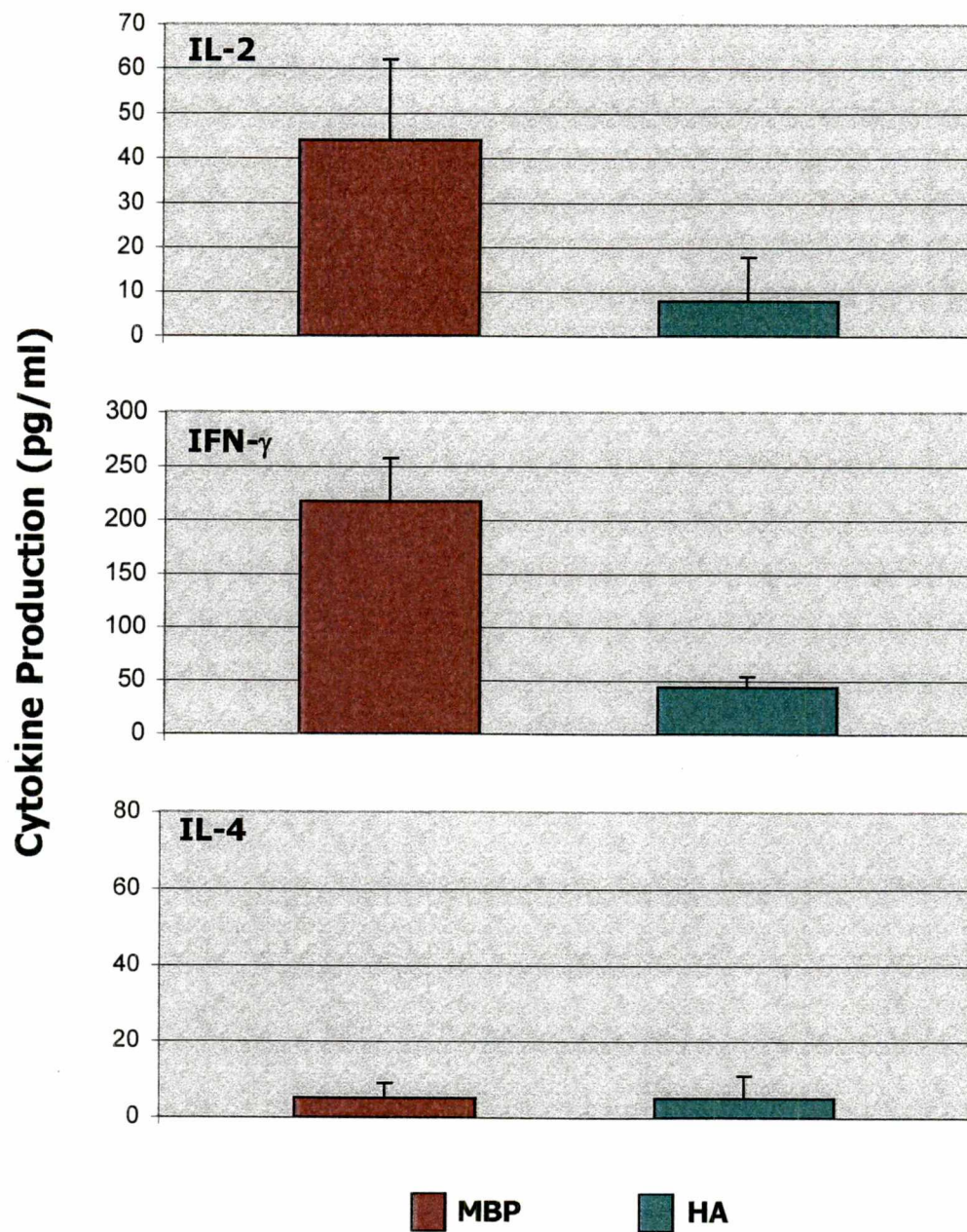


Figure 21: Cytokine profile of the spleen cells in mice immunized with Ig-MBP3A

IL-2, IFN- γ , and IL-4 responses were determined by ELISA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Duplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice.

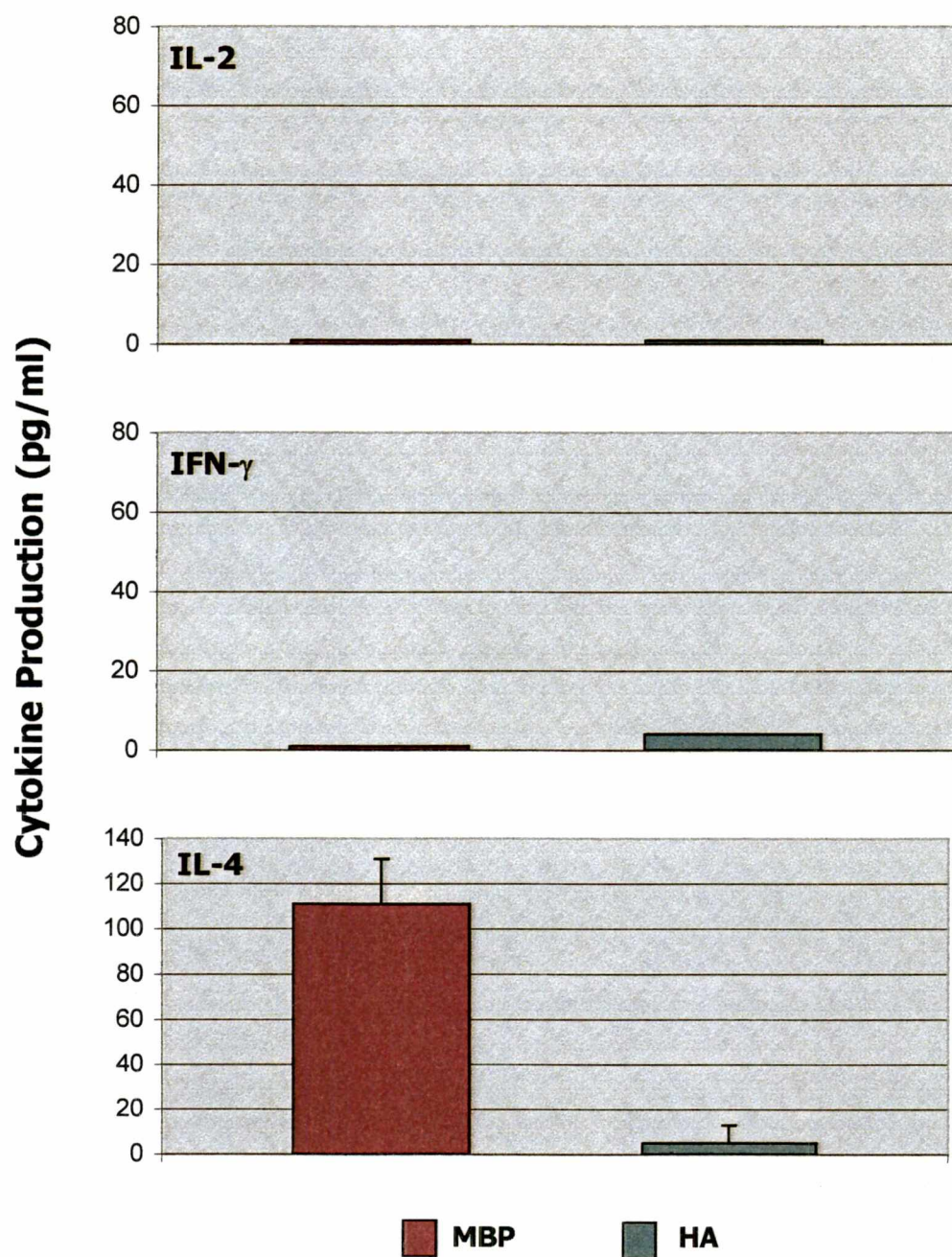


Figure 22: Cytokine profile of the spleen cells in mice immunized with Ig-W

IL-2, IFN- γ , and IL-4 responses were determined by ELISA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Duplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice.

