Demyelinating Autoimmunity: Murine T Cell Epitopes of MBP and Primate T Cell Receptor Vβ Variation

Thesis by

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Abstract

Autoimmune diseases result from inappropriate self-reactivity by lymphocytes. The long-term goal is to generate specific therapies for autoimmune diseases of humans, the success of which hinges on the definition of specific therapeutic targets. Experimental allergic encephalomyelitis (EAE) is a good animal model for the human demyelinating autoimmune disease, multiple sclerosis (MS). Risk for these diseases stratifies by major histocompatibility complex (MHC) allele, as well as by T cell receptor (TCR) locus RFLP, in the case of MS. These data suggest that (TCR-self peptide-MHC) complexes are associated, possibly causally, with pathogenesis. This work focused on the self peptide and TCR components of this complex. One specific aim was to document and characterize the T cell epitopes of the autoantigen, myelin basic protein (MBP), in the EAE-susceptible mouse strain, B10.PL. Inbred B10.PL mice which were immunized with self MBP in complete Freund's adjuvant activated lymphocytes specific for epitopes estimated by peptides MBP(NAc1-20), MBP(31-50), and MBP(121-140). These mice generated the bulk of their immune response to the MBP(NAc1-20) epitope. The responses to self MBP immunization of B10.PL wildtype and MBP null "shiverer" mice were compared, and it was found that MBP(121-140) is tolerogenic in animals which express MBP. A similar result

was observed in BALB/c wildtype and shiverer mice. These data demonstrate that MBP is not a sequestered antigen, that multiple epitopes tolerize T cells independently, and that incomplete, rather than absent, tolerance is present in mice susceptible to EAE. A second specific aim was to document the degree of variation in the primate TCR Vβ 8 subfamily, three members of which are adjacent to a BamHI RFLP restriction site linked to multiple sclerosis (MS) disease risk. Vβ 8.1 and 8.2 were compared in a number of primates. It was found that the overall coding sequences, but not the CDR coding sequences, were conserved compared with adjacent non-coding flanking sequences. CDR coding sequences were not demonstrably positively selected compared with non-coding flanking sequences or with synonymous coding sequences. A comparison of unrelated normal humans failed to demonstrate any non-synonymous substitutions within Vβ 8.1 and 8.2, and demonstrated a single non-synonymous substitution in Vβ 8.3. These data demonstrate that germline Vβ 8 gene segments are conserved and minimally polymorphic, implying that final TCR protein diversity derives from other mechanisms. Occasional allelism has been demonstrated in other Vβ subfamilies, and our data does not rule out that certain TCR Vβ alleles may ultimately be found to contribute to autoimmunity disease risk.

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INTRODUCTION

Vertebrate survival hinges on the ability of lymphocytes to anticipate, distinguish and destroy pathogens (Klein 1990). Because this set of pathogens is diverse, the set of clonal antigen specificities of lymphocytes is expected to be diverse as well. It is predicted that this diverse set of antigen specificities of lymphocytes is generated in a random fashion without regard to self-nonself distinctions. Lymphocyte antigen specificities includes self-specificities, so that tolerance to self must be acquired. Tolerance occasionally fails and lymphocyte self-reactivity, or autoimmunity, results. Risk for autoimmunity stratifies by MHC allele and, in some diseases, by T cell antigen receptor (TCR) loci RFLPs. This suggests that pathogenic (MHC/TCR/self-peptide) complexes exist which are necessary, although not necessarily sufficient, for self-reactive T cell activation and T cell-mediated injury. Unanswered questions include the degree to which self-proteins and germline TCR V gene segments can be utilized in disease induction and progression. The purpose of this thesis was to attempt to answer these questions for the autoimmune demyelinating diseases. Specifically, it sought to determine the nature of the murine response to myelin basic protein (MBP), the auto-antigen of experimental allergic encephalomyelitis (EAE), and the breadth of variation in human TCR Vβ 8, a subfamily which has been linked epidemiologically to a similar demyelinating disease

of humans, multiple sclerosis (MS).

Normal Lymphocytes

Lymphocytes are mobile, antigen-specific, non-phagocytic leukocytes. In mammals, lymphocytes are derived from hemopoietic stem cells which mature in one of two primary lymphoid organs, the bone marrow (for B lymphocytes) and thymus (for T lymphocytes). Mature lymphocytes circulate from bloodstream to lymphatics via the secondary lymphoid organs, the spleen and lymph nodes. Splenic lymphocytes are exposed to blood-borne antigens, and lymph node lymphocytes are exposed to lymph-borne antigens. After exiting the secondary lymphoid organs into efferent lymphatics, they eventually re-enter the bloodstream. This traffic pattern of mobile lymphocytes (Gowans 1964) ensures exposure of a variety of antigen-specific cells to different potential sources of antigens.

Resting B and T lymphocytes are morphologically similar, but functionally distinct. Both cell types express monoclonal cell surface antigen receptors, and these antigen receptors (immunoglobulin (Ig) in B lymphocytes, α/β T cell antigen receptor (TCR) in T lymphocytes) define the antigen specificities of the individual lymphocytes (Klein 1990). The B cell antigen receptor, immunoglobulin, recognizes a three-dimensional ligand. The α/β TCR, on the other hand, recognizes short peptides bound to major histocompatibility complex (MHC) proteins on the surface of cells. Binding of TCR to correct ligand is necessary but not sufficient for

functional activation, since T cell activation appears to require additional T cell costimulation (Gimmi et al. 1991). Two subsets of α/β T cells can be defined by the co-expression of one of the two accessory molecules, CD4 and CD8 (Bierer 1989). $CD4^+$ ("helper") T cells make cell-cell contact with antigen-presenting cells (APCs) (macrophages, activated B cells, and dendritic cells) bearing surface peptide-class II MHC (HLA-D in humans) complexes. $CD8^+$ ("cytotoxic") T cells make cell-cell contact with cells bearing surface peptide-class I MHC (HLA-A,B,C in humans) complexes. When TCR-peptide-MHC trimolecular complex binding, lymphokine levels and co-stimulatory signals are sufficient, a lymphocyte becomes activated, expressing growth factor receptors, proliferating, secreting lymphokines and, in the case of $CD8^+$ cells, destroying target cells (Klein 1990).

The diverse antigen specificities of T lymphocytes are defined by their cell surface antigen receptors (Meuer et al. 1984; Dembic et al. 1986). A clonal population of receptors is found expressed on the lymphocyte surface, and single lymphocytes have single antigen specificities. The antigen set is both large and unpredictable so, *a priori*, it would seem necessary to generate a highly diverse set of antigen specificities in each individual. Single genes encoding each of these receptor proteins would quickly exhaust the available germline complement of DNA. For both

B and T lymphocytes, multiple germline gene segments exist for different portions of the variable region, and these gene segments rearrange in a presumably random fashion to form variable region genes prior to transcription. Lymphocyte antigen receptor diversity, therefore, accrues from germline gene segment repertoire, random junctional variation, random combinatorial joining, and combinatorial (heterodimeric protein) association (Kronenberg 1986).

The generation of a diverse set of receptor specificities gives the individual the ability to recognize and clear pathogens. The spectrum of antigen specificities includes self-antigens, allowing the possibility of inappropriate self-reactivity. To limit this possibility, lymphocytes are selected for absence of self-reactivity through mechanisms of tolerance.

Lymphocyte Tolerance and Self-reactivity

A random mechanism for generating antigen specificity of lymphocyte cell surface receptors offers no means for lymphocyte distinction between self and non-self antigens. The mechanisms by which self-reactive lymphocytes are prevented from causing injury to normal self tissues are varied, but collectively referred to as mechanisms of "tolerance". Tolerance to self can result from deletion of self-reactive clones (von Boehmer 1990) and from induction of non-responsiveness in self-reactive clones (Schwartz

1990). Deletion can occur in primary lymphoid organs or in the periphery, and can occur with both immature and mature lymphocytes. T cell unresponsiveness due to anergy induction follows inadequate co-stimulation by the antigen-presenting cell, whereas unresponsiveness can also follow from TCR "desensitization" or clonal "exhaustion" (Lo et al. 1991). B cell unresponsiveness follows inadequate receptor occupancy and/or receptor-ligand affinity (Goodnow 1990). In summary, both primary and secondary lymphoid organs have mechanisms by which lymphocyte self-reactivity can be minimized.

In spite of varied mechanisms of tolerance, mature self-reactive lymphocytes can be found in normal individuals and lymphoctye-mediated autoimmune diseases are observed (Kuby 1992). The rarity of spontaneous autoimmunity reflects on the generally effective mechanisms of tolerance in normal animals. Possible explanations of self-reactivity assume an otherwise normal system of tolerance which is occasionally misled or "lifted" (Klein 1990). Lymphocytes might activate in response to antigens from which the maturing set of lymphocytes has been segregated ("antigenic sequestration") (Barker 1977). Lymphocytes might activate appropriately in response to non-self, but then cross-react with self determinants which mimic the non-self determinant ("molecular mimicry") (Klein 1990). Lymphocytes might fail to activate in response to physiologic concentrations of

antigen, but activate in response to supra-physiologic concentration of antigen which they encounter acutely (Gammon 1989). T lymphocytes might activate in the presence of inappropriate co-stimulation by antigen-presenting cells (Gimmi et al. 1991). These mechanisms are not due to flaws in the mechanisms of tolerance, but rather to untoward side effects.

Risk for T cell autoimmune disease stratifies by MHC allele for a variety of diseases in both mice (Fritz 1989) and humans (Klein 1990), and key residues in MHC ligand-binding sites can be identified (Todd et al. 1988). Risk for T cell autoimmune disease also stratifies by TCR loci RFLPs for a variety of diseases in humans (Posnett 1990). The prevention or resolution of autoimmune disease in experimental allergic encephalomyelitis (EAE) following neonatal thymectomy (Arnason et al. 1962), anti-class II MHC antibody (Ab) (Wraith et al. 1989a), anti-TCR Ab (Acha-Orbea 1989; Zaller 1990), anti-CD4 Ab (Waldor et al. 1985), and blocking peptide treatment (Wraith et al. 1989b) suggest that these epidemiologic MHC and TCR correlations imply causal roles for T cells in pathogenesis. One model is that self-peptide binding is necessary but not sufficient for T cell self-reactivity, that allelic substitutions within MHC ligand-binding sites limit the probabilities of self-peptides binding to MHC, and that pathogenic trimolecular (MHC/self-peptide/TCR) complexes are present in affected

Definition of the self-peptide epitopes which bind to a given MHC allele, definition of the TCR clonotypes which recognize these self-peptide/MHC complexes, and development of antibody and peptide reagents which interfere with the pathogenic trimolecular complex(es) are the obvious long-term therapeutic goals.

At a more basic level, however, the identity of the self-protein auto-antigen, the relationships between multiple T cell epitopes within the auto-antigen, the rules by which disease risk can be predicted from epitope-specific responses, the nature of "immunologically privileged" self antigen as auto-antigen, and the possibility that TCR V gene segment alleles could contribute to disease risk, need to be clarified. We have chosen to explore these questions for the demyelinating autoimmune diseases, experimental allergic encephalomyelitis (EAE) and human multiple sclerosis (MS). Similarities of pathologic and clinical features of these two diseases suggest that EAE represents a useful animal model for the human disease (Alvord 1987). Risk for both diseases stratifies by MHC (HLA) haplotype; H-2^u mice are highly susceptible to induction of EAE (Fritz 1989), and the HLA-DR2 allele is over-represented in caucasian humans with MS (Veneroni 1988). Activated T cells are found at sites of perivascular infiltration and demyelination in both diseases (Arnason et al. 1988; Mokhtarian 1984), and adoptive transfer of MBP-specific T_H cells in mice is sufficient for

disease induction in mice (Mokhtarian 1984). These data suggest that EAE and MS are T cell-mediated autoimmune diseases which target components of self central nervous system (CNS) myelin.

In the EAE model, the best-studied auto-antigen is MBP, and synthetic MBP peptides have allowed the identification of specific MBP epitopes which stimulate lymphocyte activation in different H-2 haplotypes (Kuby 1992). In strains where multiple epitopes exist, certain epitopes stimulate more lymphocyte activation following immunization with whole MBP than others, and have been called "major determinants" or "dominant epitopes". The basis for this "epitope dominance" has not been defined. Similarly, most mice get disease from these dominant-epitope specific T cells, although T cell responses to "subdominant" epitopes have been held responsible for a small percentage of affected animals in one study (Zaller 1990). For the dominant epitope response, the number of responder α/β TCR clonotypes appears to be limited in number (Acha-Orbea et al. 1988), raising the possibility that specific immunotherapeutic strategies can be designed.

Since therapeutic strategies for EAE must consider all potentially pathologic trimolecular complexes, it would be important to define the number and characteristics of T cell epitopes within MBP, to test the ability of epitope-specific T cell responses *in vitro* to predict risk for EAE and,

ultimately, to define all of the components of the trimolecular complexes which must be targeted. In addition, determination of the basis for the breakdown in tolerance may offer insight into the limitations on the normal mechanisms of tolerance. Chapter 1 of this thesis confirms the immunogenicity of self MBP in B10.PL mice, describes the immunogenic T cell epitopes of MBP, and defines their class II MHC presentation. Chapter 2 determines the effect of endogenous MBP expression in BALB/c ($H\text{-}2^d$) and B10.PL ($H\text{-}2^u$) mice on lymphocyte responses to self MBP immunization. In MS, risk for disease has been associated with certain TCR Vβ RFLP haplotypes (Beall 1989; Seboun 1989). The basis for this association is unknown, but could be analagous to that for the MHC locus, where particular alleles carry an intrinsically increased risk for disease. Chapter 3 describes a comparison of orthologous Vβ 8 gene segments between different primates, and between unrelated normal humans, to define the normal germline variation present in Vβ gene segments which are adjacent to an MS-associated TCR RFLP.

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T Cell Epitopes of Self Myelin Basic Protein in B10.PL Mice

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I. Summary

Experimental autoimmune encephalomyelitis (EAE') is a T cell-mediated autoimmune disease which serves as a good model for the demyelinating human autoimmune disease, multiple sclerosis. EAE can be induced in susceptible individuals by immunization with murine myelin basic protein (mMBP) or by adoptive transfer of activated MBP-specific T cells. Risk for development of EAE stratifies by H-2 haplotype, and B10.PL ($H\text{-}2^u$) mice are highly susceptible to induction of the disease. The basis for this H-2 association with EAE disease risk is unknown, but may be related to the ability of particular MHC alleles to bind processed MBP self-peptides. This study sought to define the T cell epitopes within MBP for B10.PL ($H\text{-}2^u$) mice. Following mMBP immunization, T cell proliferative responses could be recalled with the component mMBP peptides MBP(NAc1-20), MBP(31-50), and MBP(121-140). The bulk of the response following mMBP immunization was directed towards the MBP(NAc1-20) peptide. Using epitope-specific T cell hybridomas and class II transfected L cell lines, it was shown that MBP(NAc1-20) and MBP(121-140) are ($I\text{-}A^u$)-restricted, and that MBP(31-50) is ($I\text{-}E^u$)-restricted. This confirms the epitope status and class II restriction found for NAc(1-20) and (35-47) in another $H\text{-}2^u$ mouse strain, PL/J, and demonstrates a third epitope for these $H\text{-}2^u$ mice. Multiple T cell epitopes of varying dominance have been

found within self MBP for H-2^u mice, facilitating the study of their roles in normal tolerance and inducible autoimmunity.

[1] *Abbreviations used in this paper:* EAE, experimental autoimmune (allergic) encephalomyelitis; MBP, myelin basic protein; mMBP, murine MBP; MHC, major histocompatibility complex; TCR, T cell (lymphocyte) antigen receptor; CFA, complete Freund's adjuvant.

II. Introduction

The mammalian response to most pathogens relies on the specific immune response of lymphocytes, and this response hinges on the abilities of B and T lymphocytes to distinguish self from non-self (Goodnow 1990; von Boehmer 1990). This distinction is occasionally unclear, as mice and humans both develop diseases in which the targets of the specific immune response are self proteins (Klein 1990; Kuby 1992). Risk for some of these diseases stratifies by MHC allele (Klein 1990) or by TCR loci RFLPs (Posnett 1990), suggesting that particular MHC/TCR/self peptide complexes may play a role in the pathogenesis of these autoimmune diseases.

The molecular basis for autoimmune pathogenesis can best be studied in a defined model system. Experimental autoimmune encephalomyelitis (EAE) is considered a good model of the human demyelinating disease, multiple sclerosis (MS) (Alvord 1987; Zamvil 1990). In both cases, risk for disease stratifies by MHC (HLA) allele (Fritz 1989; Veneroni 1988), and both clinical and pathologic features are comparable (Mokhtarian 1984; Arnason 1988). In EAE, the components of the disease-associated trimolecular complex (MHC, TCR, and peptide) have been defined for a variety of H-2 haplotypes (Zamvil 1990). Strains which are susceptible to EAE (e.g., B10.PL ($H\text{-}2^u$)) (Fritz 1989) respond well to immunization with purified MBP, whereas those which are not

susceptible to EAE (e.g., BALB/c ($H\text{-}2^d$)) do not respond well to immunization with purified MBP. Overlapping MBP peptides can be used to restimulate T lymphocytes initially activated following mMBP immunization. These peptides estimate, and allow mapping of, the actual (unknown) T cell epitopes of MBP. The predominant *in vitro* T cell response is found to be directed towards the epitope within MBP(NAc1-20), and this response is used by the majority of affected animals (Acha-Orbea 1988; Zaller 1989). Adoptive transfer of EAE with dominant epitope-specific T cell clones (Zamvil 1985), and reversal of EAE by using monoclonal antibodies to deplete the animal of dominant epitope-specific T cells (Acha-Orbea 1988; Zaller 1990), suggests that these epitopes and their trimolecular complexes are in fact pathogenic.

Although the dominant epitope predicted the pathology in the majority of animals in one study (Zaller 1990), one animal in twenty remained ill, and this was attributed to a T cell response directed towards the subdominant epitope, MBP(121-140). The epitopes involved in pathogenesis may therefore include subdominant as well as dominant epitopes. It is clear that the number of epitopes and the nature of the T cell responses to them is important in devising therapies designed to delete or tolerize small portions of the peripheral T cell repertoire.

The goal of this study was to define the number, characteristics, and restriction elements of the T cell

epitopes of MBP recognized in the EAE-susceptible H-2^u mouse strain, B10.PL (H-2^u). Five peptides (MBP(NAc1-20), MBP(31-50), MBP(101-120), MBP(121-140), and MBP(131-150)) were individually immunogenic, three of these (MBP(NAc1-20), MBP(31-50), and MBP(121-140)) could recall a proliferative response in lymph node cells following self (murine) MBP immunization, and MBP(NAc1-20) predominated in the proliferative response. The two adjacent peptides, MBP(121-140) and MBP(131-150), shared a single epitope. The T cell epitopes of MBP, defined from responses following MBP immunization, were also found to be encephalitogenic. The dominant epitope response predicted the highest probability for disease induction. MBP(101-120) was incapable of recalling a proliferative response following mMBP immunization, and was also found to be non-encephalitogenic. MBP(NAc1-20) and MBP(121-140) were found to be presented by class II I-A^u, and MBP(31-50) was found to be presented by class II I-E^u. Given knowledge of the auto-antigen and its amino acid sequence, derived epitope maps for disease-susceptible MHC or HLA haplotypes may allow prediction, and possibly prevention, of the pathologic trimolecular complex interactions which characterize T cell-mediated autoimmune diseases.

III. Materials and Methods

Mice

B10.PL mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or derived from Jackson mating pairs at Caltech. Immunization and disease induction experiments utilized 6-10 week old animals.

Myelin Basic Protein (MBP)

Murine MBP was prepared from frozen mouse brains (Pel-Freeze Biologicals Co., Rogers, AR) according to a previously published protocol (Smith 1969).

Peptides

Peptides were synthesized by the stepwise solid-phase technique (Sluka 1990). The numbering scheme is based on the amino acid sequence of the 18.5 kD form of MBP, an isoform translated from a transcript of exons I, III, IV, V, VI, and VII of the MBP gene (deFerra 1985) (Table 1).

Mouse MBP Immunization Regimen

Murine MBP or MBP peptides were emulsified with an equal volume of complete Freund's adjuvant (CFA, Gibco Laboratories, Grand Island, NY) supplemented with 4 mg/ml M. tuberculosis H37 RA (Difco Laboratories, Detroit, MI). Mice were immunized with a total of 55 - 75 μg MBP, or 50 - 100

nmoles of MBP peptide, in equal 50 μl volumes SC in the hind footpads. For disease induction, mice were also given pertussigen 75-250 ng IV at 24 and 72 h after immunization.

EAE Disease Severity

Clinical EAE was graded on a 5 point scale: grade 1, flaccid tail; grade 2, hind limb weakness; grade 3, hind limb paralysis; grade 4, fore/hind limb paralysis; grade 5, death.

In vitro lymph node cell proliferation assay

Mice were sacrificed by cervical dislocation ten days after immunization. Inguinal and popliteal lymph nodes were sharply dissected and excised, and were suspended in Ventrex HL-1 serum free medium (Ventrex Laboratories, Portland, ME) or complete RPMI (for the (81-100) immunization experiment) after crush dissociation. Viable lymph node cells were then plated at 3 - 4 x 10^5/well in 200-250 microliter/well in Falcon 3077 96 well round bottom plates (Becton Dickinson & Co., Lincoln Park, NJ), and murine MBP (mMBP) (from the same preparation as that used in the immunization) or HPLC-purified synthetic peptides were added. Four days later, 1 uCi [methyl-^{3}H] thymidine was added to each well and cells were collected 16-24 hours later on Whatman 934-AH glass fiber filters (Whatman Co., Maidstone, England) using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA).

Counts per minute (CPM) from triplicate wells were determined by liquid scintillation counting, and calculated disintegrations per minute (DPM) were used for analysis where noted.

Class II MHC transfectants

The I-A^u L cell transfectant, 6A2, was a generous gift from Dr. Pat Jones. I-A^u expression is HAT-selected. The I-E^u L cell transfectant, 4D, was a generous gift from Dr. Ginnie Appel. I-E^u expression is G418-selected. Cells were grown in complete αMEM (FCS (10%), glutamine (2 mM), and penicillin (100 U/mL)/streptomycin (100μg/mL), cαMEM with 1x HAT (50X = hypoxanthine 5 x 10^{-3} M, aminopterin 2 x 10^{-5} M, and thymidine 8 x 10^{-4} M), or in cαMEM with G418 (400μg/mL).

MHC-specific monoclonal antibodies

MoAb Y-3P (ATCC HB 183) binds to H-2 I-A^u molecules. MoAb 14-4-4S (ATCC HB 32) binds to H-2 I-E^u molecules. MoAb 30-5-7 binds to H-2^d class I molecules. MoAb 11-4.1 (ATCC TIB 95) binds to H-2 K^k molecules.

FACS Analysis

The above antibodies were used as cell culture supernatants to stain ca. 1 x 10^6 cells/sample, on ice, for 30-45 minutes. Free antibody was washed off, and FITC-GAM secondary Ab was used to detect bound primary Ab.

IV. Results

Immunogenicity of murine myelin basic protein (mMBP)

To rule out the possibility that the observed responses to mMBP and/or mMBP peptides were due to cross-reactivity with adjuvant components, B10.PL mice immunized with Freund's adjuvant (ICFA), Freund's adjuvant with M. tuberculosis (CFA), or murine MBP in Freund's adjuvant with M. tuberculosis (mMBP/CFA), were compared. As shown in Figure 1, mice immunized with ICFA failed to respond to restimulation with mMBP, mMBP peptides, or PPD ("Mtb", tuberculin). Mice immunized with CFA responded to restimulation with PPD, but did not respond to restimulation with mMBP or mMBP peptides. Mice immunized with mMBP/CFA, however, responded to restimulation with mMBP, mMBP peptides, and PPD, demonstrating that there is no false positivity from cross-reactivity associated with the observed responses to peptide stimulation *in vitro*.

Immunogenic peptides in B10.PL mice

To determine the T cell proliferative response to all of the MBP peptides, two sets of peptides, each representing one half of the total MBP length, were separately pooled and used to immunize 3 - 4 naive B10.PL mice. As shown in Figure 2, significant (stimulation index > 3) activation of B10.PL lymph node cells resulted from re-stimulation with

peptides NAc(1-20), (31-50), (101-120), (121-140), and (131-150). These results do not rule out the possibility that some peptides failed to stimulate T cells because of competition for presentation.

Response to Murine Myelin Basic Protein (mMBP) in B10.PL Mice

To determine the response of B10.PL mice to the intact self protein, mMBP, purified MBP emulsified in CFA/M.Tb. was used to immunize 28 naive B10.PL mice. Lymph node cells from these animals were compared for responsiveness to all of the individual mMBP peptides (n=16), mMBP (n=26), NAc(1-20) (n=28), and (31-50) (n=28). Figure 3 illustrates the fraction of mice in the sample able to respond above background ($P<0.05$) and ($P<0.01$) to peptide stimulation in vitro following mMBP immunization, and shows that B10.PL mice can respond to 4 - 5 different epitopes within the 18.5 kD mMBP molecule. Averaged *in vitro* lymph node proliferation (incorporated ^{3}H-TdR DPM) is given in Table 2 for the individual mice studied. Averages of the net DPM responses to individual mMBP peptides are illustrated in Figure 4, and demonstrate that B10.PL mice respond predominately to MBP(NAc1-20), with significant ($P<0.01$) responses to MBP(31-50) and MBP(121-140). The immunogenicity of peptide MBP(101-120) shown in Figure 2 contrasts with the absence of response to (101-120) following mMBP immunization, and suggests that this epitope is cryptic in vivo, i.e., not

presented by antigen-presenting cells to peripheral T cells.

H-2^u presentation of MBP(NAc1-20)

To define the response to restimulation with MBP(NAc1-20) as being T cell-mediated, B10.PL (H-2^u) and B10.T(6R) (H-2^q) mice were compared for responsiveness to mMBP immunization. These mice are near-isogenic save for the H-2 locus, so their antigen processing and germline TCR/Ig repertoires should be identical. As shown in Figure 5, B10.T(6R) mouse lymph node cells fail to be restimulated by MBP(NAc1-20), suggesting the observed proliferative response is a function of the ability of H-2^u MHC molecules to present MBP(NAc1-20).

Peptides (121-140) and (131-150) contain a single T cell epitope

To clarify the apparent responsiveness of B10.PL mice to the two adjacent peptides, MBP(121-140) and MBP(131-150), mice were immunized with each peptide and restimulated in vitro with both peptides individually. As shown in Table 3, T cells specific for MBP(121-140) or MBP(131-150) cross-react with the adjacent peptide, suggesting that a single T cell epitope is shared by these two peptides.

Incidence of EAE after Peptide Immunization

The incidence, time of onset, and severity of clinical EAE following immunization with individual mMBP peptides in

adjuvants are shown in Table 4. B10.PL mice routinely got (severe) EAE after MBP(NAc1-20) immunization, occasionally got (less severe) EAE after MBP(31-50) and MBP(121-140) immunization, and failed to get EAE after MBP(101-120) immunization. These outcomes parallel the observations made on *in vitro* restimulation with these peptides following mMBP immunization, and suggests that self MBP processing and presentation of an epitope mimicked by the peptide is required for the peptide to be an effective encephalitogen.

Class II Restriction

The MHC class II (-) L cell line, L929, the class II I-A^u L929 transfectant "6A2", and the class II I-E^u L929 transfectant "4D", were used as antigen-presenting cells for determination of the class II restricting elements for the peptides NAc(1-20), (31-50), and (121-140). Figure 6 shows that L929 is class II (-), that 6A2 expresses I-A^u (detected with the MoAb Y3P), and that 4D expresses I-E^u (detected with the MoAb 14-4-4). Figure 7 shows that MBP(NAc1-20) is presented by 6A2 (I-A^u), that MBP(31-50) is presented by 4D (I-E^u), and that MBP(121-140) is presented by 6A2 (I-A^u).

V. Discussion

MBP as an immunogenic protein.

The T cell autoimmune response has been attributed to cross-reactivity with microbial pathogens in both EAE (Fujinami 1985) and experimental autoimmune uveitis (EAU) (Sing 1989). Data in this paper shows that the observed response to MBP is not due to a cross-reactivity with components of Freund's adjuvant or M. tuberculosis. This suggests that T cells are not tolerized to MBP, rather than being (inappropriately) cross-reactive to MBP following a xenoprotein response. B10.PL mice are therefore not tolerized to MBP, and the basis for this (e.g., antigenic sequestration, epitope-specific tolerance, incomplete tolerance, dose-specific tolerance) is unknown.

MBP as a multiple-epitope immunogenic protein.

Synthetic peptides were used in this report to document the number and characteristics of the epitopes within the self-protein, MBP. MBP(101-120) is immunogenic, but fails to recall a response following mMBP immunization and fails to trigger EAE. This implies that MBP(101-120) binds to self MHC class II molecules, that MBP(101-120)-specific T cells exist, and that these T cells can expand in response to synthetic peptide on H-2^u APCs. They do not expand, however, in response to processed and presented MBP, and

this suggests that the peptide epitope estimated by MBP(101-120) is altered during MBP processing by APCs, or fails to compete successfully for presentation by surface MHC class II molecules.

MBP(NAc1-20), MBP(31-50) and MBP(121-140) are immunogenic peptides, recall proliferative responses following mMBP immunization, and trigger EAE. This implies that they bind to self MHC molecules, that they are a good estimate of the actual peptide epitopes resulting from processing of MBP by APCs, and that T cells exist which can expand in response to APCs presenting these peptides.

In B10.PL mice, T cells can recognize five MBP-derived peptides, APC processing provides peptides similar to three of these, and one of these three (MBP(NAc1-20)) dominates as a target for the response to mMBP immunization.

Class II MHC presentation of mMBP epitopes.

Our data suggest that the epitope estimated by MBP(NAc1-20) is presented to T cells by the I-A^u class II MHC molecule, and that the epitope estimated by MBP(31-50) is presented to T cells by the I-E^u class II MHC molecule. These observations are consistent with the class II restriction element data on MBP epitopes in PL/J, another H-2^u strain (Zamvil 1990). In addition, we have determined that MBP(121-140) is presented to T cells by the I-A^u class II MHC molecule. Since MBP(NAc1-20) and MBP(121-140) are

both presented by I-A^u, this may offer a model to study the factors involved in T cell activation and tolerance to epitopes of a well-defined self protein.

Basis for subdominance of epitopes.

The basis for the reduced proliferative responses to some synthetic MBP peptides following mMBP immunization is unknown. We have shown that the immunogenicity of MBP, rather than that of other antigens present in the adjuvants, is responsible for the proliferative responses recalled by synthetic peptides following mMBP immunization. We have shown that pooled peptide immunization results in comparable proliferative responses upon in vitro restimulation with individual peptides within the pool (Table 2). This suggests that competitive inhibition by MBP(NAc1-20) does not account for the subdominant response to MBP(31-50) following mMBP immunization, and that competitive inhibition by MBP(121-140) does not account for the absent response to MBP(101-120) following mMBP immunization. Additional experiments (data not shown) have failed to demonstrate competitive inhibition by MBP(NAc1-20) for presentation of MBP(121-140) by I-A^u, have failed to demonstrate cross-reactivity between epitopes MBP(NAc1-20), MBP(31-50), and MBP(121-140), and have failed to demonstrate substantially increased responses for MBP(31-50) by varying the length and frame of the peptide. These data suggest that subdominance

is due not to the absence of specific T cells, but rather to the inability of APCs to generate adequate densities of surface (class II MHC - peptide) complexes. The subdominance of MBP(31-50) is particularly prominent, given its immunogenicity as a peptide, suggesting abnormalities of MBP processing.

Risk for EAE is correlated with epitope dominance

The prevalence and severity of EAE following immunization with peptides in adjuvants is roughly correlated with the proportion of the proliferative response attributed to each peptide following mMBP immunization, i.e., most mice get EAE after MBP(NAc1-20) immunization, and fewer get EAE after immunization with MBP(31-50) or MBP(121-140) (Table 5). The proliferative response appears to predict the risk for disease, but subdominant (lower-level) responses do not rule out risk for disease. No disease resulted following immunization with MBP(101-120), the peptide which was immunogenic but not presented following immunization with intact mMBP. This is consistent with a model in which presentation of processed self MBP peptide is necessary, but not sufficient, for disease induction and progression. This emphasizes the need for determination of the antigen and its component epitopes in the study of autoimmune diseases.

VI. References

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TABLE LEGENDS

Table 1:

Text references to these peptides are given as "MBP(*peptide*)". "NAc1-20" refers to the N-acetylated derivative of the amino-terminal 20-mer. This numbering system does not take the exon 2-encoded amino acid residues (n=26) into account. Peptide (104-113)/(155-164) ("E6(-)) is an (exon 5, exon 7)-encoded fusion peptide made to mimic the 14 kD MBP isoform (E6(-)) protein sequence.

Table 2:

B10.PL ($H\text{-}2^u$) mice were immunized with 75-200 μg mMBP in CFA. Lymph nodes were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with murine MBP for 5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Data points reflect the mean of triplicate samples, and are given as mean DPMs (^{3}H-TdR) for each peptide (30. μM) or (-) antigen control. Three of these animals were tested at both 3. and 30. μM [peptide]; for these mice, the larger of the two proliferative responses are listed. The Wilcoxson signed rank test and Student's paired T test were used to determine statistically significant differences of peptide-stimulated samples from paired background control samples, as depicted in Figure 4.

Table 3:

B10.PL response to immunization with MBP(121-140)/CFA or MBP(131-150)/CFA. Lymph node cell *in vitro proliferation* assays were performed as described in Table 2. Proliferation in response to 30. μM peptide (immunogen or adjacent overlapping peptide) was estimated by net DPM (^{3}H-TdR) above (-) antigen (medium only) background control.

Table 4:

B10.PL mice were immunized with 50-100 nmoles SC of peptide in CFA. Pertussigen 75-400 ng IV was given 24 and 72 hours after immunization. Incidence indicates the proportion of mice which developed detectable signs of EAE. Onset indicates the average day of first signs of EAE, with the associated sample range. Severity indicates the average maximal grade of disease observed in each sample.