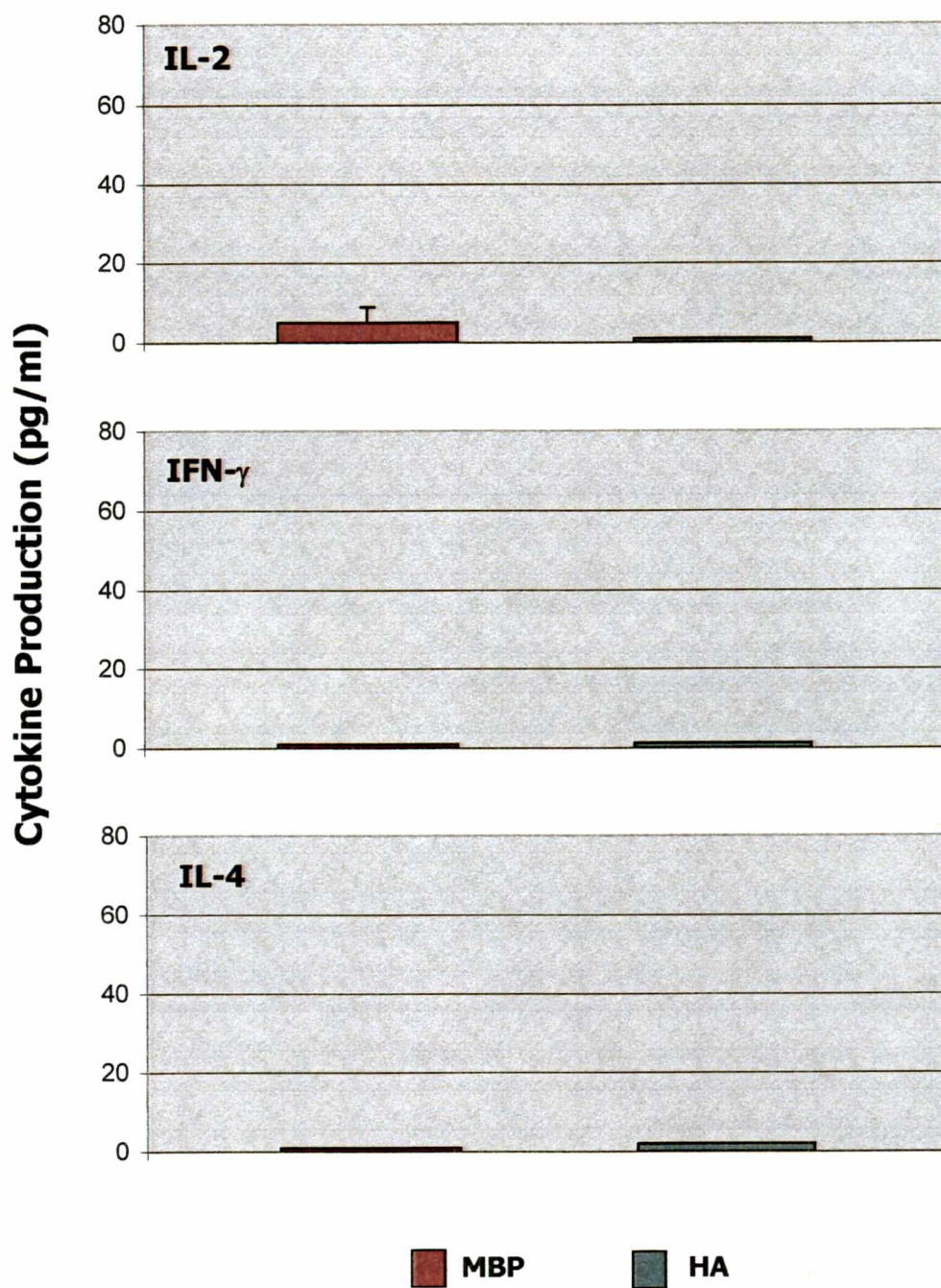


Figure 23: Cytokine profile of the lymph node cells in mice immunized with Ig-MBP3

IL-2, IFN- γ , and IL-4 responses were determined by ELISPOT as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Triplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice. SPF, spot forming unit.

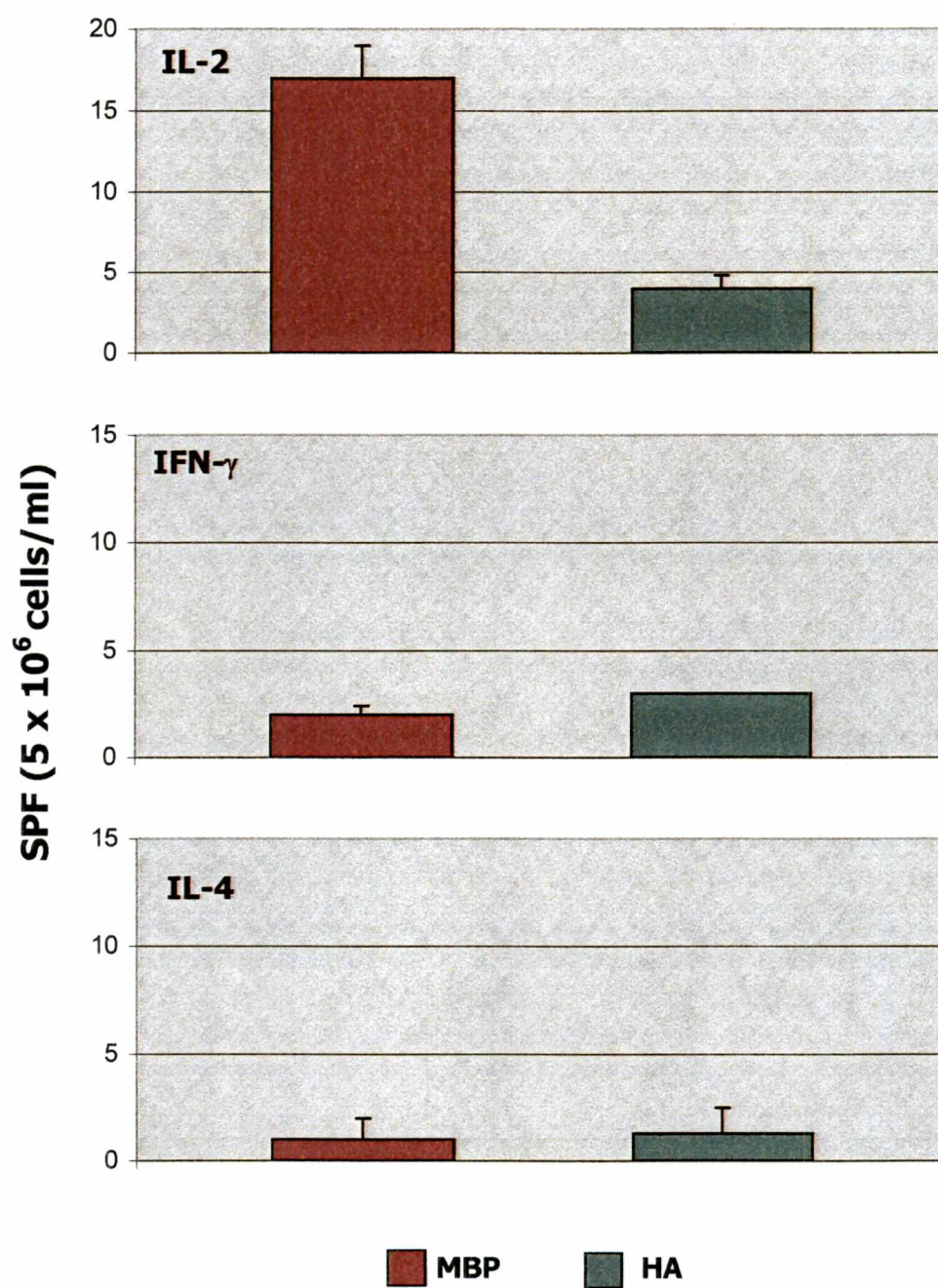


Figure 24: Cytokine profile of the lymph node cells in mice immunized with Ig-MBP3A

IL-2, IFN- γ , and IL-4 responses were determined by ELISPOT as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Triplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice. SPF, spot forming unit.