Table 1. Overlapping Peptides from the 18.5 kD Form of Murine Myelin Basic Protein

Peptide Name	Peptide Sequence	Coded by Exon
NAc1-20	ASQKRPSQRSKYLATASTMD	I
11-34	KYLATASTMDHARHGFLPRHRDTG	I
22-40	ARHGFLPRHRDTGILDSIG	I
31-50	RDTGILDSIGRFFSGDRGAP	I
41-58	RFFSGDRGAPKRGSGKDS	I, III
49-70	APKRGSGKDSHTRTTHYGSLPQ	I, III
61-80	RTTHYGSLPQKSQHGRTQDE	III
68-91	LPQKSQHGRTQDENPVVHFFKNIV	III, IV
81-100	NPVVHFFKNIVTPRTPPPSQ	III, IV
87-114	FKNIVTPRTPPPSQGKGRGLSLSRFSWG	III, IV, V
101-120	GKGRGLSLSRFSWGAEGQKP	IV, V, VI
111-130	FSWGAEGQKPGFGYGGRASD	V, VI
121-140	GFGYGGRASDYKSAHKGFKG	VI
131-150	YKSAHKGFKGAYDAQGTLSK	VI
141-160	AYDAQGTLSKIFKLGGRDSR	VI, VII
151-168	IFKLGGRDSRSGSPMARR	VI, VII
104-113/ 155-164	RGLSLSRFSWGGRDSRSGSP	V, VII

Table 2: B10.PL ($H\text{-}2^{u}$) Lymph Node Cell Proliferative Responses Following mMBP Immunization

Animal #	(-) Ag	mMBP	NAc(1-20)	(11-34)	(22-40)	(31-50)	(41-58)	(49-70)	(61-80)	(68-91)
1	6651	NT	26495	10093	9388	9741	7035	11180	8375	9014
2	9451	NT	29832	29832	29832	29832	29832	29832	29832	29832
3	11047	39933	69073	8077	8817	21468	5395	4096	6974	9515
4	6035	26171	34699	18771	6820	14357	5594	4839	4077	7920
5	7951	36524	63586	10355	11203	14151	5984	21369	14782	17859
6	4535	23268	43062	4337	4378	14762	4780	6225	2692	10522
7	3805	13920	11646	3926	3553	3618	3783	2470	3534	11062
8	4835	22631	23049	6341	6706	4809	6271	4573	6277	11358
9	3849	12736	17292	5113	4784	3278	5495	3834	3655	17108
10	11160	59907	56036	10379	13851	13213	14841	12358	13432	36341
11	4913	36890	71892	7514	4455	4453	7950	6896	6132	6921
12	10844	54661	108910	12125	12953	10519	29901	11099	10871	13706
13	5494	55128	94815	5044	6286	6806	10442	4560	7827	8859
14	3404	NT	41443	1123	785	1055	1175	887	767	700
15	3191	NT	51147	7384	2857	2823	4474	3421	2682	3806
16	12188	NT	69743	16297	18070	14165	6690	7603	11230	9193
20	2876	10420	17964			6357				
21	1760	3252	8523			3214				
22	1999	8433	12514			4236				
23	3043	14971	57900			5113				
24	2085	4286	36196			4752				
25	1227	1212	5603			2833				
26	1563	1348	28895			5878				
27	1733	1649	17610			5222				
28	2319	15699	93898			19842				
29	1480	2198	45861			4912				
30	720	1256	4354			811				
31	2475	1831	26668			7238				

Animal #	(-) Ag	(81-100)	(87-114)	(101-120)	(111-130)	(121-140)	(131-150)	(141-160)	(151-168)	E6(-)
1	6651	16004	6640	13897	8529	11853	11716	12234	12825	8516
2	9451	29832	29832	29832	29832	29832	29832	29832	29832	11182
3	11047	9813	2332	8967	6342	14375	7047	3791	7505	9087
4	6035	8636	2507	11148	5137	15885	7841	4438	9448	5117
5	7951	20506	11885	10213	7135	20879	13070	8158	12680	11028
6	4535	7729	2980	13339	7667	8265	6786	3113	7398	9715
7	3805	3849	1697	2489	3674	4087	5806	3435	1937	2865
8	4835	5041	4296	5172	4686	6712	14311	12585	13829	10334
9	3849	3947	4126	5261	6096	4732	14291	17158	14126	10119
10	11160	12461	10085	12701	12722	12119	13917	11648	12674	11680
11	4913	6863	3756	5154	3939	14442	9857	6611	8228	13105
12	10844	11924	10360	14813	9482	32084	17024	8590	18686	16327
13	5494	8579	4822	5111	6734	15313	9200	6717	7694	6780
14	3404	887	NT	NT	NT	NT	NT	NT	NT	NT
15	3191	4509	NT	NT	NT	NT	NT	NT	NT	NT
16	5909	11602	NT	NT	NT	NT	NT	NT	NT	NT
17	2652	4172	3382	7006	3519	2961	4949	3992	6282	NT
18	2308	1378	1003	2493	1517	2615	11480	1800	3915	NT
19	1772	2491	1737	1803	1117	2896	5801	1680	5108	NT
20	2876			2068						
21	1760			2129						
22	1999			2134						
23	3043			1457						
24	2085			7431						
25	1227			8723						
26	1563			2020						
27	1733			988						
28	2319			4486						

Table 3: Cross-reactivity between MBP(121-140) and MBP(131-150)

Immunogen	Animal	(-) Ag	MBP(121-140)	MBP(131-150)
MBP(121-140)	1	1496	50712	19490
	2	1366	33115	8235
	3	1958	11377	6880
MBP(131-150)	1	1891	41235	37803
	2	2018	42685	37702
	3	2393	20188	24298

Table 4: EAE Induction in B10.PL Mice with Synthetic Peptides

Immunogen	Incidence	Onset (Range)	Severity
MBP(NAc1-20)	5/5	11 (10-12)	3.4
MBP(31-50)	15/27	13 (7-25)	1.0
MBP(101-120)	0/11	--	--
MBP(121-140)	2/7	11 (11-12)	2.5
MBP(131-150)	2/9	13 (12-14)	3.0

FIGURE LEGENDS

Figure 1.

Responses of B10.PL mice to immunization with incomplete Freund's adjuvant, complete Freund's adjuvant with M. tuberculosis (CFA), or murine MBP (75. μg) in CFA. Mice were immunized SC in the hind footpads with 100.μL of each emulsion. Lymph nodes were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with murine MBP, individual overlapping MBP peptides (30 μM each), or PPD for for 5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Data points reflect the mean of triplicate samples, and error bars reflect the standard error of the difference of the means.

Figure 2.

Response of B10.PL mice to immunization with pools of synthetic peptide. Six mice were immunized with a pool of 8 or 9 peptides containing 20. nmoles of each peptide, components of the two pools shown in the abscissas of the upper and lower histograms. *In vitro* proliferative responses upon restimulation of lymph node cells (see Figure 1 legend) with each of the peptides (30. μM) individually are given as net DPM (^{3}H-TdR).

Figure 3.

Proportion of B10.PL mice which demonstrate statistically significant proliferation to mMBP or mMBP peptide stimulation following mMBP/CFA immunization. Proliferative responses to stimulation with each MBP peptide for each mouse listed in Table 2 were compared statistically (unpaired Student's T test) to (-) antigen background control samples. Increased proliferation was noted by significance level, and proportion of mice responding to each peptide at $P<0.05$ and $P<0.01$ significance were tabulated.

Figure 4.

Response of B10.PL mice to immunization with mMBP/CFA. The proliferative responses listed in Table 2 were compared (using both the paired Student's T test and the Wilcoxson signed rank test) to determine statistically significant difference from background for mMBP and each mMBP peptide. The mean and standard error of the mean are shown, and peptides which re-stimulate significantly above background are shown for $P<0.05$ and $P<0.01$ significance levels.

Figure 5.

Response of B10.PL and B10.T(6R) mice to immunization with mMBP/CFA. Mice immunized with 75. μg mMBP/CFA and assayed as in the Figure 1 legend. This antigen dose-

proliferative response curve indicates the mean and standard error of the difference of the means for triplicate samples, and each curve represents the response of one mouse.

Figure 6.

L cell transfectant expression of I-A^u or I-E^u. L929 (class II (-)), 6A2 (I-A^u (+)), and 4D (I-E^u (+)) were stained on ice with cell supernatant MoAbs 30-5-7 (irrelevant control), 11-4-1 (class I (+) control), Y3P (I-A^u-specific), or 14-4-4 (I-E^u-specific). Antibody binding was detected with FITC-conjugated goat anti-mouse secondary antibody, and fluorescence per cell is indicated on a linear scale.

Figure 7.

L cell transfectant presentation of MBP peptides to MBP-specific T cell hybridomas. L cell transfectants (10^5/well) 6A2 (I-A^u (+)) or 4D (I-E^u (+)) were mixed with T hybridomas (10^5/well) specific for MBP(NAc1-20) (172.10), MBP(31-50) (122.13), or MBP(121-140) (B27.1), and the net IL2 release in response to increasing concentrations of cognate ligand was measured. Bar height indicates the mean for triplicate samples, and error bars indicate the standard error of the difference of the means.

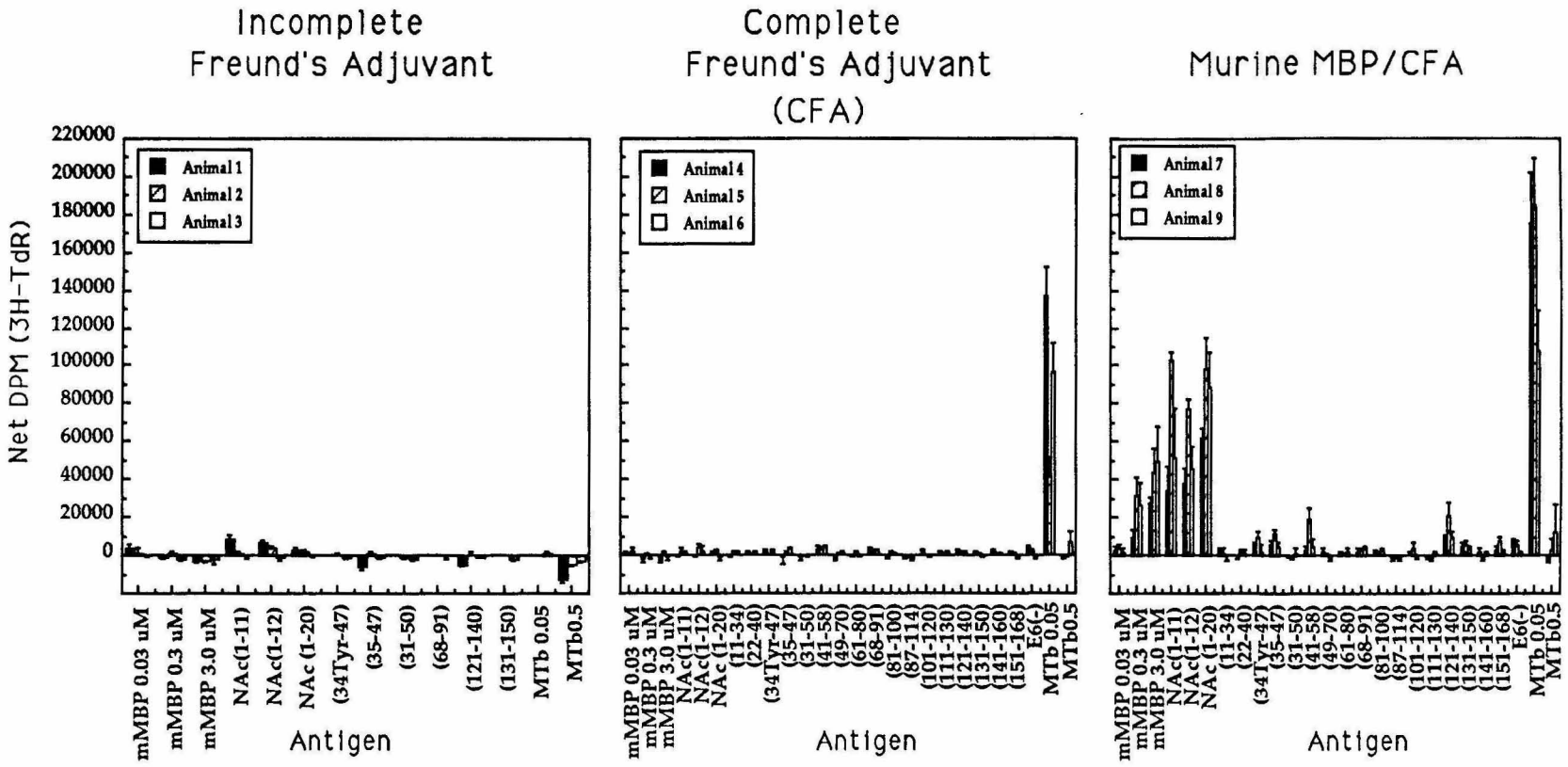
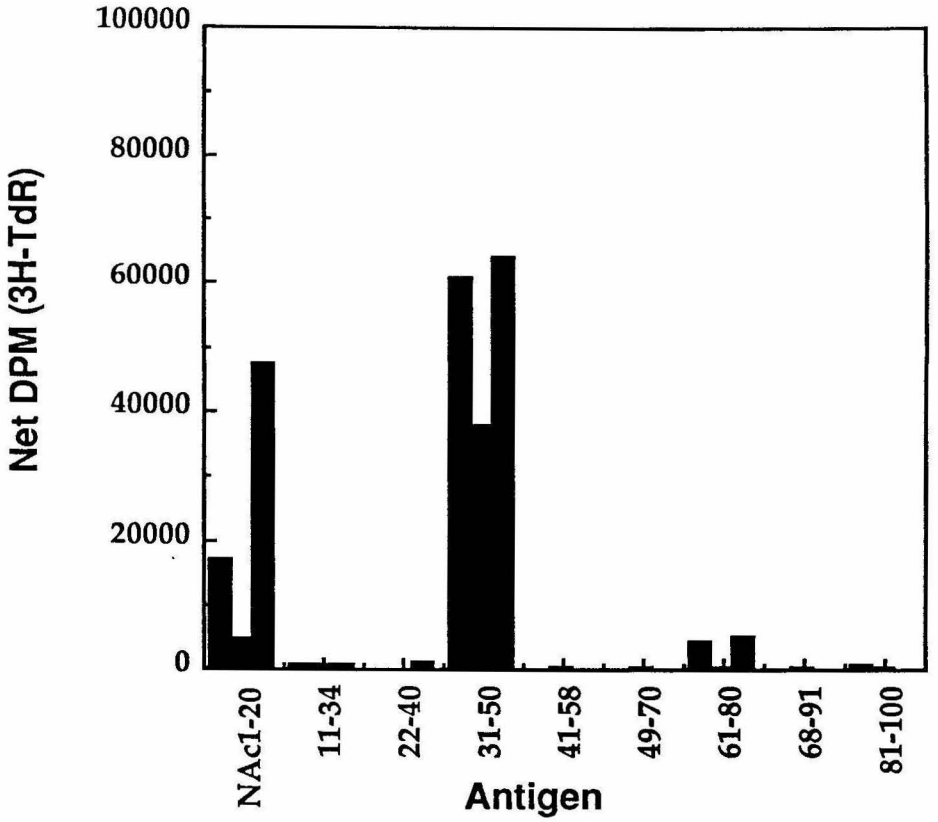

Incomplete
Freund's Adjuvant
Complete
Freund's Adjuvant
(CFA)
Murine MBP/CFA
Net DPM (3H-TdR)
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Animal 1
Animal 2
Animal 3
Animal 4
Animal 5
Animal 6
Animal 7
Animal 8
Animal 9
mMBP 0.03 uM
mMBP 0.3 uM
mMBP 3.0 uM
NAc(1-11)
NAc(1-12)
NAc (1-20)
(34Tyr-47)
(35-47)
(31-50)
(68-91)
(121-140)
(131-150)
MTb 0.05
MTb0.5
(11-34)
(22-40)
(41-58)
(49-70)
(61-80)
(81-100)
(87-114)
(101-120)
(111-130)
(121-140)
(131-150)
(141-160)
(151-168)
E6(-)
Antigen

Figure 1


Net DPM (3H-TdR)
100000
80000
60000
40000
20000
0
NAc1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
Antigen

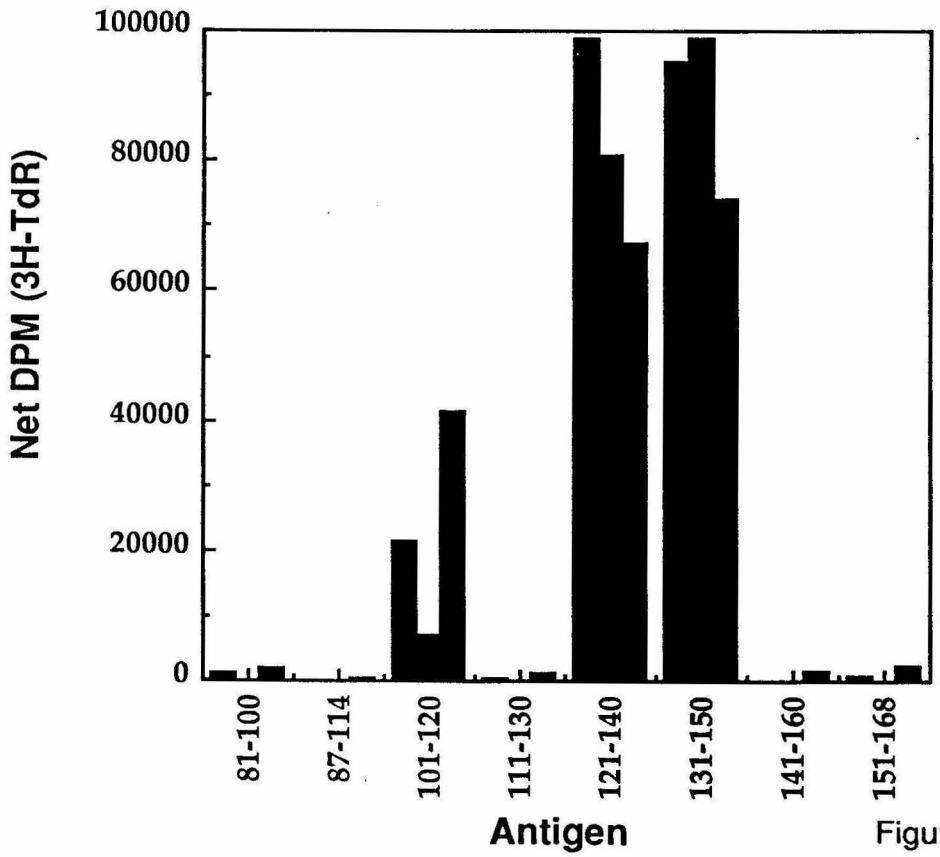

Net DPM (3H-TdR)
100000
80000
60000
40000
20000
0
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
Antigen

Figure 2

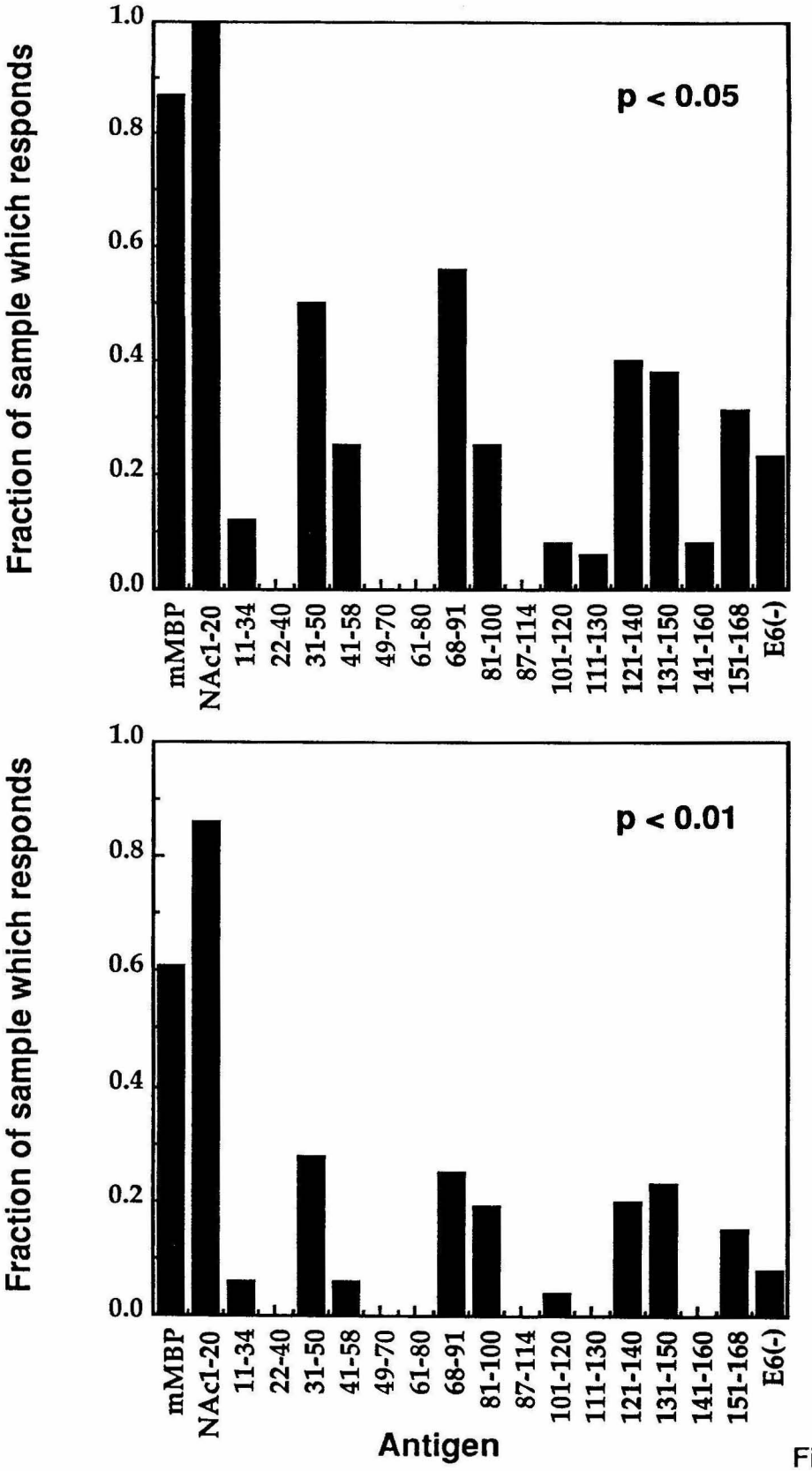

Fraction of sample which responds
1.0
0.8
0.6
0.4
0.2
0.0
p < 0.05
mMBP
NAc1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Fraction of sample which responds
1.0
0.8
0.6
0.4
0.2
0.0
p < 0.01
mMBP
NAc1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Antigen

Figure 3

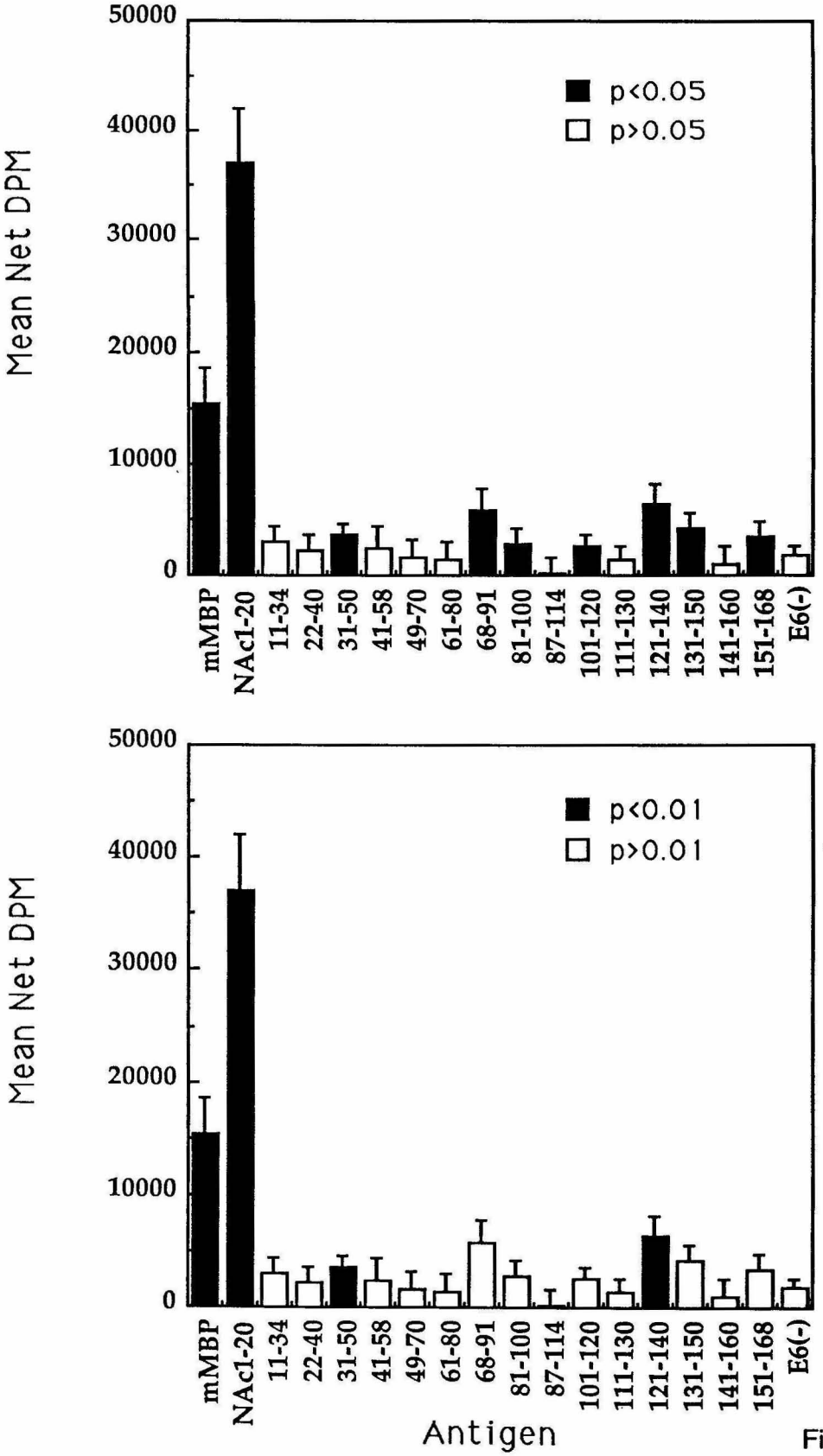

Mean Net DPM
50000
40000
30000
20000
10000
0
p<0.05
p>0.05
mMBP
NAc1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Mean Net DPM
50000
40000
30000
20000
10000
0
p<0.01
p>0.01
mMBP
NAc1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Antigen

Figure 4

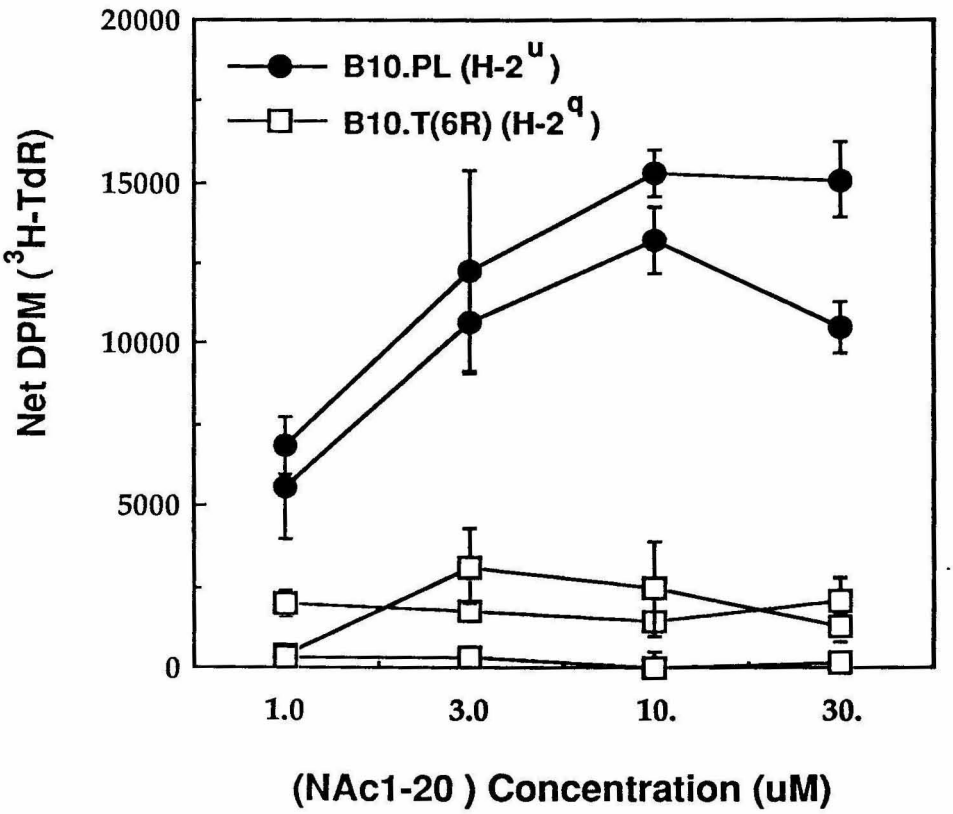

20000
15000
10000
5000
0
B10.PL (H-2u)
B10.T(6R) (H-2q)
Net DPM (3H-TdR)
1.0
3.0
10.
30.
(NAc1-20) Concentration (uM)

Figure 5

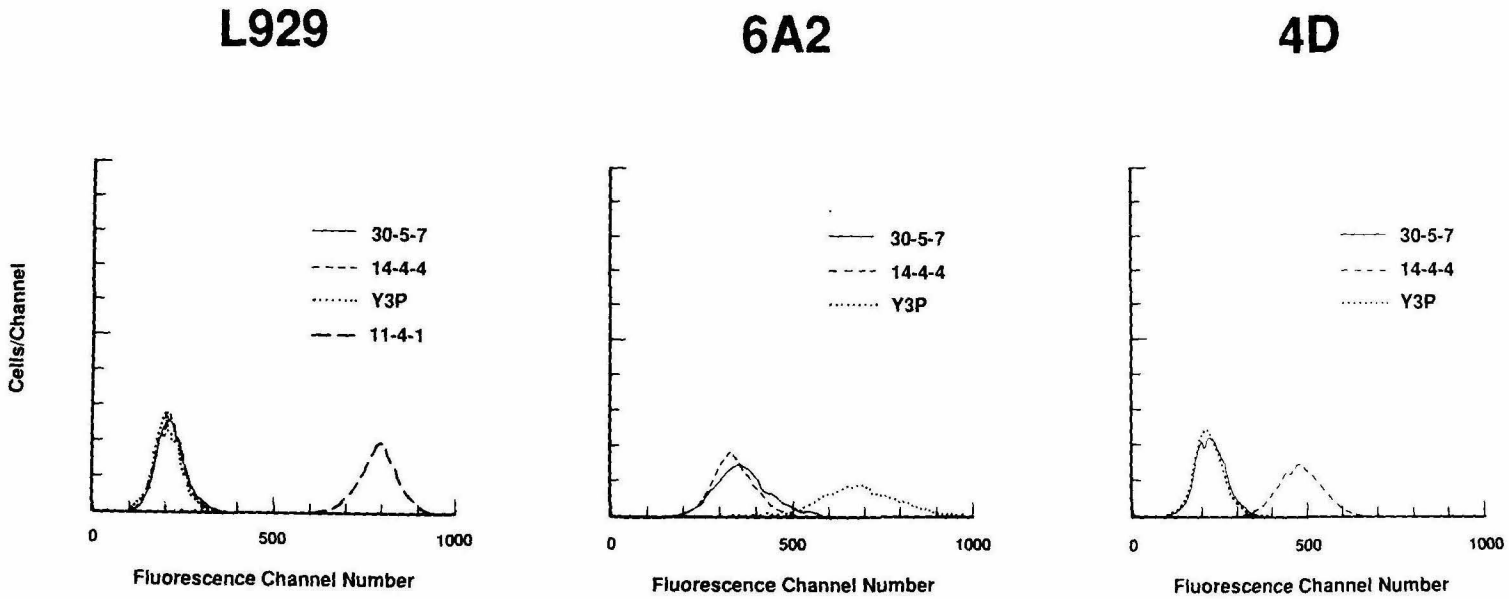

L929
6A2
4D
Cells/Channel
30-5-7
14-4-4
Y3P
11-4-1
0
500
1000
Fluorescence Channel Number

Figure 6

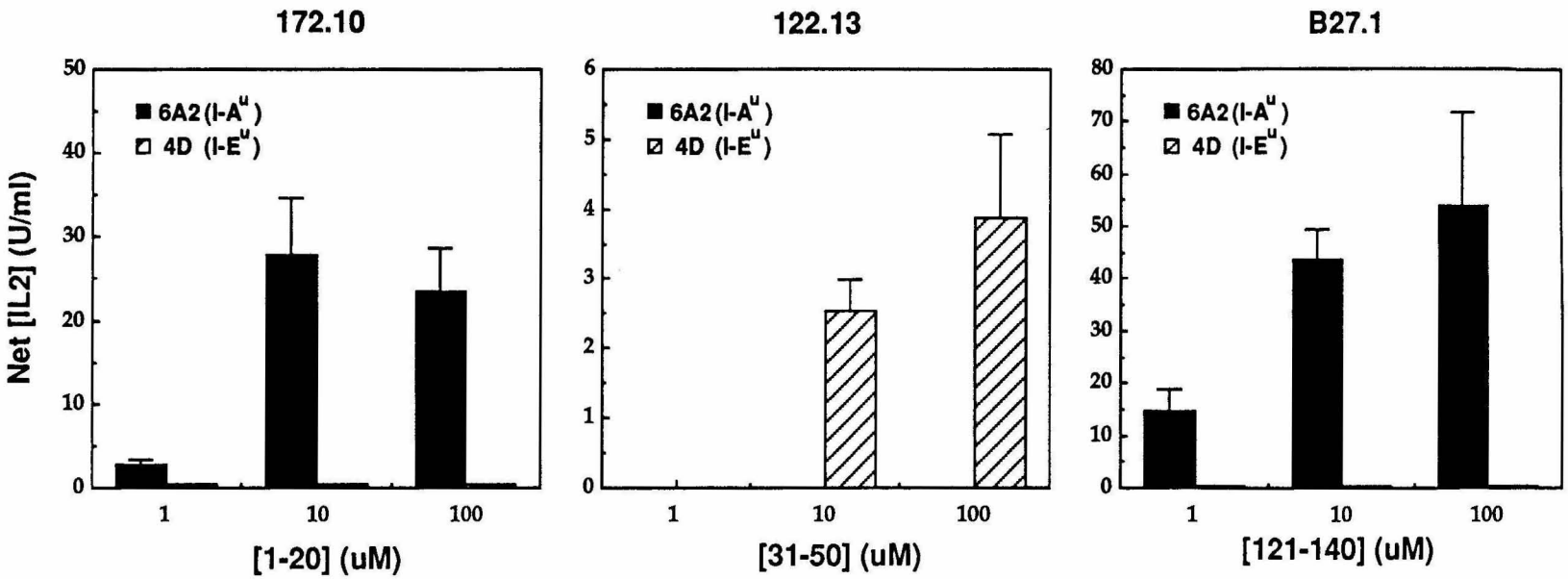

172.10
122.13
B27.1
Net [IL2] (U/ml)
6A2 (I-A^u)
4D (I-E^u)
[1-20] (uM)
[31-50] (uM)
[121-140] (uM)
1
10
100

Figure 7

The Immune Response to Myelin Basic Protein is Elevated in Myelin Basic Protein-Deficient Shiverer Mice

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I. Summary

The immune system is generally tolerant to self proteins. The breakdown of self-tolerance can lead to autoimmune diseases such as multiple sclerosis, insulin-dependent diabetes mellitus, and rheumatoid arthritis. Experimental allergic encephalomyelitis (EAE)[1] is considered a good animal model for the human demyelinating disease, multiple sclerosis. EAE can be induced by immunization with myelin basic protein (MBP) in adjuvants and by adoptive transfer of MBP-specific CD4+ T lymphocytes. Inbred strains of mice with different H-2 haplotypes vary in their responsiveness to immunization with MBP and in their susceptibilities to EAE. For example, H-2^d strains are tolerant to MBP, but H-2^u strains develop EAE upon immunization with MBP. The basis for the correlation of MHC haplotype with risk for EAE is unknown, but may be related to the ability of MBP-derived peptides to bind to specific MHC class II molecules. In this study, mouse MBP-specific lymph node proliferative responses of normal and MBP-deficient "shiverer" mice were compared. H-2^d shiverer mice were hyper-responsive to both intact MBP and peptide MBP(81-100) compared to non-shiverer littermates. H-2^u shiverer mice were hyper-responsive to both intact MBP and peptide MBP(121-140) compared to non-shiverer littermates. We conclude that MBP is not a sequestered self antigen, since its expression induces tolerance to specific T cell epitopes in wildtype H-2^d and

H-2^u mice. H-2^u wildtype and shiverer littermates were equally responsive to peptides MBP(NAc1-20) and MBP(31-50). Thus, tolerance to MBP in H-2^u mice appears to be epitope-specific. Residual lymph node cell responses to MBP(81-100) in normal H-2^d mice, and to MBP(121-140) in normal H-2^u mice, indicate that this tolerance is incomplete. Incomplete tolerance to peripheral self antigens may account for the presence of autoreactive cells in adult mice.