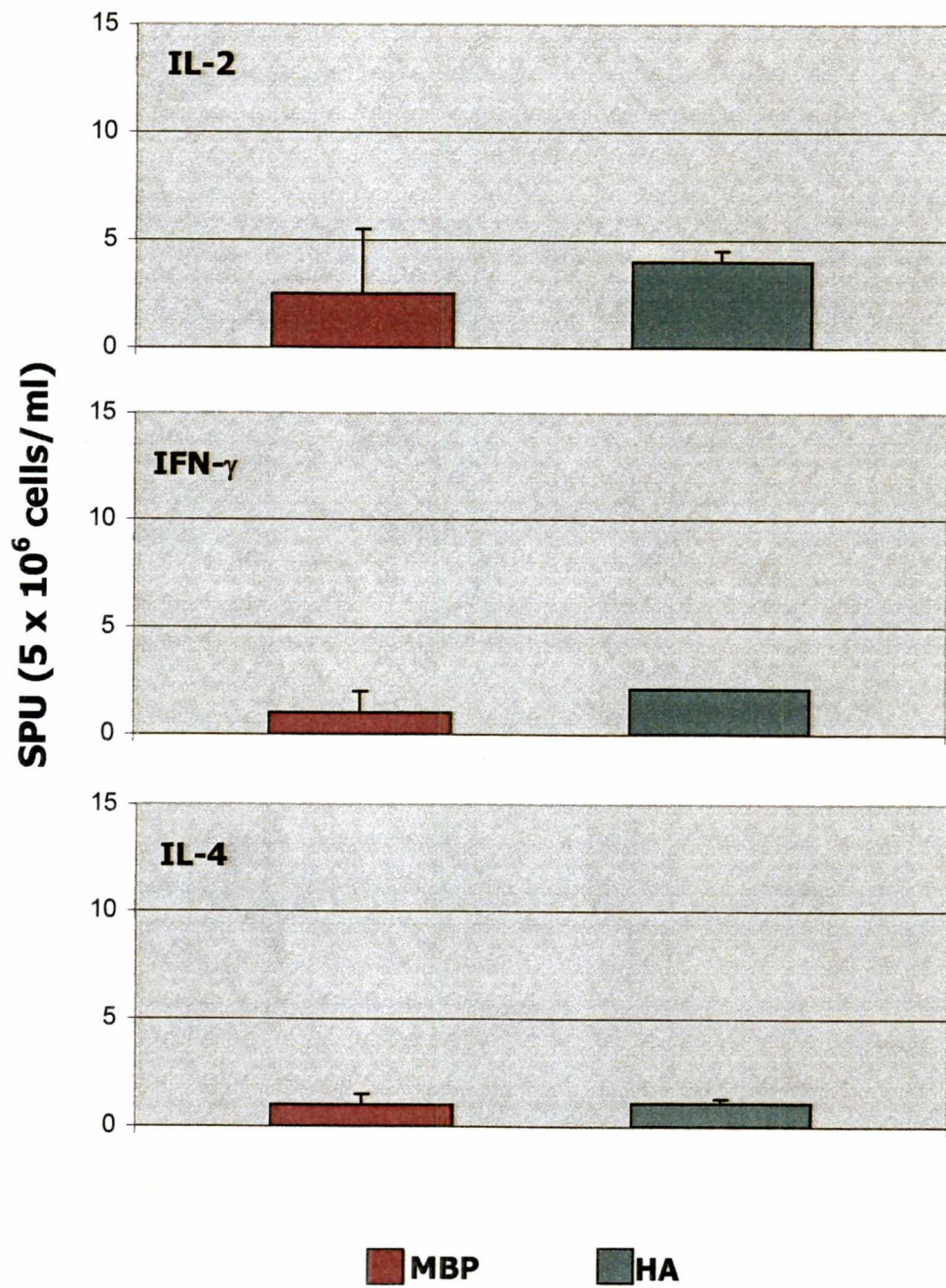
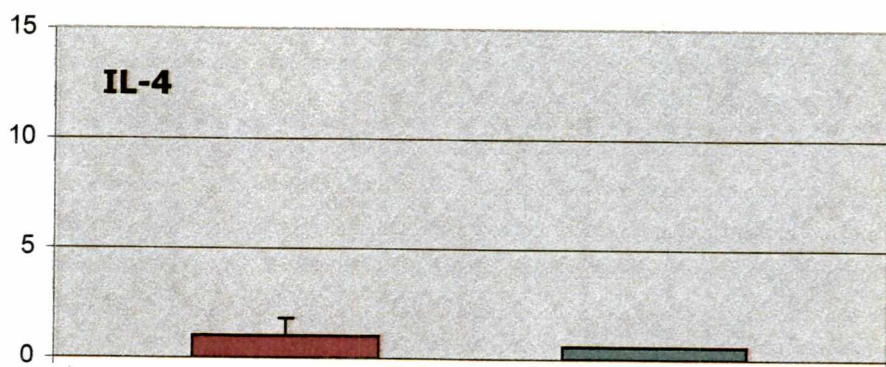
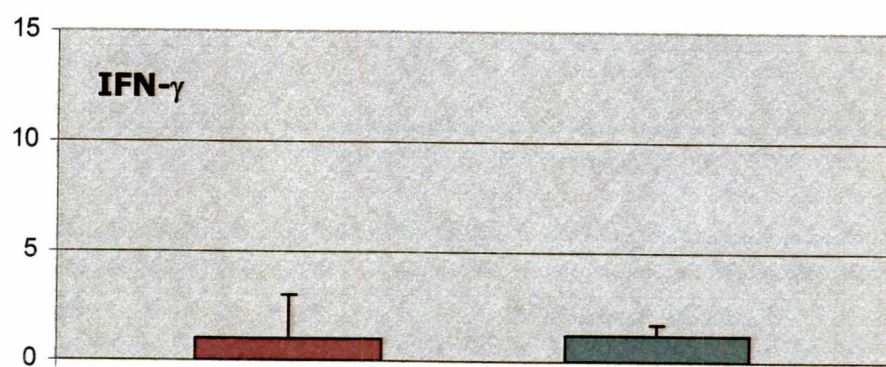
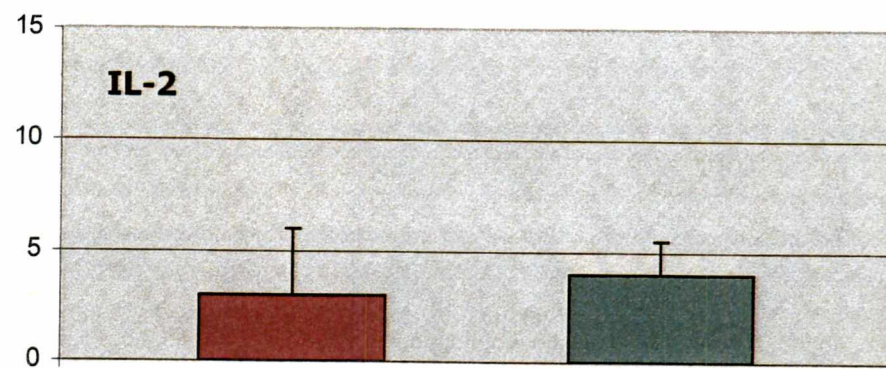


Figure 25: Cytokine profile of the lymph node cells in mice immunized with Ig-W

IL-2, IFN- γ , and IL-4 responses were determined by ELISPOT as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Triplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice. SPF, spot forming unit.

SPU (5 x 10⁶ cells/ml)



■ MBP

■ HA

proliferation assays were performed using the lymph node cells as well as the spleens. In addition, ELISAs and ELISPOTs were performed to reveal cytokine profiles of each group.

Mice co-immunized with the agonist and antagonist showed conflicting results with respect to the lymph node cells' and spleen cells' proliferative response to the protein. Spleen cells in co-immunized mice showed a reduction in proliferation in response to MBP (Figure 26), while the lymph node response was no different than that of mice co-immunized with Ig-MBP3 and Ig-W, the negative control (figure 27). ELISAs (Figure 28) conducted on the spleen cells in co-immunized mice revealed that the IL-2 response was reduced, the IFN- γ response was completely diminished, and the IL-4 levels were non-existent, in a comparison to the control group co-immunized with Ig-MBP3 and Ig-W (Figure 29). ELISPOTs were inconclusive in that there was no significant cytokine production evident for either co-immunization (data not shown).

4. Discussion

The intent of these experiments was to reveal the *in vivo* recognition of the Ig constructs by T cells. Mice immunized with Ig-MBP3 did in fact respond to stimulation with MBP protein *in vitro*. Therefore, subsequent to immunization, these mice developed T cells specific to MBP3. These T cells

Figure 26: Proliferative spleen cell response in mice co-immunized with Ig-MBP3 and Ig-MBP3A

Five mice were each immunized with 50 μ g Ig-MBP3 and 150 μ g Ig-MBP3A in CFA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Each value represents the mean $\pm$ SD of triplicates after deduction of BG cpm obtained with no stimulator in the medium.