[1] *Abbreviations used in this paper:* EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; mMBP, murine MBP; OLA, oligonucleotide ligation assay; HEL, hen egg lysozyme; NAc-, N-acetyl.

II. Introduction

T cells can rearrange their TCR α/β loci in a highly diverse fashion (1,2), generating a set of T cell specificities which includes reactivities to self proteins (3). In adult mice, however, spontaneous T cell self-reactivity is unusual (4), so mice are generally self-tolerant. Tolerance of mature T cells to self antigens may result from one of several mechanisms, including thymic deletion of self-reactive immature T cells (5,6), peripheral inactivation (anergy) of self-reactive mature T cells (7,8,9), peripheral deletion of self-reactive mature T cells (10), and conceivably from active suppression of self-reactive mature T cells by CD8(+) regulatory cells (11).

T cell tolerance to self antigens occasionally fails, as evidenced by a variety of human (12) and murine (13) autoimmune diseases. A number of mechanisms for this breakdown in tolerance have been postulated. T cells might come into contact with sequestered self antigens to which they have never become tolerant (14). T cells might activate in response to a foreign antigen and then cross-react with a self antigen which mimics the nonself T cell epitope (15). T cells which are tolerant to low levels of antigen might activate in response to higher levels (16). Finally, inappropriate costimulation by antigen-presenting cells (APCs) might lead to inappropriate T cell activation, rather than anergy, upon antigen contact (17,18).

Mature T cells recognize a ligand comprised of short peptides bound to self MHC surface proteins. Class I MHC molecules present 8-9 residue peptides (19) processed from cytoplasmic proteins (20). Class II MHC molecules present 13-17 residue peptides (21) processed from endocytosed proteins (22). Self proteins are apparently processed and presented by APCs in the same ways as non-self proteins (23). A processed self peptide derived from hemoglobin can be found on the surface of antigen-presenting cells (APCs) of most tissues (24), suggesting that T cell self-tolerance is due to an absence of T cell responsiveness rather than to self/non-self discrimination at the level of APC function.

A good model for studying the cellular and molecular processes of self-tolerance would be one in which the components of the TCR/MHC/self-peptide trimolecular complex have been well-defined, in which T cells recognize multiple epitopes within the same self protein, and in which the level of the tolerizing self protein can be varied. The T cell response to myelin basic protein (MBP) represents such a model. The role of MBP as a target self antigen in the T cell-mediated autoimmune disease, EAE, is well-documented (25). Proteolytic processing of MBP is required for T cell activation (26), and multiple epitopes are recognized by some strains of mice (15). MBP expression is limited to the nervous system, comprising 30-40% of CNS myelin and 5-15% of PNS myelin (27). Expression of the *Mbp* gene varies

naturally due to two alleles, wildtype (+) and shiverer *(shi)*(27, 28,29). The wildtype allele of *Mbp* (*Mbp*$^{+}$) has seven exons (30, 31), of which the last five exons are deleted in the *shi* allele (*mbp*^{shi}) (32, 33). Although the MBP gene is largely deleted in the *shi* allele, the remaining exons 1 and 2 are transcribed (34). However, they do not appear to be translated, since no protein is detected (27,28,29). Mice that are homozygous for the *shi* allele (shiverer, *mbp*^{shi} mice) are therefore considered MBP null. These animals have a hypomyelinated CNS, motor tremors, tonic seizures, and a shortened lifespan (27).

In this study, T cell responses to MBP immunization in shiverer and non-shiverer mice of H-2^{d} and H-2^{u} haplotypes were compared. Enhanced strain-specific responsiveness to certain epitopes of MBP was observed in shiverer mice. MBP is therefore not sequestered from the immune system, since its expression in normal mice leads to partial tolerance. Epitope-specific tolerance was suggested by comparison of responses to mMBP-derived peptides in H-2^{u} shiverer and non-shiverer mice. MBP represents a good model for the study of tolerance to a self protein which is etiologic to a well-defined autoimmune disease.

III. Materials and Methods

Shiverer mouse breeding

Outbred shiverer mice were crossed with BALB/c mice, progeny intercrossed, and shiverer offspring backcrossed with BALB/c mice. The mice used in the experiment were from the 12th backcross (generous gift of Dr. Richard L. Sidman).

Outbred shiverer mice were crossed with B10.PL mice, progeny intercrossed, and shiverer offspring backcrossed with B10.PL mice. The mice used in the experiments were the offspring from intercrosses following the first or second backcrosses. First intercross offspring were screened for H-2 haplotype (see below), and only $H\text{-}2^{u/u}$ mice were studied. Mice were maintained in a clean, non-sterile environment during the period before and after immunization.

Shiverer mice are homozygous for the shiverer allele of the gene encoding MBP. We propose to identify this gene as *Mbp*, the alleles of this gene as Mbp^{+} and mbp^{shi}, and the genotypes of the mice as Mbp^{+} (homozygous, normal phenotype), Mbp^{+}/mbp^{shi} (heterozygous, normal phenotype), and mbp^{shi} (homozygous, shiverer phenotype).

Mouse MBP purification

Murine MBP was prepared from frozen mouse brains (Pel-Freeze Biologicals Co., Rogers, AR) according to a previously published protocol (35).

Peptide synthesis

Peptides were synthesized by the stepwise solid-phase technique (36). The numbering scheme is based on the amino acid sequence of the 18.5 kD form of MBP, an isoform translated from a transcript of exons I, III, IV, V, VI, and VII of the MBP gene (31) (Table 1).

MHC screening with the Oligo Ligation Assay (OLA)

For experiments involving intercross offspring from first generation backcross matings of outbred shiverer mice ($H\text{-}2^{unknown}$) with B10.PL ($H\text{-}2^{u}$) mice, DNA polymorphisms within coding sequences for class II I-A proteins were used to distinguish $H\text{-}2^{u}$ and $H\text{-}2^{unknown}$ mice. The region containing the polymorphism was PCR-amplified using oligos (5'- TATCA GTCTC CTGGA GACAT TGGCC -3') and (5'- GGTAG CTGGG GTGGA ATTTG ACCTC -3'). The oligo ligation assay (OLA) (37,38) used the oligos (5'- AAAAC ACAAC TTGGG A -3') (common to all alleles) and (5'- TGCAA AACAT AGCTA CAGG -3') ($I\text{-}A^{u}$-specific) to determine the presence of the $I\text{-}A^{u}$ allele.

MBP allele screening with polymerase chain reaction (PCR)

Phenotypically, $MBP^{shi/+}$ mice are indistinguishable from wildtype mice. Normal littermates were screened for the presence of the shiverer MBP allele using oligonucleotide primer pairs flanking the *shi* allele deletion site (33) (5'-CAGGG GATGG GGAGT CAGAA GTGAG -3',5'-ATGTA TGTGT GTGTG TGCTT

ATCTA GTGTA -3') were synthesized and used for PCR amplification (39). PCR product indicated the presence of the *shi* MBP allele.

Mouse MBP immunization regimen

Murine MBP or peptide MBP(81-100) was emulsified with an equal volume of complete Freund's adjuvant (CFA, Gibco Laboratories, Grand Island, NY) supplemented with 4 mg/ml M. tuberculosis H37 RA (Difco Laboratories, Detroit, MI). Mice were immunized with a total of 55-75 μg MBP, or 100 nmoles of MBP(81-100), in equal 50 μl volumes SC in the hind footpads.

In vitro lymph node cell proliferation assay

Mice were sacrificed by cervical dislocation ten days after immunization. Inguinal and popliteal lymph nodes were sharply dissected and excised, and were suspended in Ventrex HL-1 serum free medium (Ventrex Laboratories, Portland, ME) or complete RPMI (for the MBP(81-100) immunization experiment) after crush dissociation. Viable lymph node cells were then plated at 3 - 4 x 10^5/well in 200-250 μl/well in Falcon 3077 96 well round bottom plates (Becton Dickinson & Co., Lincoln Park, NJ), and murine MBP (mMBP) (from the same prep as that used in the immunization) or HPLC-purified synthetic peptides were added. Four days later, 1 μCi [methyl-^{3}H] thymidine was added to each well

and cells were collected 16-24 hours later on Whatman 934-AH glass fiber filters (Whatman Co., Maidstone, England) using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Counts per minute (CPM) from triplicate or quadruplicate wells were determined by liquid scintillation counting, and calculated disintegrations per minute (DPM) were analyzed where noted.

IV. Results

Elevated response to murine MBP in shiverer mice.

To determine the effect of endogenous MBP expression on the immune response to MBP, non-shiverer and shiverer H-2^d mice were immunized with MBP, and lymph node proliferative responses upon restimulation with MBP were compared. Two experiments involving a total of 10 mice were performed. Figure 1 illustrates the lymph node proliferative responses of H-2^d *Mbp*$^+$ and *mbp*^{shi} mice observed in experiment 1 (Table 2a). *Mbp*$^+$ mice responded poorly to restimulation with mMBP, whereas *mbp*^{shi} mice responded well. In a second experiment, *Mbp*$^+$/*mbp*^{shi} littermates were also studied, and these mice responded similarly to *Mbp*$^+$ mice (Table 2a). Restimulation with purified protein derivative (PPD) of M. tuberculosis (25μg/ml), a positive control for immunization, gave similar responses (ca. 500,000 net dpm in expt. 1, ca. 200,000 net dpm in expt. 2) for all animals tested in each experiment. Overall, H-2^d MBP-deficient shiverer mice were hyper-responsive to restimulation with MBP compared with MBP-expressing littermates ($P < 0.05$).

Because wildtype B10.PL (H-2^u) mice respond well to MBP immunization and are susceptible to EAE induction, similar experiments were performed with H-2^u mice. Intercross offspring following 1 or 2 backcrosses of shiverer mice with B10.PL mice were screened for MBP and H-2 genotypes. The response of H-2^u mice to mMBP immunization

was determined as described above. Four experiments involving a total of 26 mice were performed. Figure 2 illustrates the lymph node proliferative responses of H-2u *Mbp*$^{+}$, *Mbp*$^{+}$/*mbp*^{shi}, and *mbp*^{shi} mice observed in experiment 1 (Table 2b). *Mbp*$^{+}$ and *Mbp*$^{+}$/*mbp*^{shi} mice responded poorly to restimulation with mMBP, whereas *mbp*^{shi} mice responded well. Responses to PPD within each experiment were similar for all three MBP genotypes (data not shown). Overall, H-2^{u} myelin-deficient shiverer mice were hyper-responsive to restimulation with MBP compared with MBP-expressing littermates ($P < 0.05$), and *Mbp*$^{+}$/*mbp*^{shi} mice responded indistinguishably from *Mbp*$^{+}$ mice (P = NSD) (Table 2b).

In both H-2^{d} and H-2^{u} mice, expression of endogenous MBP led to a statistically significant reduction in responsiveness to mMBP restimulation following mMBP immunization. *Mbp*$^{+}$ and *Mbp*$^{+}$/*mbp*^{shi} mice of both haplotypes showed similar responsiveness to mMBP restimulation *in vitro*.

Elevated responses in shiverer mice map to particular epitopes of MBP.

To determine the basis for the enhanced responsiveness to self MBP immunization in H-2^{d} shiverer mice, lymph node cells from mMBP-immunized shiverer and non-shiverer littermates were stimulated in vitro with individual overlapping synthetic peptides derived from the 18.5 kD MBP sequence (Table 1). Figure 3 illustrates proliferative responses to these peptides, and shows that the enhanced

responsiveness to mMBP immunization in H-2^d shiverer mice is largely accounted for by an increased response to a single peptide, MBP(81-100) ($P < 0.05$). In addition, two of four shiverer mice studied showed a significant, but lower, response to MBP(61-80). As shown in Table 2a, *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} H-2^d mice responded similarly to MBP(81-100). Thus, the hyper-responsiveness of H-2^d shiverer mice to mMBP immunization is mainly due to hyper-responsiveness, or intolerance, to MBP(81-100).

The ability of MBP(81-100) to prime T cell responses in H-2^d mice was then studied. *Mbp*$^+$, *Mbp*$^+$/*mbp*^{shi}, and *mbp*^{shi} littermates were immunized with MBP(81-100), and *in vitro* proliferative responses to mMBP and MBP(81-100) were compared. As shown in figure 4, lymph node cells from *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} mice responded poorly to *in vitro* stimulation with both mMBP and MBP (81-100), while those from *mbp*^{shi} mice responded well to both mMBP and (81-100). It appears that *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} mice are similarly tolerant to self MBP and MBP(81-100), and that H-2^d shiverer mice are not tolerant to an epitope estimated by MBP(81-100). Whereas wildtype BALB/c (H-2^d) mice respond poorly to murine (self) MBP immunization, wildtype B10.PL (H-2^u) mice respond well. A typical epitope map for a B10.PL mouse is shown in figure 5a, and demonstrates that most of the T cell response to mMBP immunization of inbred B10.PL is directed towards the N-terminal portion of mMBP, MBP(NAc1-20). Response to this

"dominant" epitope is frequently accompanied by lesser responses to other, "subdominant", epitopes including MBP(31-50) and MBP(121-140) (see chapter 1, "T cell epitopes of self myelin basic protein in B10.PL mice"). To determine whether the enhanced response to MBP in H-2^u shiverer mice is directed towards the dominant epitope, the subdominant epitope, or new epitopes, lymph node cells from MBP-immunized H-2^u wildtype and shiverer mice were stimulated in vitro with the overlapping synthetic peptide series described above. Figure 5b shows that the enhanced responsiveness to mMBP in H-2^u shiverer mice is largely accounted for by an increased response to the wildtype subdominant epitope, MBP(121-140) ($P < 0.01$). No enhanced response to the other wildtype subdominant epitope, MBP(31-50), was observed in H-2^u shiverer mice. There was no evidence for tolerization of MBP(NAc(1-20))-specific T cells in MBP-expressing mice, and responsiveness to MBP(NAc1-20) was not demonstrably different between shiverer and non-shiverer mice (P = NSD).

In both H-2^d and H-2^u mice, the observed T cell hyper-responsiveness to mMBP in shiverer mice is directed towards single epitopes, i.e., MBP(81-100) in H-2^d shiverer mice and MBP(121-140) in H-2^u shiverer mice. In wildtype H-2^u mice, the response to mMBP(121-140) is tolerized and the response to mMBP(NAc1-20) is not, suggesting that these epitopes are tolerized independently.

V. Discussion

MBP as a sequestered self antigen.

In H-2^d and H-2^u mice, the immune response to mMBP is reduced when myelin basic protein (MBP) is expressed endogenously. MBP is therefore not a sequestered self antigen, since its expression leads to tolerance induction in normal mice.

The brain is one of several organs which are considered to be "immunologically privileged" sites (14,40). The blood-brain barrier of tight endothelial junctions prevents access of both cells and dyes into the brain under normal circumstances. Normal cerebrospinal fluid (CSF), for example, has $\leq$ 1 lymphocyte per microliter, while normal blood has an average of 2200 per microliter (41). In addition, the brain lacks conventional lymphatic drainage, the usual means for trafficking of antigens to the secondary lymphoid organs. An effective anatomic barrier, plus a lack of efferent lymphatics, may account for the observed prolonged survival of tumor and embryonic allotransplants in the brain. These observations have given rise to the hypothesis that the brain and its component tissues are sequestered from routine lymphoid surveillance. Thus, myelin is expected to be a sequestered tissue and MBP is expected to be a sequestered antigen. The data presented in this report, however, indicate that MBP is tolerogenic, so some mechanism must exist for presentation of endogenous MBP

to the immune system. Soluble protein exchange between CSF and lymph, as has been observed for albumin (42), or incomplete integrity of the blood-peripheral nerve barrier (43), may provide routes by which endogenous MBP could traffic to sites of tolerance induction.

Both *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} H-2^u mice have a non-shiverer phenotype. No statistical difference between their responses to mMBP immunization was demonstrable. The MBP concentration in the brain varies linearly with normal MBP gene copy number, but morphometric analysis on *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} brains has shown no significant difference in the thickness of myelin in the myelin sheaths (44). The data presented in this report suggest that the amount of MBP generated in *Mbp*$^+$/*mbp*^{shi} mice is sufficient for tolerance equivalent to that seen in *Mbp*$^+$ mice.

Epitope-specific tolerance to self MBP in H-2^u mice.

Compared to H-2^u non-shiverer littermates, H-2^u shiverer mice are hyper-responsive to MBP(121-140), but similarly responsive to MBP(NAc1-20) and MBP(31-50). The response to MBP(121-140) in non-shiverer mice is well-tolerized, whereas the response to MBP(NAc1-20) is not well-tolerized. The response to MBP(31-50) is poor in both shiverer and non-shiverer mice. Thus, T cell tolerance to self MBP in non-shiverer H-2^u mice appears to be epitope-specific.

Shiverer mice have been found to generate small amounts of polyadenylated transcripts of exons 1 and 2 from the normal MBP promoter (34). This observation suggests that a truncated, 83 amino acid polypeptide that encompasses MBP(NAc1-20) and MBP(31-50) could in theory be translated from the exon (1,2) partial MBP transcript. This polypeptide has not been observed in shiverer brain using anti-human MBP polyclonal antibodies in an RIA with a sensitivity of 0.5 ng/ml (44). However, a truncated polypeptide could be present at a low level sufficient to induce tolerance. It is possible that a completely MBP null mouse might prove to be hyper-responsive to MBP(NAc1-20) and MBP(31-50). In any case, normally myelinated H-2^{u} mice are poorly tolerized to MBP(NAc1-20), but well-tolerized to MBP(121-140). In addition, the MBP(31-50) recall response is poor in both shiverer and non-shiverer mice, suggesting that processed and presented MBP on the APC surface is of marginal sufficiency for MBP(31-50)-specific T cell expansion.

The murine immune response to the xenoprotein, hen egg lysozyme (HEL), also involves epitope-specific tolerance (16). Following immunization with HEL in CFA, B10.A mice respond to restimulation with at least four different HEL peptides, with the greatest proliferative responses found due to stimulation with peptides HEL(20-35) and HEL(46-61). Interestingly, induced tolerance to HEL via IV injection of

HEL was epitope-specific; tolerant mice did not respond to stimulation with HEL(20-35) or HEL(46-61), but did respond to minor determinants. These data are similar to our observations in MBP-deficient shiverer mice, in that the predominant response in shiverers is the best tolerized in non-shiverers.

The basis for these examples of epitope-specific T cell tolerance is not known. The TCR/MHC/peptide complex might have a threshhold affinity for T cell tolerization and activation which is satisfied by some epitopes, but not others (16). Alternatively, epitopes might be transported with different efficiencies to sites of tolerization (45). Finally, central and peripheral APCs might use different mechanisms of antigen processing and/or presentation to T cells. In this model, certain epitopes would be presented by thymic APCs, leading to tolerance, whereas other epitopes would be presented in the periphery, leading to activation.

Although these data suggest that T cell tolerance to MBP occurs in mice expressing the protein, we have not identified the sites or mechanisms of tolerance to MBP. This model does, however, offer the opportunity to correlate tolerance to specific self epitopes with their presence at sites of tolerization and their chemical affinities for MHC and TCR.

Incomplete tolerance to tolerogenic epitopes in H-2^d and H-2^u mice.

Tolerance to MBP(81-100) in H-2^d mice and to MBP(121-140) in H-2^u mice is frequently incomplete, as 4 of 6 MBP-expressing H-2^d mice responded to MBP(81-100), and 9 of 17 MBP-expressing H-2^u mice responded to MBP(121-140). In addition, MBP(121-140)-specific T cells have been isolated from normal B10.PL (H-2^u) mice (data not shown).

The basis for incomplete tolerance to these MBP epitopes is not known. A similar effect was noted in the mouse HEL tolerance model (16). One model to explain this phenomenon envisions a set of epitope-specific T cell clones with different ligand-binding affinities. T cell clones with high, but not low, ligand binding affinities would be tolerized, resulting in incomplete tolerance to the epitope in normal mice. This model would predict that shiverer mice would contain epitope-specific T cells expressing unique TCRs that are not found in wildtype mice. Alternatively, epitope-specific T cells in wildtype and shiverer mice may express the same set of TCRs with ligand-binding affinities which are borderline for the induction of tolerance. Borderline ligand-binding affinities of epitope-specific T cells would lead to incomplete tolerance in normal mice.

We have demonstrated that normal mice are incompletely tolerant to particular epitopes of the nervous system-specific self antigen, myelin basic protein. Incomplete tolerance to self proteins has important implications for

understanding the etiology of autoimmune disease.

VI. References

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TABLE LEGENDS

Table 1:

Text references to these peptides are given as "MBP(*peptide*)". "NAc1-20" refers to the N-acetylated derivative of the amino-terminal 20-mer. This numbering system (31) does not take the exon 2-encoded amino acid residues (n=26) into account. Peptide (104-113)/(155-164) ("E6(-)") is an (exon 5, exon 7)-encoded fusion peptide made to mimic the 14 kD MBP isoform (Exon 6(-)) protein sequence.

Table 2:

Lymph node proliferative responses of a) H-2^d and b) H-2^u mice following mMBP immunization. Responses are given as net DPMs ($DPM_{net} = DPM_{ag} - DPM_{bkgd}$) with associated standard error of the difference of the means (SED), and as stimulation indices (SIs) ($SI = (DPM_{ag} / DPM_{bkgd})$. Means are given as an estimate of central tendency for each group. SI is assumed to be a non-parametric variable, so the Wilcoxson rank sum test was used to determine statistical significance. "*" means that $P < 0.05$ in the comparison of *Mbp*$^+$ and *mbp*^{shi} mice. "ǂ" means that $P < 0.01$ in the comparison of *Mbp*$^+$ and *mbp*^{shi} mice. No statistical difference is demonstrable between *Mbp*$^+$ and *Mbp*$^+$/*mbp*^{shi} mice. Intra-group variation may be due to the use of non-isogenic mice, different batches of mMBP, and a calculated variable (SI) for normalization of experiments.

Table 1. Overlapping Peptides from the 18.5 kD Form of Murine Myelin Basic Protein

Peptide Name	Peptide Sequence	Coded by Exon
NAc1-20	ASQKRPSQRSKYLATASTMD	I
11-34	KYLATASTMDHARHGFLPRHRDTG	I
22-40	ARHGFLPRHRDTGILDSIG	I
31-50	RDTGILDSIGRFFSGDRGAP	I
41-58	RFFSGDRGAPKRGSGKDS	I, III
49-70	APKRGSGKDSHTRTTHYGSLPQ	I, III
61-80	RTTHYGSLPQKSQHGRTQDE	III
68-91	LPQKSQHGRTQDENPVVHFFKNIV	III, IV
81-100	NPVVHFFKNIVTPRTPPPSQ	III, IV
87-114	FKNIVTPRTPPPSQGKGRGLSLSRFSWG	III, IV, V
101-120	GKGRGLSLSRFSWGAEGQKP	IV, V, VI
111-130	FSWGAEGQKPGFGYGGRASD	V, VI
121-140	GFGYGGRASDYKSAHKGFKG	VI
131-150	YKSAHKGFKGAYDAQGTLSK	VI
141-160	AYDAQGTLSKIFKLGGRDSR	VI, VII
151-168	IFKLGGRDSRSGSPMARR	VI, VII
104-113/ 155-164	RGLSLSRFSWGGRDSRSGSP	V, VII

Table 2a: H-2^d Lymph Node Proliferation in Vitro After mMBP Immunization

	Expt	Animal	mMBP Net DPM	mMBP SED	SI	MBP(81-100) Net DPM	MBP(81-100) SED	SI
MBP +/+	1	1	43412	±7753	3.3	31519	±6910	2.7
		2	31519	±6910	2.9	30025	±6099	2.7
	2	3	10595	±7840	1.9	-1019	±2543	0.9
		4	-6645	±693	0.1	7906	±2734	2.1
		Mean			2.1			2.1
MBP shi/+	2	1	-517	±2149	0.9	3284	±2226	1.5
		2	-39	±287	1.0	1929	±280	1.8
		Mean			1.0			1.7
MBP shi/shi	1	1	183122	±11775	11.7	153311	±15694	10.0
		2	235001	±7402	11.8	189041	±4617	9.6
	2	3	128075	±19108	25.4	155767	±10778	30.6
		4	11125	±3622	4.3	123313	±36114	37.8
		Mean			13.3*			22.0*

Table 2b: H-2^{u} Lymph Node Proliferation in Vitro After mMBP Immunization

	Expt	Animal	mMBP			MBP(NAc1-20)			MBP(121-140)		
			Net DPM	SED	SI	Net DPM	SED	SI	Net DPM	SED	SI
MBP +/+	1	1	77957	±3263	19.4	89706	±3470	22.1	8347	±752	3.0
		2	59509	±2571	11.5	84057	±2339	50.5	4008	±698	3.6
		3	40622	±2511	8.4	71107	±4063	15.8	-1534	±1268	1.7
	2	4	98485	±2453	4.8	153016	±9415	7.0	30158	±1688	2.2
		5	119288	±7905	4.9	158748	±4102	6.2	27515	±5732	1.9
	3	6	7042	±1168	3.2	3297	±1964	2.0	754	±1474	1.2
	4	7	1893	±1196	1.2	775	±1139	1.1	-3146	±1014	0.8
		8	3462	±1073	1.4	7653	±1691	1.9	1890	±1975	1.2
		Mean			6.9			13.3			2.0
MBP shi/+	1	1	20029	±1613	6.4	27176	±2147	8.4	3422	±2038	1.9
		2	90062	±2643	24.9	93215	±6646	25.7	3231	±840	1.9
		3	58475	±2604	12.3	55895	±1152	11.8	991	±1228	0.9
	2	4	217275	±30302	8.5	195297	±15258	7.7	31990	±7807	2.1
		5	255051	±10126	10.6	227558	±18993	9.6	33616	±6223	2.3
	3	6	24474	±6738	6.3	29140	±6293	7.3	-91	±1824	1.0
		7	13355	±2124	5.1	4974	±1820	2.5	1572	±519	1.5
	4	8	2546	±673	1.6	4458	±1687	2.0	3464	±979	1.8
		9	16552	±6754	2.5	36997	±4570	4.3	2795	±931	1.3
		Mean			8.7			8.8			1.6
MBP shi/shi	1	1	187625	+5502	70.3	101333	+4665	38.4	140635	+1615	52.9
		2	187985	+1843	49.7	56185	+2961	15.6	88061	+410	23.8
		3	208340	+4413	33.4	101430	+3835	16.8	113264	+5646	18.6
	2	4	412614	+52433	11.2	222493	+3109	6.5	299541	+13127	8.4
		5	427742	+14663	20.9	206468	+3963	10.6	335326	+11754	16.6
	3	6	129377	+9473	9.3	49096	+5659	4.2	170388	+9998	11.9
		7	41639	+2650	20.8	385	+293	1.2	33727	+7792	17.0
	4	8	44484	+966	4.5	8073	+1722	1.6	68962	+4040	6.4
		9	113839	+6594	14.8	12865	+1678	2.6	127032	+14202	16.4
		Mean			26.1*			10.8			19.1†

FIGURE LEGENDS

Figure 1. Effect of endogenous MBP expression in H-2^d mice on lymph node cell proliferative response to mMBP restimulation. Littermate H-2^d *Mbp*$^+$ and *mbp*^{shi} mice were immunized with 50-70 μg murine MBP in CFA. Lymph nodes were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with murine MBP for 5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Data points reflect the mean of triplicate samples, and error bars reflect the standard error of the difference of the means. Each dose response curve represents the responses of one mouse in experiment 1 (Table 2a).

Figure 2. Effect of endogenous MBP expression in H-2^u mice on lymph node cell proliferative response to mMBP restimulation. Littermate H-2^u *Mbp*$^+$, *Mbp*$^+$/*mbp*^{shi} and *mbp*^{shi} mice were immunized with murine MBP in CFA. Lymph nodes were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with murine MBP for 5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Data points relect the mean of triplicate samples, and error bars reflect the standard error of the

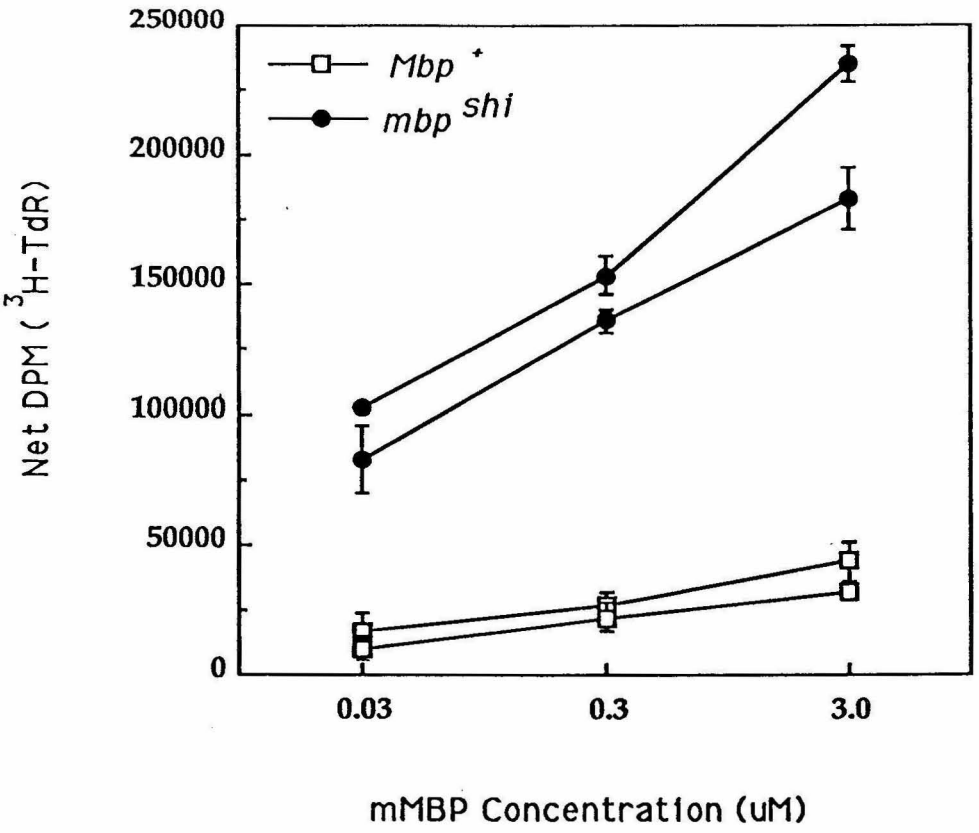
difference of the means. Each dose response curve represents the responses of one mouse in experiment 1 (Table 2b). *Mbp*$^{+}$/*mbp*^{thi} mouse results were indistinguishable from *Mbp*$^{+}$ mouse results (see Table 2b), and were not plotted.

Figure 3. Effect of endogenous MBP expression in H-2^{d} mice on lymph node cell response to MBP peptide stimulation following mMBP/CFA immunization. Littermate H-2^{d} *Mbp*$^{+}$, *Mbp*$^{+}$/*mbp*^{thi} and *mbp*^{thi} mice were immunized with 50-70 μg murine MBP in CFA. Lymph nodes were harvested 10 days later, and 3-4 x 10^{5} viable cells/well were incubated with each of the overlapping MBP peptides at 30 μM for 4-5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Bar heights reflect the mean of triplicate samples, and error bars reflect the standard error of the difference of the means. Responses of two mice of *Mbp*$^{+}$ and *mbp*^{thi} genotypes (Experiment 2, Table 2a) are represented here. Responses of *Mbp*$^{+}$/*mbp*^{thi} mice were similar to those of *Mbp*$^{+}$ mice, and were not plotted.

Figure 4. Effect of endogenous MBP expression in H-2^{d} on lymph node cell response to mMBP and MBP(81-100) stimulation following MBP(81-100)/CFA immunization. Littermate H-2^{d} *Mbp*$^{+}$, *Mbp*$^{+}$/*mbp*^{thi} and *mbp*^{thi} mice were immunized with 100 nmoles

MBP(81-100) peptide in CFA. Lymph nodes were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with 1 μM mMBP or 30 μM MBP(81-100) for 4-5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Bar heights reflect the mean of triplicate samples, and error bars reflect the standard error of the difference of the means. Responses of two mice of each genotype are represented here.