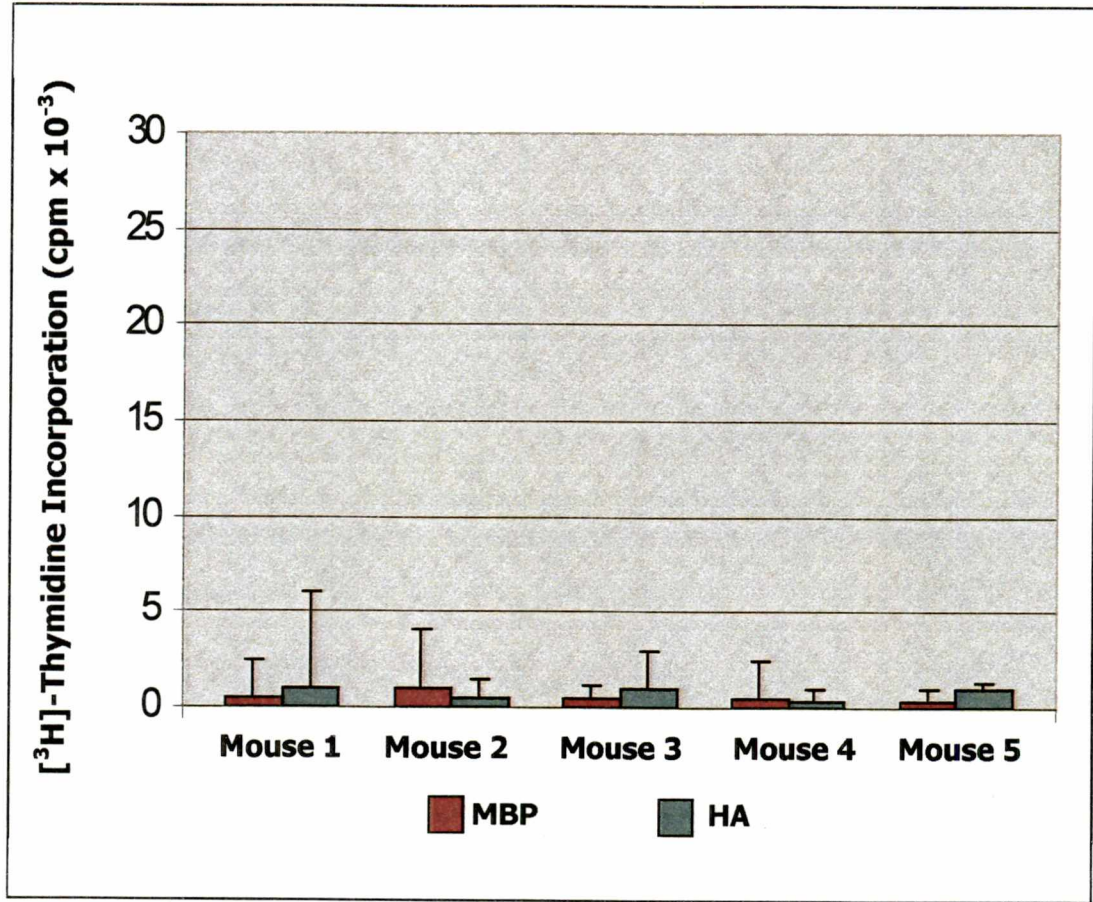


Figure 27: Proliferative spleen cell responses in mice co-immunized with Ig-MBP3 and Ig-W

Five mice were each immunized with 50 μ g Ig-MBP3 and 150 μ g Ig-W in CFA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Each value represents the mean $\pm$ SD of triplicates after deduction of BG cpms obtained with no stimulator in the medium.

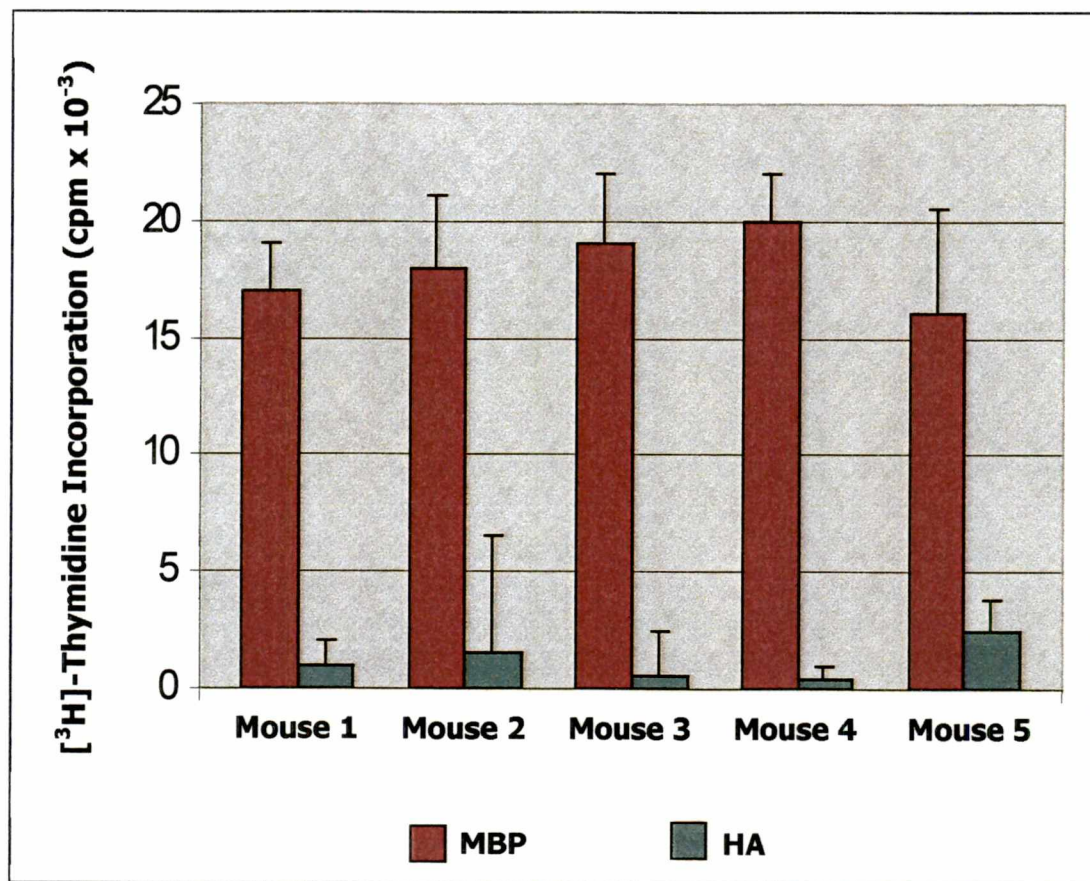


Figure 28: Cytokine profile of the spleen cells in mice co-immunized with Ig-MBP3 and Ig-MBP3A

IL-2, IFN- γ , and IL-4 responses were determined by ELISA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Duplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice.

Cytokine Production (pg/ml)

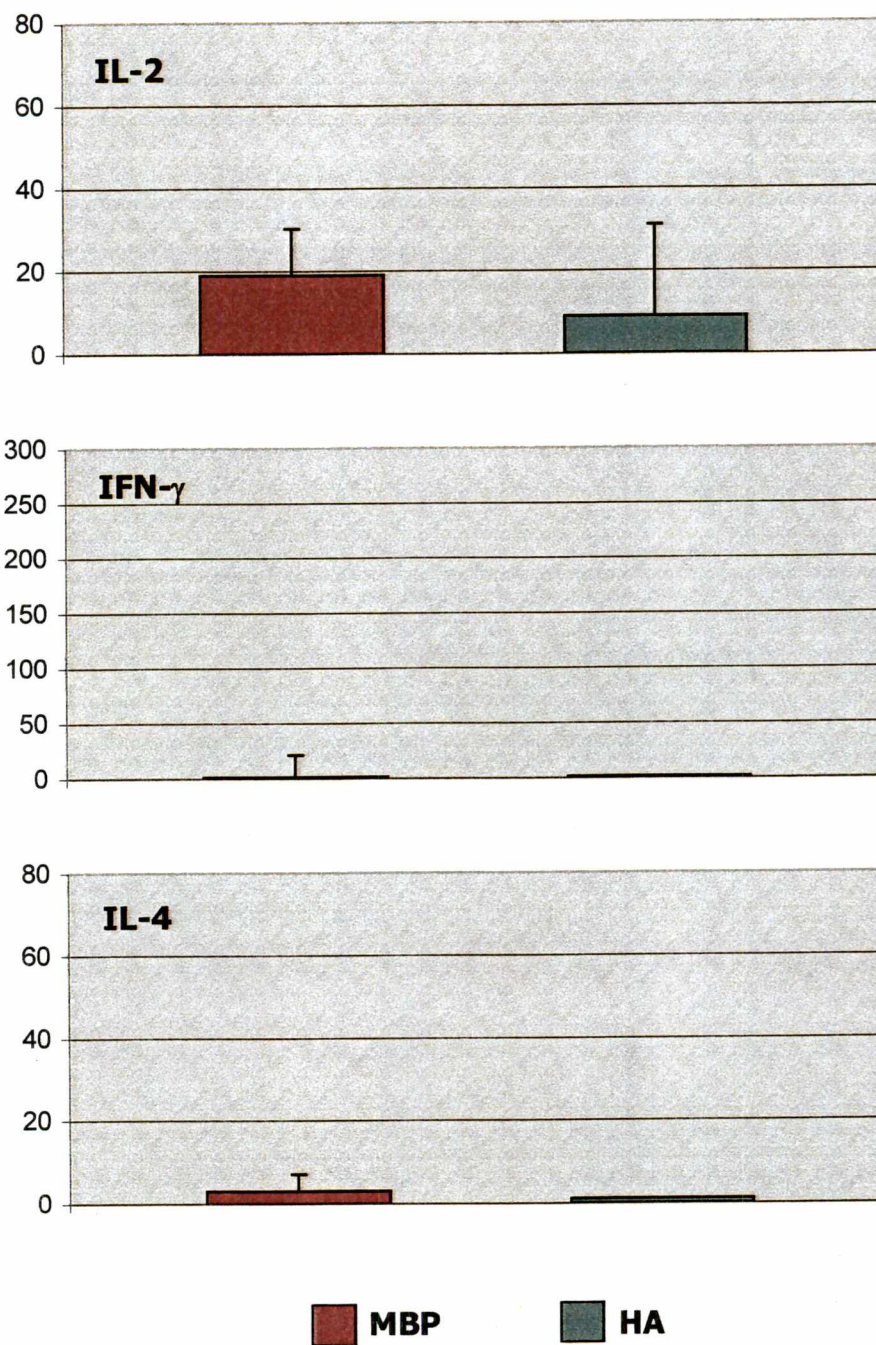
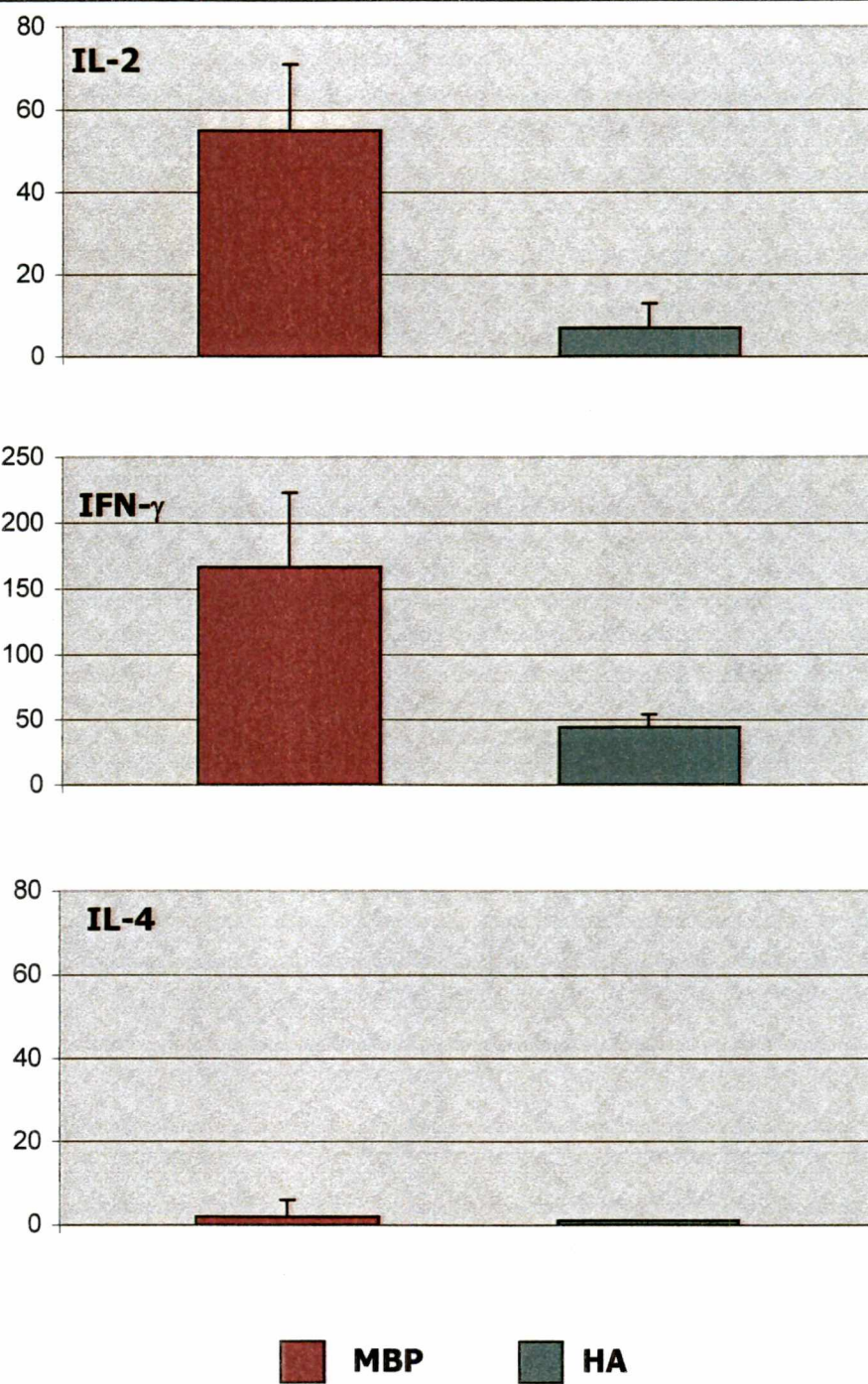


Figure 29: Cytokine profile of the spleen cells in mice co-immunized with Ig-MBP3 and Ig-W

IL-2, IFN- γ , and IL-4 responses were determined by ELISA as described in materials and methods. The stimulators, MBP and HA were used at 0.5 μ M and 15 μ g/ml respectively. Duplicate wells were used for each peptide stimulation. Cells incubated with media not containing any stimulus were used as BG. The indicated amounts represent the mean $\pm$ SD of 5 individually tested mice.

Cytokine Production (pg/ml)



specific for MBP3 were also able to recognize MBP protein, as revealed by their strong proliferative response to the protein, as well as elevated levels of IL-2 and IFN- γ production, inflammatory cytokines characteristic of an autoimmune response. Therefore, injecting SJL/J mice with Ig-MBP3 induced prominent MBP-specific lymph node and splenic T cell proliferative and cytokine responses.

Mice immunized with Ig-MBP3A, the altered peptide, had slightly different responses to the protein stimulus. These mice did reveal a similar response in proliferation, meaning that mice with T cells specific for the altered peptide, also recognized the protein. However, upon viewing the cytokine profiles, it is evident that these cells respond to the protein stimulus in a different manner. Different in that they produce only IL-4 in response to the protein, a non-inflammatory cytokine.

Due to the opposing natures of the Ig constructs with respect to cytokine profile induced upon protein stimulation, a series of co-immunizations were conducted. These experiments were performed in an attempt to view a mutual down regulation of cells of the Th1 type and those of the Th2 type. Mice immunized with a mixture of both Ig-MBP3 and Ig-MBP3A demonstrated a high proliferative response in the lymph nodes, yet all proliferative responses in the spleen were diminished. This information is quite puzzling. One would expect to see a diminished proliferative response in mice co-immunized with these Igs in that the Th1 cells secreting IL-2 and IFN- γ would turn off the Th2

cells producing IL-4 and vice-versa. The diminished levels of IFN- γ produced in the spleen further prove this theory. Therefore, in the spleen, immunization of both immunoglobulins most likely yielded two initial responses, Th1 and Th2, both of which acted against each other to silence the overall effect. The reason as to why there was a high proliferative response in the lymph nodes is not fully understood. Often when immunizations include CFA, cells become activated and are induced to proliferate and produce cytokines.

As in all other experiments, Ig-MBP3 co-immunized with Ig-W was used as a negative control. Results indicated that the proliferative and cytokine responses were no different in these mice than in those immunized with Ig-MBP3 alone.

The preceding data are of great importance in that they demonstrate the ability of Ig-MBP3A to act as a partial agonist, which can successfully silence the inflammatory effects of the agonist, Ig-MBP3.

Part V: Induction of EAE and Possible Treatment

1. Introduction

The previous experiment demonstrated mutual downregulation of Th1 and Th2 cells in mice co-immunized with agonist and partial agonist immunoglobulins. Therefore, a series of immunizations and co-immunizations were designed to demonstrate whether or not the Ig carrying the altered peptide could treat mice committed to developing EAE.