Figure 5. Response of MBP-primed H-2^u lymph node cells to restimulation with mMBP peptides. A B10.PL (H-2^u) mouse (a) or H-2^u *Mbp*$^+$, *Mbp*$^+$/*mbp*^{shi} and *mbp*^{shi} mice (b) were immunized with 55-75 μg murine MBP in CFA. Lymph node cells were harvested 10 days later, and 3-4 x 10^5 viable cells/well were incubated with each of the overlapping MBP peptides (30 μM each) for 4-5 days. One μCi ^{3}H-TdR was added during the last day, cells were filtered, and CPM were counted. Bar heights reflect the mean of triplicate samples, and error bars reflect the standard error of the difference of the means. Responses of three mice of *Mbp*$^+$ and *mbp*^{shi} genotypes (Experiment 2, Table 2b) are represented here. Responses of *Mbp*$^+$/*mbp*^{shi} mice were similar to those of *Mbp*$^+$ mice, and were not plotted.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150000
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mbp ^{shi}
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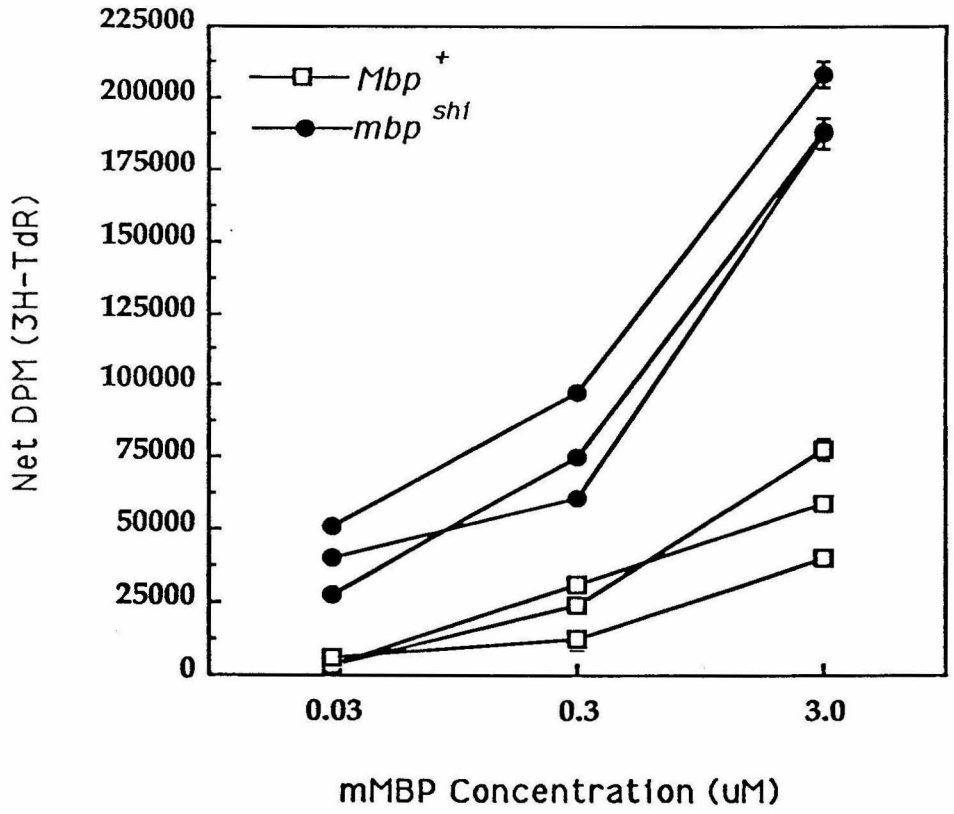
0.3
3.0
mMBP Concentration (uM)

Figure 1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50000
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mbp shi
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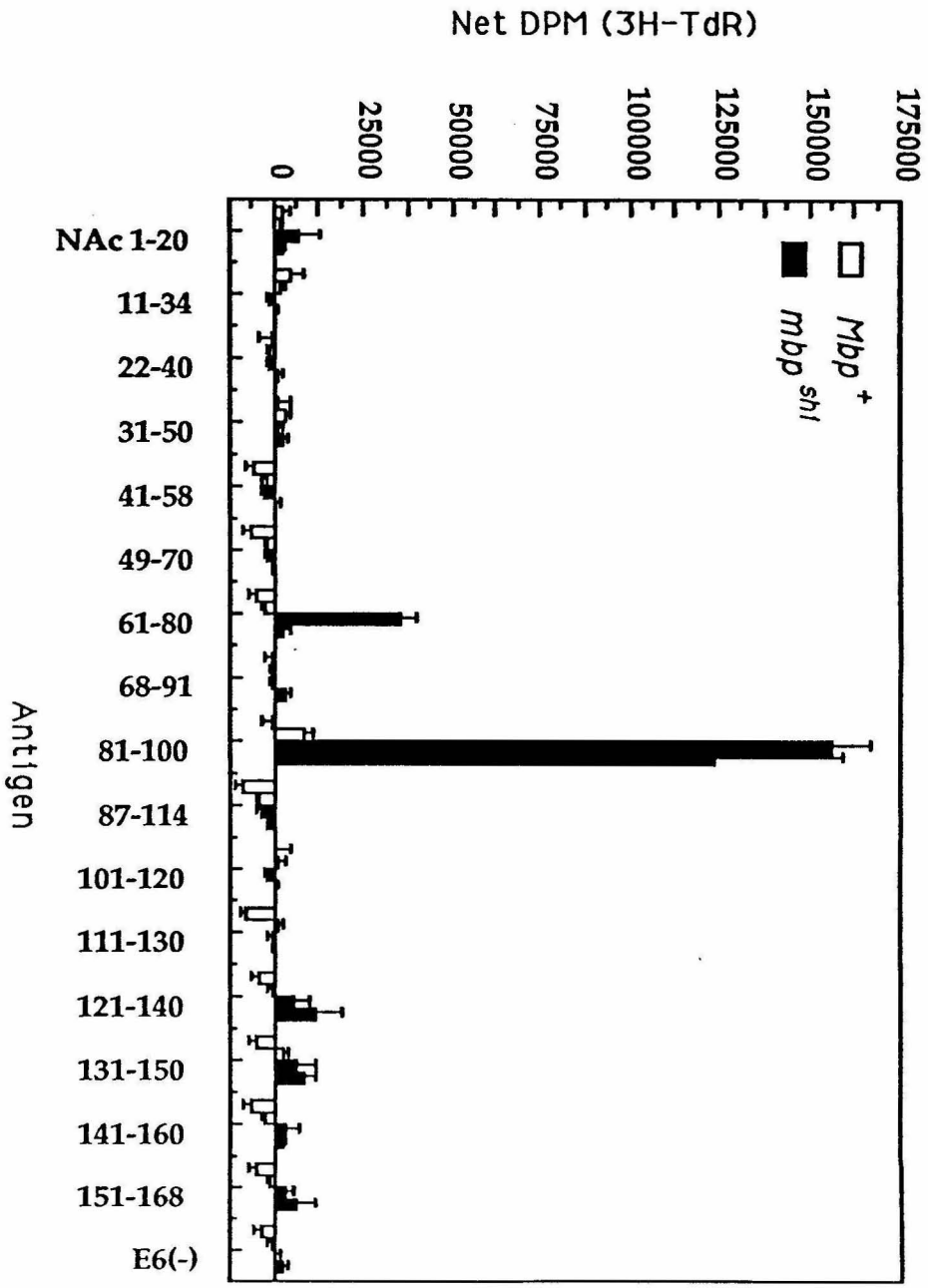
0.3
3.0
mMBP Concentration (uM)

Figure 2

Figure 3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50000
75000
100000
125000
150000
175000
Mbp +
mbp sh1
Antigen
NAc 1-20
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)

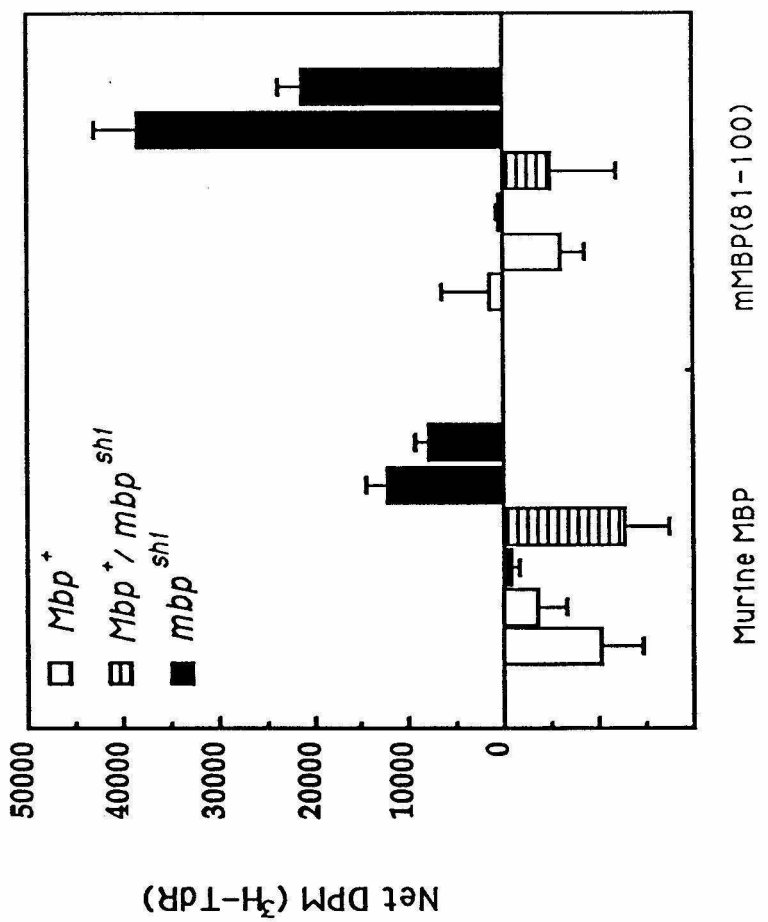
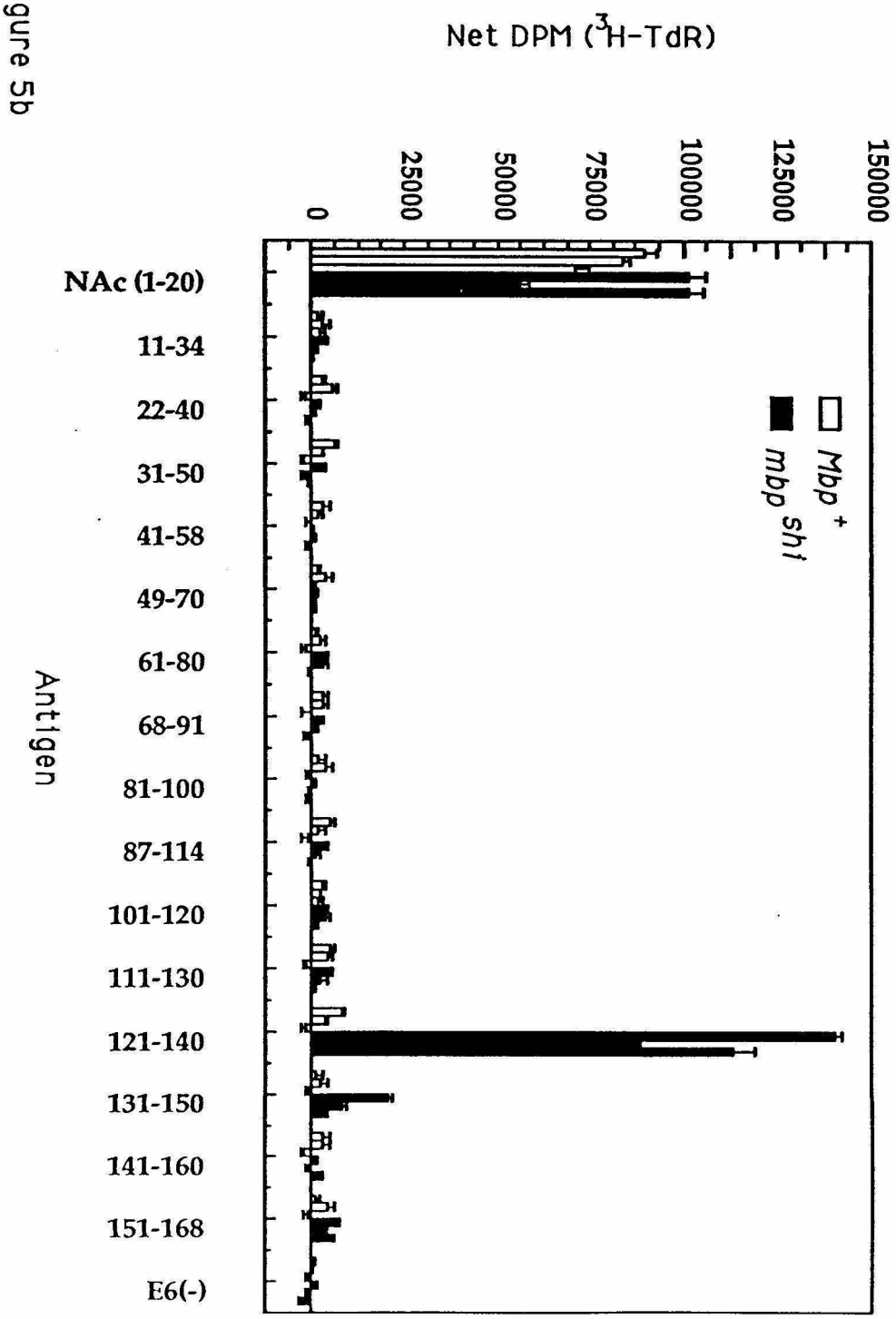

Net DPM (^{3}H-TdR)
50000
40000
30000
20000
10000
0
Mbp+
Mbp+/ mbpshi
mbpshi
Murine MBP
mMBP(81-100)

Figure 4

Net DPM (^{3}H-TdR)
75000
50000
25000
0
NAc (1-20)
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Antigen

Figure 5a

Figure 5b


Net DPM (^{3}H-TdR)
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25000
50000
75000
100000
125000
150000
Antigen
NAc (1-20)
11-34
22-40
31-50
41-58
49-70
61-80
68-91
81-100
87-114
101-120
111-130
121-140
131-150
141-160
151-168
E6(-)
Mbp +
mbp shi

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Variation of Primate T Cell Antigen Receptor Vβ 8.1 and 8.2

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I. Summary

The degree of allelic sequence variation within the T cell antigen receptor (TCR[1]) α and β loci has not been firmly established. It is known that the MHC proteins, the natural ligands for the α/β TCR proteins, are highly polymorphic due to multiple germline alleles, and that this MHC allelism is positively selected. The TCR Vα and Vβ loci might therefore also sustain a high rate of substitution and subsequent polymorphism as a mechanism for generating variation for normal selection processes of tolerance and response to pathogens. To test this hypothesis, human Vβ8.1 and 8.2 gene segments and their non-coding flanking regions were sequenced and compared to their orthologous sequences in chimpanzee, gorilla, pig-tailed macaque, and squirrel monkey. In addition, Vβ 8.1, 8.2, 8.3, and 8.5 gene segments from non-related humans were sequenced and analyzed for polymorphism. Inter-species comparisons show that these gene segments have sustained duplication, conversion, and deletion during 35 million years of anthropoid primate evolution. Vβ 8.1 and 8.2 coding sequences are generally conserved with respect to their flanking non-coding sequences, but the regions coding for putative complementarity-determining (ligand-binding) regions (CDRs) substitute at the same rate as their non-coding flanking sequences. Unrelated humans demonstrate minimal Vβ 8.1,

8.2, 8.3, or 8.5 coding sequence polymorphism, with only one non-synonymous nucleotide substitution (in Vβ8.3) noted in thirteen individuals studied. We conclude that germline substitutions and polymorphism in TCR Vβ 8.1 and 8.2 probably do not play a major role in generating TCR β chain protein variation, and infer that the extensive variation predicted for T cell antigen receptors must derive primarily from junctional and combinatorial diversity.

[1] Abbreviations used in this paper: TCR, T cell antigen receptor; MHC, major histocompatability complex; MYA, millions of years ago; NWM, new world monkey; OWM, old world monkey.

II. Introduction

T cells express monoclonal heterodimeric antigen receptors (TCRs) on their surfaces (Meuer 1984; Haskins 1984). The antigen specificity of the T cell is determined by the specificity of the T cell receptor (Meuer 1984; Dembic 1986), the function of the T cell is defined by T cell CD4 or CD8 coexpression (Meuer 1984), and activation of the T cell is rate-limited by a B7/CD28 costimulatory signal (Gimmi 1991).

T cells appear to be selected on the basis of their TCR specificities. Negative selection, whether deletional (Kappler 1987; Kisielow 1988) or anergic (Schwartz 1990; Lo 1991) in nature, is contingent on TCR specificity, as is positive selection, both for T cell maturation (von Boehmer 1990) and T cell activation in response to pathogens (Brown 1991; Hahn 1991).

The wide range of antigens recognized by the TCR requires some means of generating diversity in the ligand-binding regions of the TCR proteins. To generate this diversity, several mechanisms have been proposed, including germline V gene segment polymorphism (Plaza 1991; Robinson 1989; Li 1990), combinatorial V(D)J rearrangement (Kronenberg 1986; Kimura 1987), and N region diversity (Concannon 1986). Somatic mutation has not been observed for rearranged TCR genes, as it has for immunoglobulin genes (Tonegawa 1983).

MHC proteins, the ligands for the TCRs, are known to be highly polymorphic (Klein 1983, 1990). DNA sequence polymorphism in these MHC genes is extensive, with non-synonymous (amino acid-changing) substitution rates exceeding synonymous (silent) substitution rates for the region coding for the antigen binding cleft of both the MHC class I and class II molecules (Hughes 1988, 1989). This overdominant (positive) selection (Maruyama 1981) is felt to result from a potential heterozygous advantage in terms of pathogen xenoprotein presentation and subsequent disease resistance. This rationale suggests that the T cell receptors for these MHC class I and II proteins may also be highly diverse in order to prevent failures of recognition, or "holes", in the T cell repertoire of an individual (Schaeffer 1989; Ogasawara 1987). The degree to which this diversity is supplied by variation in individual gene segments is not clear.