2. Materials and Methods

Animals

Six to eight week old SJL/J mice were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in the University of Tennessee animal facility for the duration of the experiments.

Peptides

All peptides used in this experiment were purchased from Research Genetics, Inc (Huntsville, Alabama) and purified by HPLC to > 90% purity. PLP1 peptide (HSLGKWLGHDPKF) corresponds to amino acid residues 139-151 of proteolipid protein (PLP). PLP1 is a T cell epitope presented by I-A^s class II molecules and is encephalogenic in H-2^s mice (33). MBP3 peptide has been described previously in this text.

Immunoglobulins

Ig-MBP3A has been described previously in this report. Ig-W is an immunoglobulin construct containing wild type anti-arsonate heavy and light chain expression. Ig-W is used as a negative control in the following experiments.

Immunization for Disease

Three groups of seven mice were immunized sub-cutaneously in the footpads and at the base of the limbs with 100 μ g of MBP3 emulsified in a 200 μ l mixture containing PBS, H37Ra *Mycobacterium Tuberculosis* (Difco Laboratories, Detroit, MI) and IFA. Approximately 7 hrs post immunization, each mouse was injected intravenously with 4.5×10^9 microorganisms of *Bordatella Pertussis* (Michigan Department of Public Health, Lansing, MI).

Immunization for Multiple Epitope Disease

Three groups of seven mice were immunized for disease with multiple epitopes by sub-cutaneous injection in the footpads and at the base of the limbs with 100 μ g MBP3 and 100 μ g of PLP1 emulsified in a 200 μ l mixture containing PBS, H37Ra *Mycobacterium Tuberculosis* and IFA. Injections with *B. Pertussis* cells were initiated as above.

Treatment Injections

After mice in two of the immunized groups above reached a mean clinical score (see below) of 0.5, they were each treated IV with 200 μ g of either Ig-MBP3A or the control, Ig-W in PBS. Mice in the two groups received 3 or 4 treatment injections 5 days apart, depending on clinical scores.

Grading

Mice were scored daily after initial immunization for disease. The assignment of clinical scores to the mice is according to the following scale: 0, no clinical disease; 1, loss of tail muscle tone; 2, incomplete paralysis of one or two hindlimbs; 3, complete paralysis of one or two hindlimbs; 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead animals.

3. Results

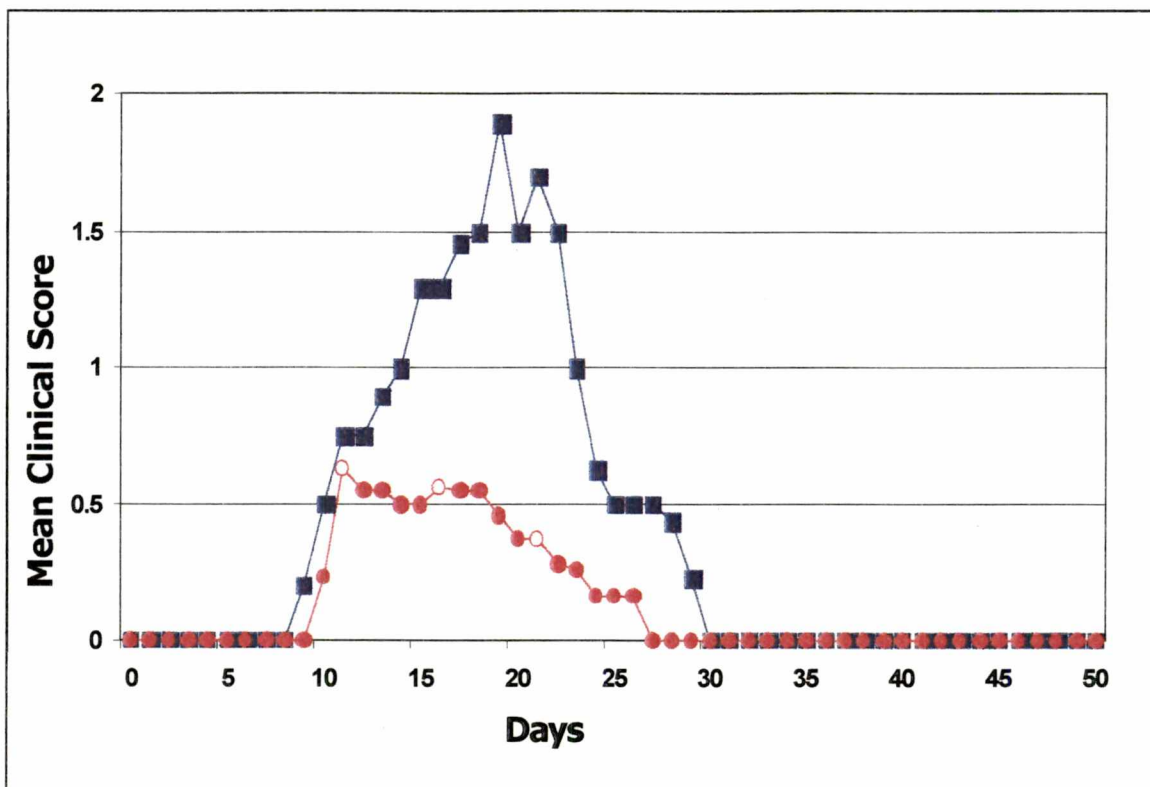
Mice were immunized primarily with MBP3 to demonstrate that this peptide can induce EAE in SJL/J mice. After immunization, clinical signs became evident by day 10 (Figure 30). A peak in the disease occurred at day 17 with a mean clinical score of 2. Also, disease induced with MBP3 was monophasic. These results were conclusive with results obtained by Brocke, et al (24). Therefore, administration of MBP3 peptide resulted in a mild, monophasic form of EAE in SJL/J mice.

Figure 30: Induction of disease with MBP3, and treatment with Ig-MBP3A

For disease, mice were immunized with 100 μ g MBP3 peptide as described in materials and methods. Mean clinical score was recorded daily according to the following scale: 0, no clinical disease; 1, loss of tail muscle tone; 2, incomplete paralysis of one or two hindlimbs; 3, complete paralysis of one or two hindlimbs; 4, paraplegia, with forelimb weakness or paralysis; 5, moribund or dead animals.

For treatment animals, mice were immunized with 100 μ g MBP3 peptide as described in materials and methods. After mean clinical score reached 0.5, mice were each treated with 200 μ g Ig-MBP3A as described in materials and methods. A total of three treatment injections were given to each mouse (as shown by the arrows). Mean clinical score was recorded daily as above.

Mice immunized with MBP3, blue squares; mice immunized with MBP3 and then treated with Ig-MBP3A, red circles. The open circles indicate days of treatment.



In the hopes that administration of Ig-MBP3A would deviate T cells specific for MBP *in vivo*, mice were first immunized with MBP3, and then treated with Ig-MBP3A (Figure 30). EAE was induced in mice with MBP3. Disease was allowed to progress until the mean clinical score reached 0.5. At that point, Ig-MBP3A was administered intravenously. Subsequent treatment injections were given every five days after the initial injection, for a total of three IV injections per mouse. As a result, treated mice had a peak mean clinical score of 0.6, less than half of the peak disease score in mice immunized with MBP3 alone. Therefore, administration of Ig-MBP3A significantly decreased the severity of disease induced with MBP3.

Mice were immunized for disease and treated with Ig-W as a negative control (Figure 31). As shown, administration of Ig-W as treatment did not reduce the severity of the disease.

A group of mice were co immunized for disease with multiple epitopes. Mice were immunized with MBP3 and PLP1 to demonstrate the induction of an additive disease by causing disease with T cells specific for two epitopes (Figure 32). Clinical signs in mice immunized with both peptides were evident at day 7. The peak in mean clinical score occurred at day 12 and reached a value of 4.3. This peak in mean clinical score is significantly higher than those induced in mice immunized with MBP3 or PLP1. Therefore, the disease induced by MBP3 and PLP1 was additive in that it was greater than disease induced by either if the two peptides.

Figure 31: Treatment of disease with Ig-W

Mice were immunized with 100 μ g MBP3 peptide as described in materials and methods. After mean clinical score reached 0.5, mice were each treated with 200 μ g Ig-W as described in materials and methods. A total of three treatment injections were given to each mouse (as shown by the arrows). Mean clinical score was recorded daily according to the following scale: 0, no clinical disease; 1, loss of tail muscle tone; 2, incomplete paralysis of one or two hindlimbs; 3, complete paralysis of one or two hindlimbs; 4, paraplegia, with forelimb weakness or paralysis; 5, moribund or dead animals.

Mice immunized with MBP3, blue squares; mice immunized with MBP3 and treated with Ig-W, red diamonds. Open diamonds indicate days of treatment.

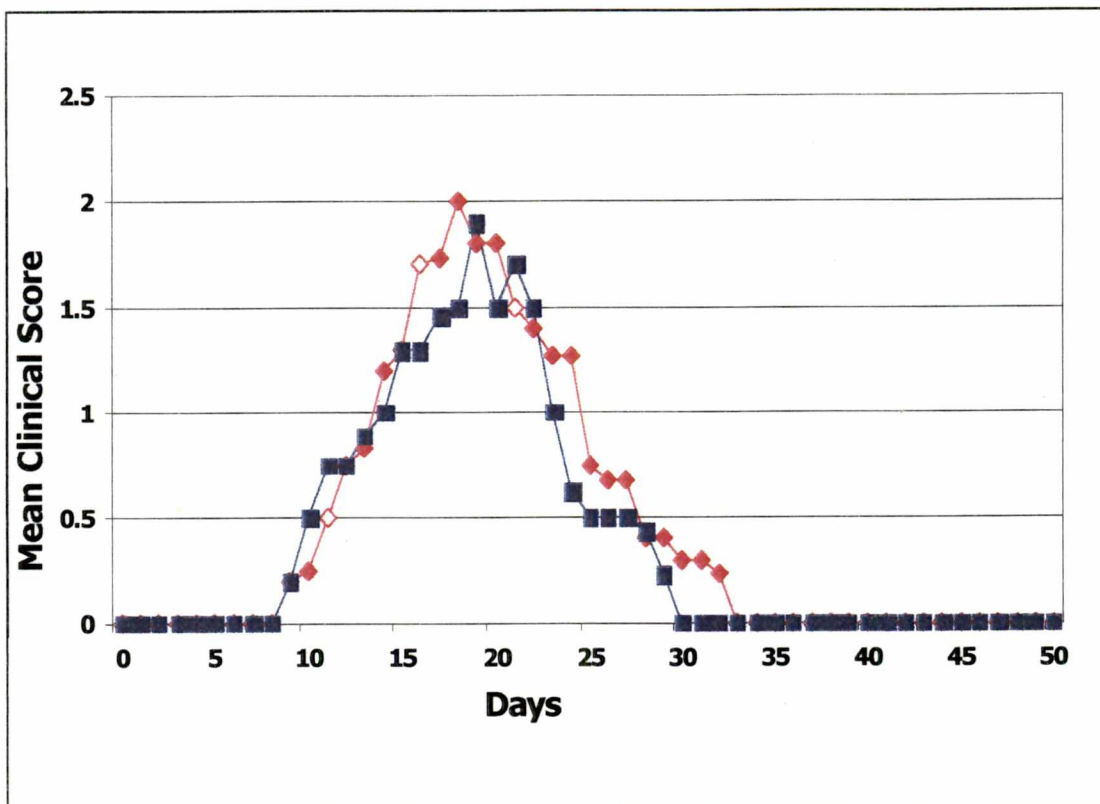
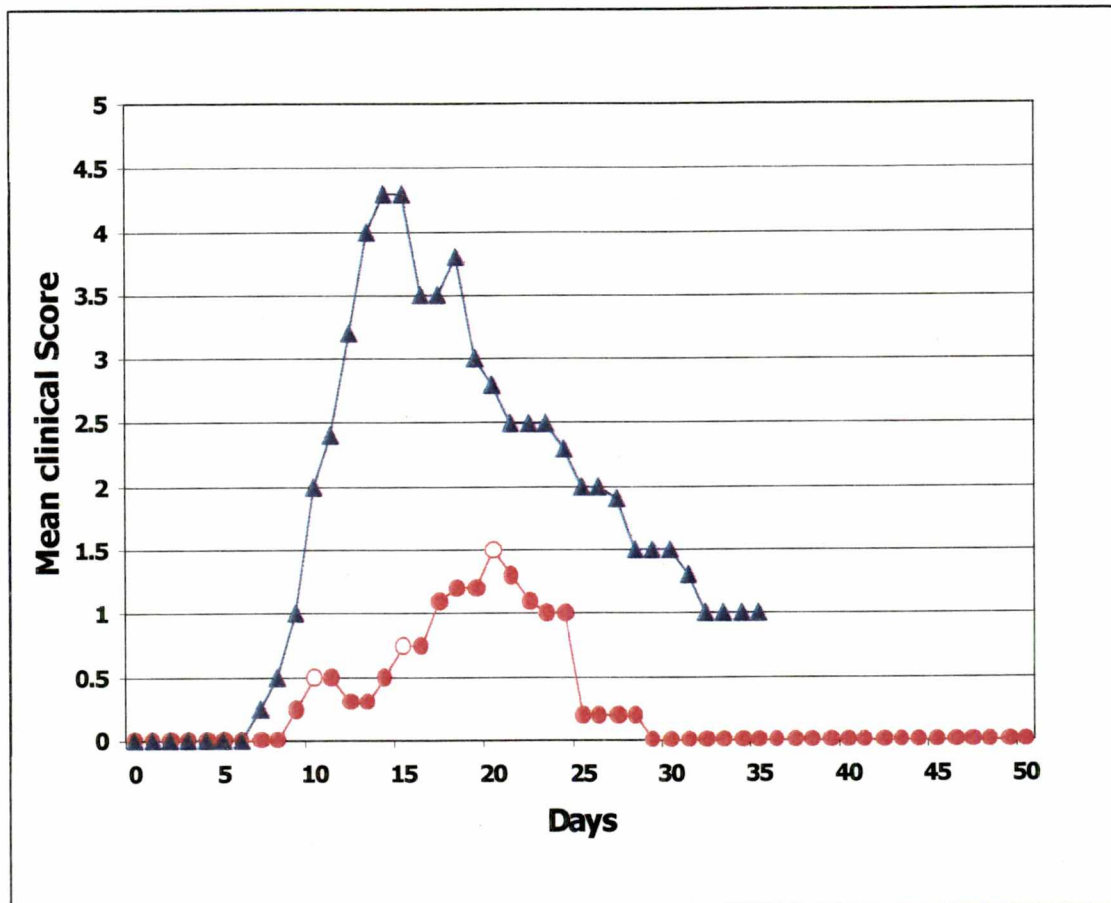


Figure 32: Induction of multiple epitope disease with MBP3 and PLP1, and treatment with Ig-MBP3A

For disease, mice were co-immunized with 100 μ g MBP3 and 100 μ g PLP1 as described in materials and methods. Mean clinical score was recorded daily according to the following scale: 0, no clinical disease; 1, loss of tail muscle tone; 2, incomplete paralysis of one or two hindlimbs; 3, complete paralysis of one or two hindlimbs; 4, paraplegia, with forelimb weakness or paralysis; 5, moribund or dead animals.

For treatment, mice were immunized with 100 μ g MBP3 and 100 μ g PLP1 as described in materials and methods. After mean clinical score reached 0.5, mice were each treated with 200 μ g Ig-MBP3A as described in materials and methods. A total of four treatment injections were given to each mouse. Mean clinical score was recorded daily as described above.

Mice co-immunized with MBP3 and PLP1, blue triangles; mice treated with Ig-MBP3A, red circles. Open circles indicate days of treatment.



The next experiment was developed with intent to show the ability of Ig-MBP3A to treat a multiple epitope disease. Mice were immunized with MBP3 and PLP1 to induce disease with multiple epitopes, and then treated with Ig-MBP3A (Figure 32). Following induction of disease and a mean clinical score of 0.5, mice were treated intravenously with Ig-MBP3A. Subsequent treatment injections were given 5 days apart for a total of 4 injections. Treatment with Ig-MBP3A resulted in a peak mean clinical score of 1.5, less than half of the peak clinical score in mice co-immunized with MBP3 and PLP1 alone (PLP1 immunization data not shown). Thus, administration of Ig-MBP3A to mice with multiple epitope disease significantly decreased the severity of the disease.

Mice committed for disease with multiple epitopes were also treated with Ig-W as a negative control. As shown, Ig-W treatment did not reduce the severity of the disease (Figure 33).

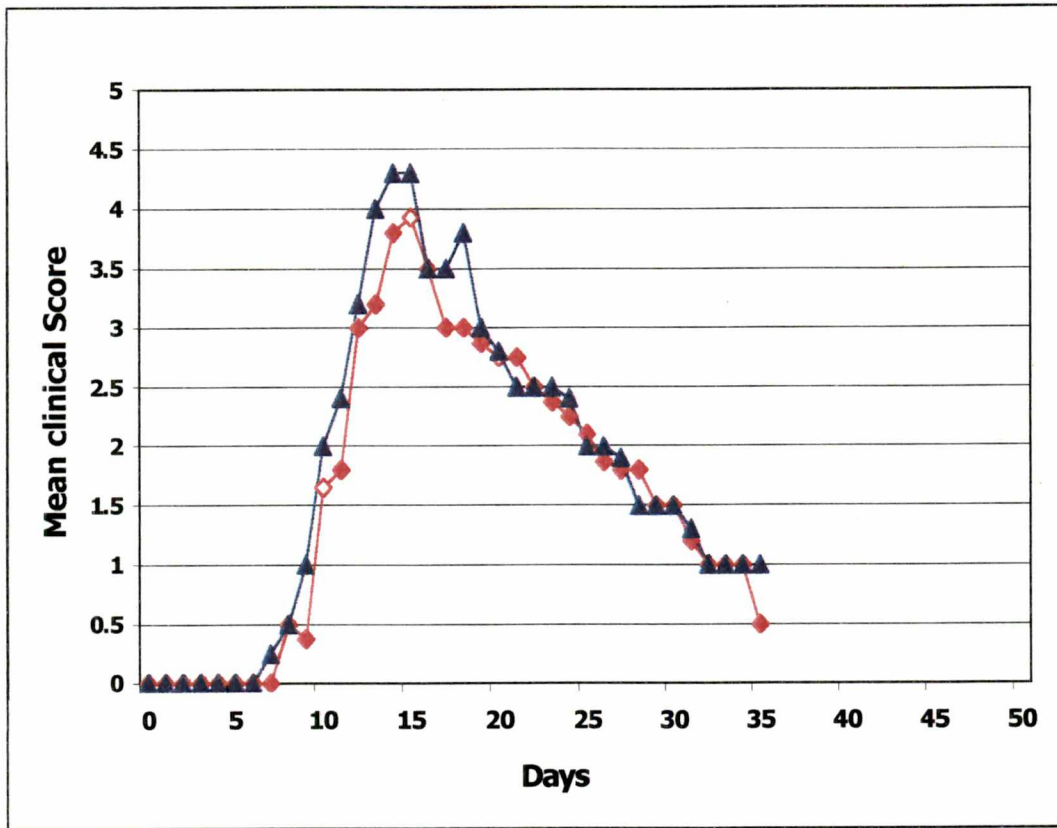
4. Discussion

Mice were initially immunized with MBP3 peptide to demonstrate the course of disease that occurred through induction with this peptide. Results indicated that MBP3 was able to induce EAE in SJL/J mice. Treatment of disease induced with MBP3 was achieved through injection with Ig-MBP3A. Therefore, it was speculated that the administration of the altered peptide in the context of immunoglobulin was able to deviate the T cells specific to MBP3 in vivo and

Figure 33: Treatment of multiple epitope disease with Ig-W

Mice were immunized with 100 μ g MBP3 and 100 μ g PLP1 as described in materials and methods. After mean clinical score reached 0.5, mice were each treated with 200 μ g Ig-W as described in materials and methods. A total of four treatment injections were given to each mouse (as shown by the arrows). Mean clinical score was recorded daily according to the following scale: 0, no clinical disease; 1, loss of tail muscle tone; 2, incomplete paralysis of one or two hindlimbs; 3, complete paralysis of one or two hindlimbs; 4, paraplegia, with forelimb weakness or paralysis; 5, moribund or dead animals.

Mice co-immunized with MBP3 and PLP1, blue triangles; mice treated with Ig-W, red diamonds. Open diamonds represent the days of treatment.



lead to the reversal of the disease. These data reveal the possibility of utilizing altered peptides inserted into immunoglobulins for therapeutic ideas regarding autoimmunity.

The next series of immunizations were perhaps the most crucial of all experiments performed. It is well understood that spontaneous disease in humans occurs against multiple epitopes, not simply one. As time and disease progress the T cells become specific for additional myelin epitopes. This is called active disease. In active disease, the encephalogenic T cells are specific for a multitude of epitopes at various times during the course of disease. Mice immunized with one epitope (MBP3) are representatives of passive disease, one in which the T cells induced are specific for that particular epitope and no others. In an attempt to address the issue of disease induced with multiple epitopes, mice were first immunized with MBP3 and PLP1. The results indicated that these mice experienced a disease that was additive. Additive in that the disease was greater than those induced by MBP3 or PLP1 alone (PLP1 data not shown). The immunizations yielded T cells that were specific in the destruction of two different epitopes, therefore these mice had an active disease that was elevated in comparison to those receiving a single epitope.

The next issue addressed was the ability of Ig-MBP3A to treat active disease induced with multiple epitopes. It was already demonstrated that administration of Ig-MBP3A was able to reverse disease induced with MBP3 peptide. There was the existing possibility that administration of Ig-MBP3A

could treat multiple epitope disease. Treatment would be the result of an induced IL-4 production from deviated T cells acting to inhibit the production of IFN- γ . Experiments were designed with the intent to treat active disease induced with MBP3 and PLP1. Results indicated that indeed, administration of Ig-MBP3A lead to the significant reversal of EAE in these mice, once again demonstrating the importance in using altered peptide ligands in the context of immunoglobulins to treat autoimmunity.

Part VI: Summary

Antigens that stimulate T cells must first undergo a degradation process to generate peptides capable of associating with MHC. The peptide-MHC complex is translocated to the APC surface where it is displayed for recognition by T cells. It is generally accepted that T cells of the CD8 subset recognize antigen in the context of MHC class I generated in the endogenous pathway, whereas those of the CD4 subset respond to antigens in the context of MHC class II generated in the endocytic pathway (35, 36).

An endocytic antigen presentation system was designed and the fundamental mechanisms were evaluated as to whether TCR partial agonist peptides could overcome antigens that generate large quantities of encephalogenic peptides in autoimmunity. In the created system, MBP3 and an altered form of it, MBP3A, a known partial agonist, were expressed on the anti-arsonate immunoglobulin, 91A3. The resulting Ig-MBP3 and Ig-MBP3A chimeras were used to evaluate T cell partial agonism in an antigen system requiring endocytic processing as it might occur in autoimmune situations.

Immunoglobulin constructs were designed to provide a more efficient and effective way to deliver peptides to autoreactive T cells. Briefly, the chimeras were designed to contain mutated diversity regions within the variable region of the heavy chain of an anti-arsonate antibody. The D region was altered to encode the agonist peptide, MBP3 and the altered peptide or partial agonist, MBP3A.

T cell hybridomas were generated specific for MBP3 to prove the efficient and effective presentation of the peptides grafted into the immunoglobulins. Also demonstrated was success in the T cell recognition of these peptides. Cytokine profiles of the hybridomas revealed a Th1 response to the agonist peptide and immunoglobulin and a Th2 response to the partial agonist immunoglobulin.

In vivo experiments were conducted to reveal the T cells' ability to recognize and respond to immunoglobulin constructs as shown in vitro. It was shown that mice immunized with the agonist Ig responded to MBP protein in a Th1 type fashion, whereas mice immunized with partial agonist Ig yielded a T cell response of the Th2 type. These results were conclusive with the previous hybridoma experiments.

A series of immunizations were developed to demonstrate the ability of the partial agonist Ig to treat mice committed to EAE. It was discovered that mice immunized with MBP3 were treated with Ig-MBP3A. Also discovered was the ability of Ig-MBP3A to reverse active disease induced with multiple epitopes.

In conclusion, the aforementioned experiments demonstrate a means of deviating autoreactive T cells to become near harmless. These experiments may lead to the eventual treatment of other autoimmune diseases in animals, and hopefully will shed light on the treatment and prevention of human autoimmune disease.

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VITA

Aimee Elizabeth Cestra was born in Brookhaven, New York on August 29, 1973. She grew up in both Southhaven, New York and Long Valley, New Jersey. She is a graduate of the West Morris Central High School of Chester, New Jersey. Currently, she is from Harvard, Massachusetts, her parents' newest location. Aimee entered The University of Tennessee, Knoxville in the fall of 1991. She received the Bachelor of Science degree in winter 1995 with a double major in Biology and Zoology. She entered the graduate school at the same university in the fall of 1996 and is currently seeking a Master's degree in Microbiology, scheduled for completion August 1998.

Aimee has accepted a job in Boston, Massachusetts, where she will be working for Dr. Gerald L. Waneck at the Massachusetts General Hospital Transplantation Biology Research Center. She will be conducting research on xenotransplantation. Included is the strategy for inhibiting lysis by human NK cell based on the expression of human MHC class I molecules in swine tissues.