To determine the extent of evolutionary divergence and extant polymorphism of a model Vβ gene segment, human Vβ8.1 and Vβ8.2 gene segments were compared to orthologous sequences in other primates and to homologous sequences in other humans. Non-synonymous nucleotide substitution rates were found to be slightly lower than those for non-coding flanking sequences, but coding sequence substitution rates for the putative complementarity-determining (ligand-binding) regions (CDRs) 1 and 2 were found to be similar to

those of non-coding flanking sequences. Furthermore, study of samples of fourteen and ten unrelated humans revealed no non-synonymous substitutions within Vβ8.1 or Vβ8.2, respectively, suggesting minimal polymorphism for these Vβ gene segments. Although certain TCR Vβ gene segment alleles may be found to be pathologically relevant, our observations together suggest that the predominant basis for TCR protein diversity is due not to germline diversity, but rather to other mechanisms, such as combinatorial and junctional diversity.

III. Materials/Methods

Human DNA Samples

Human cosmid clones H7.1 and H12.18 were derived from a HeLa cell cosmid library. 12.18.1 is an M13 subclone of H12.18 containing hVβ 8.1 on a 4.6 kb EcoRI fragment. Unrelated human peripheral blood specimens were used (some extracted directly, some extracted after B cell line transformation) to isolate the germline genomic DNA used for PCR templates. Blood donors were normal or RA patients, and did not have MS.

Non-Human Primate DNA Samples

Chimpanzee (Pan troglodytes) liver from animal YN86-189 at Yerkes Primate Center was used to obtain the germline genomic DNA used for Southern blots and PCR templates. Gorilla (Gorilla gorilla) placenta from animal YB84-131 at Yerkes Primate Center was used to obtain the germline genomic DNA used for Southern blots and PCR templates. Pig-tailed macaque (Macaca nemestrina) liver from animal YN88-210 at Yerkes Primate Center was used to obtain the germline genomic DNA used for Southern blots and PCR templates. DNA from Rhesus monkey (M. mulatta) YN88-221 was used for some Southern blots, as noted. Squirrel monkey (Saimiri sciureus) DNA was a gift from Dr. W. H. Li, and was used for Southern blots and PCR templates. Cebus monkey (Cebus albifrons) DNA was a gift from Dr. R. Higuchi, and was used

for Southern blots as noted.

Southern Blotting and DNA Hybridization

Ten micrograms of human, chimp, gorilla, macaque, and squirrel monkey DNA were digested for each condition for each species, and the DNA was size-separated on 0.7-1.0% agarose gel. Gels were photographed for migration rates of size markers, then transferred to Zetaprobe membranes (BioRad Labs, Richmond, CA) using 0.4 N NaOH. These blots were washed three times in 2x SSC (NaCl 0.3 M, NaCit 0.03 M) at room temperature, blotted damp, then prehybridized in (5x SSC, 1% SDS, 50% formamide, 25.mM NaH_2PO_4, 1% powdered milk, and 0.2 mg/ml boiled salmon sperm DNA) for $\geq$ 20 minutes. Random primer extension (Feinberg 1983) was used to ^{32}P-label double strand DNA probes, and the probes were individually hybridized to the blots in the above prehybridization solution plus 10% Dextran SO_4 at 37 C for $\geq$ 16 hours. Hybridized blots were washed twice in 2x SSC/0.1%SDS at room temperature for 15 minutes, then washed three times in 2x SSC/0.1%SDS at 60 C, or more stringently as noted, prior to film exposure at -70 C for various intervals.

DNA Sequencing of Human 12.18.1

Sequenase kit reagents (US Biochemical Co., Cleveland, Ohio) were used to perform dideoxynucleotide termination sequencing of single (+) strand M13 clone 12.18.1, this

clone containing hVβ8.1 and its 5' flanking region. Six percent polyacrylamide gels were poured into 38 x 50 cm Sequigen gel plates (Bio-Rad, Richmond, CA). Oligonucleotide primers were successively synthesized (ABI 380B, Caltech Biopolymer Synthesis and Analysis Resource Center) as new sequence data was generated.

The (+) strand of H12.18.1 was sequenced over a 2.0 kb region from the 3' end of the second exon of human Vβ 8.1 to 1.5 kb 5' of the first exon. From this sequence, primers were designed and synthesized to sequence the complementary strand, and it was sequenced in a similar fashion.

Cloning of Primate Vβ 8.1 & 8.2 Gene Segments

PCR amplification primers AP7 (5'-AGG TAT TGG CAG GGC TAC ATT CCT TCC-3') and AP2 (5'-AGC TTA AGG AGA AGG GGA AAC ACC GGG TTT-3') were then designed based on known 12.18.1 sequence, and these were used to obtain 2.0 kb PCR products from human cosmid H7.1 as well as from chimp and gorilla. PCR amplification primer pairs [AP8 (5'-AGG GAC TGG CTG AGC TTT GC-3') and AP2 (above)] or [AP9 (5'-AAA GCC AAC AGG GAC TCT GC-3') and AP2] were used to obtain ca. 2.0 kb PCR products containing macaque Vβ 8.1 and 8.2 orthologues. PCR amplification primer pairs [APSM5 (5'-CAA AGC CAA CAA GGA CTG GC-3') and APSM3 (5'-GAT CCA TCA GGC CTC TCA GC-3')] or [8130 (5'-GAG GTG ACA GAG ATG GGA CA-3') and SP2 (5'-TTT CTG CAC AGG AAA GGG GT-3')] were used to obtain 1.8 kb and 0.3

kb PCR products containing the squirrel monkey Vβ 8.5 orthologue. PCR amplification primers AP1 (5'-GAT CCA AGT TGG GGG TGG TGG CCC ATT CAG-3') and SP2 (above) were used to obtain 0.6 kb PCR product containing the squirrel monkey Vβ 8.1 orthologue. PCR products were filled in with T4 DNA polymerase, kinased with PNK, then blunt-end cloned into Sma-cut de-phosphorylated M13mp(10 or 18) RF. Ligation mixes were used to tranfect F' (+) competent cells (Amersham Co., Arlington Heights, IL), clear plaques were picked and expanded, and clonal single strand DNA in the supernatants was purified.

DNA Sequencing of Primate Vβ 8.1, 8.2

The 2.0 kb human AP7-AP2 PCR product was cloned into M13mp10, and individual subclones "8.4" (+) and "8.5" (-) were sequenced using dideoxy nucleotide termination methods as described above. This double strand sequence was used as the benchmark for comparison of human Vβ 8.2 and primate orthologues of these two gene segments. The 2.0 kb human AP7-AP2 PCR product containing human Vβ 8.2 was cloned into M13mp10, and individual subclones "8.6" (+) and "8.1.1" (-) were sequenced.

The 2.0 kb chimpanzee AP7-AP2 PCR product was cloned into M13mp10. Multiple individual subclones were sequenced, and only one set of sequences was observed. Representative clones "2.16" (+) and "2.1" (-) were found to be

complementary.

The 2.0 kb gorilla AP7-AP2 PCR product was cloned into M13mp10. Multiple individual subclones were sequenced, and two sets of sequences were observed. Representative clones "3.11" (+) and "3.2" (-) were complementary, and clones "3.12"(+) and "3.1"(-) were complementary.

The 2.0 kb macaque AP7-AP2 PCR product was cloned into M13mp10. Multiple individual subclones were sequenced, and multiple sites of non-complementarity were noted. Two new upstream primers differing by a deletion in one of the macaque gene segments, AP8 and AP9, were designed. The 1.9 kb macaque AP8-AP2 and AP9-AP2 PCR products were cloned into M13mp10, and multiple individual subclones were sequenced. AP8-AP2 clones "1.1.4"(+) and "1.1.8" (-) were complementary, and AP9-AP2 clones "2.4"(+) and "2.8" (-) were complementary.

The 2.0 kb squirrel monkey AP7-AP2 PCR product was sequenced, and only end sequence was obtained. The 1.7 kb AP6' (5'-CCT TGC CAC ATA ATT TAA CAC -3')-SP2 PCR product was sequenced and non-Genbank, indeterminate sequence was obtained. The 1.8 kb SM5-SM3 PCR product was sequenced, and was found to be most similar to human Vβ8.5. Selected clones were sequenced using synthetic fluorescent primers (Smith, 1987) and analyzed on the ABI 370A DNA sequenator (ABI, Foster City, CA). The 0.6 kb AP1-SP2 PCR product was sequenced, and was found to be most similar to hVβ 8.1. No

further 5' flanking sequence from the squirrel monkey hVβ8.1 orthologue was amplifiable with multiple available primers.

PCR of (Unrelated) Human Vβ 8.1,8.2,8.3, and 8.5

PCR primer pairs were designed from published sequences (Siu, 1986) to specifically amplify Vβ 8.1, 8.2, 8.3, and 8.5 gene segments. Vβ 8.1 was amplified with primer VB8.1-1 (5'-ATG CAG CCT CCT CTT AAA GAA-3') and primer VB8.1-2 (5'-GAA GGG GAA ACA CCG GGT-3'). Vβ 8.2 was amplified with primers VB8.2-1 (5'-ATG CAG CCT CCT CTT AAA GTT-3') and VB8.2-2 (5'-GAA GGA GAA ACA CCA GGG-3'). Vβ 8.3 was amplified with primers VB8.3-1 (5'-GCT CCT CTG CTG TGT GGT T-3') and VB8.3-2 (5'-GAC CAA ACC ACT AGC ACA A-3'). Vβ 8.5 was amplified with primers VB8.5-1 (5'-CTG CCT GAT TCA TCT CCC AA-3') and VB8.5-2 (5'-GCC GAG TCC CCC TGC TCT GCA-3').

PCR was performed using a Perkin-Elmer Cetus (Emeryville, CA) "DNA Thermal Cycler" in a reaction mix containing DNA polymerase (Cetus, 10U/ml final concentration), the reaction buffer recommended by the manufacturer, dNTPs (final concentration 20. uM), primers (final concentration 0.3 uM each), and DNA template (100. ng genomic DNA). The cycle profile was an initial denaturation for 3 minutes at 94 C, then 35 cycles of 1 minute at 94 C, 2 minutes at 60 C, and 3 minutes at 72 C, with a final extension for 7 minutes at 72 C.

DNA Sequencing of (Unrelated) Human Vβ 8.1, 8.2, 8.3, and 8.5

For purposes of DNA sequencing, a large scale PCR was performed in 200 uL according to the conditions described above. Mineral oil was not used during the amplification, so that ethanol precipitation could be done in the same tube as the amplification. The concentrated PCR products were purified by electrophoresis through 1% low-melting point agarose, and the amplified products excised from the gel. Sequencing of the PCR products was performed in 96 well assay plates essentially according to Kretz (Kretz 1989) The sequencing primers used were the same as the DNA primers used for the original amplification.

IV. Results

Fine restriction map of Vβ 8.1 & 8.2

Southern blots were hybridized with Vβ8.1 coding sequence (cDNA YT35) (Siu 1984) or 5'flanking sequence (AP7-8159) probes. The resulting autoradiograms allowed assignment of orthologous sequences to particular fragment sizes, and these were used to restriction site map the Vβ 8.1 and 8.2 region. Two of these autoradiographs are shown in Figures 1A and 1B in order to demonstrate the absence of Vβ8.2 in the two chimps studied here, and the absence of the second gene segment in the cebus monkey. Figure 2 shows an autoradiograph of a squirrel monkey Southern blot probed with YT35, and suggests that only one fragment in each lane hybridizes with the probe. Both gene segments are present in human, gorilla, and macaque. Figure 1C shows the derived restriction map for this region in human, chimp, gorilla, macaque, and squirrel monkey.

Sequence alignment of primate Vβ 8.1 & 8.2

Human Vβ 8.1 and 8.2 DNA coding sequences are 98% similar, appear to be the result of a recent DNA sequence duplication and, as such, are defined as paralogous sequences. Sequence overlap comparison of the complete human sequence (data not shown) demonstrates 3.3 kb duplicands, each containing one paralogue, which are juxtaposed and in the same orientation. The junction of

these duplicands is identical in human, gorilla, and macaque, arguing that the duplication event predated the divergence of these species (ca 25 MYA). Figure 3 shows the alignment of 2 kb DNA sequence fragments containing human Vβ 8.1, human Vβ 8.2, and their primate orthologues.

Phylogenetic diversity

Parsimony analysis of the nucleotide substitutions was performed, and trees requiring the least number of changes between sequences from human, chimp, gorilla, pig-tailed macaque, and squirrel monkey were made. The phylogenetic arrangement of these sequences, illustrated in Figure 4, suggests that the Vβ8.1/2 ancestral gene segment was duplicated sometime after the old world monkey (OWM)-new world monkey (NWM) divergence (ca. 35 MYA). This is supported not only by the Vβ DNA sequence phylogeny, but also by the fact that we find no evidence of a duplication product in the cebus or squirrel monkeys (see Figures 1A, 1B). The phylogenetic arrangement of primate Vβ 8.1 and Vβ 8.2 DNA sequences and the physical map indicate that the last common ancestor of chimpanzee and human had both Vβ 8.1 and Vβ 8.2 orthologous gene segments, and that at least some chimpanzees have deleted the Vβ 8.2 orthologue.

The phylogenetic tree obtained from the entire sequence suggests that the Vβ8.1 and Vβ8.2 gene segments arose independently in the hominoid common ancestor (human,

chimpanzee, and gorilla) and in a macaque ancestor. Those findings were quite strongly supported. Additional changes to the most parsimonious branching arrangement were required to group together all of the Vβ 8.1 orthologous sequences and all of the Vβ 8.2 orthologous sequences (Vβ 8.1 and Vβ 8.2 defined by physical map position). Upon further inspection of the sequence data, it became apparent that the 5' 700 bp suggested a branching arrangement that supported separate Vβ8.1 and Vβ8.2 gene lineages, while the rest of the sequence data continued to strongly support the overall phylogenetic tree (Figure 5). The incongruency between these two phylogenetic branching arrangements suggests that some additional genetic mechanism was acting on these gene sequences. A pattern similar to this has been observed in the γ-globin genes of primates. In this latter case, in the time since a tandem 5 kb duplication in an anthropoid ancestor, more than 15 separate gene conversion events have homogenized γ1 and γ2 genes in human, chimpanzee, gorilla, orangutan, and rhesus monkey (Slightom 1987). If two such gene conversion events were hypothesized, one in a hominid ancestor and one in a macaque ancestor, then the phylogenetic branching arrangement of the 3' Vβ8 region can be reconciled with that of the 5' Vβ8 region and the physical map.

With regard to the species branching arrangement suggested by these Vβ8.1 and Vβ8.2 sequences, human and

chimpanzee are most closely related, followed by gorilla, macaque, and squirrel monkey. The most controversial aspect of this arrangement is the human, chimpanzee and gorilla placement. In the phylogenetic tree shown in Figure 5, the human-chimp grouping is supported by thirteen nucleotide positions. The next most parsimonious arrangement places human with gorilla, though seven additional changes are required to accommodate this arrangement. The grouping of human with chimpanzee is consistent with a growing body of molecular evidence from rRNAs, mitochondrial sequences, immunoglobulins, and globins (Koop, 1989).

Divergence analysis

The differences in DNA sequences listed in figure 3 were analyzed for non-coding sequence (positions 1 to 700, positions 701-end), and coding sequence (non-synonymous substitutions, synonymous substitutions). These data are summarized in Table 1. Non-coding sequence divergence values obtained from these orthologous Vβ gene region comparisons among human, chimpanzee, gorilla, macaque, and squirrel monkey are similar to values obtained from other non-coding sequences, most notably those from a 10 kb stretch of DNA including the β-globin locus (Koop, 1989) (Table 2). Overall, non-synonymous coding differences are less than these non-coding differences ($p < 0.01$, Wilcoxson Signed Rank test), demonstrating relative conservation.

The boundaries of the complementarity-determining (ligand-binding) regions (CDRs) of the T cell receptor V gene segments have been estimated (Clothia, 1988) and identified in our Vβ coding sequence data. We used the polypeptide KPISGHNSLF (positions 24-33) as being consistent with CDR 1, and used the polypeptide IYFNNNVPIDDSGMPE (positions 47-63) as being consistent with CDR 2. The nucleotides coding for these residues were then compared across species. The divergence values for these comparisons are given in Table 2, and indicate that non-synonymous differences within CDRs 1 & 2 were indistinguishable from non-coding differences (p = NSD, Wilcoxson Signed Rank test). This suggests that substitutions in functionally critical regions of the Vβ 8.1 and 8.2 gene segment products occur freely, but are neither positively (Maruyama, 1981) nor negatively selected.

Human Vβ 8.1 polymorphisms

Two alleles of human Vβ 8.1 from a single individual (from cosmids 7.1 and 12.18) were compared across the entire 2 kb fragment. Three polymorphisms were found, all in non-coding sequence, and are illustrated in the sequence comparison in Figure 3. The first polymorphism (C/T) is located 1.28 kb 5' to Vβ8.1 exon 1 at position 235. The second polymorphism (C/T) is located 0.98 kb 5' to Vβ8.1 exon 1 at position 542. The last polymorphism (A/G) is

located 1.05 kb 5' to Vβ8.1 exon 1 at position 466. This A/G polymorphism involves a Bam HI restriction site, has previously shown to be present at a bi-allelic frequency of 50% (Concannon 1987), and is relevant in human disease correlations (see discussion). As shown in the fine restriction map in Figure 2, this Bam HI site is present in all the other species studied, arguing that the second human allele lost the site through an A -> G substitution.

Vβ 8.1 coding sequences from fourteen (14) unrelated humans (28 chromosomes) were compared, and no polymorphisms were observed.

Human Vβ 8.2 polymorphisms

Vβ 8.2 coding sequences from ten unrelated humans (20 chromosomes) were compared, and two polymorphisms were observed. The first is located in the intron at position 1773, and involves a C <-> T substitution. The allelic frequency was 90% C and 10% T. The second is located in the second exon at position 1875 and involves a C <-> T substitution. This change is synonymous with respect to translated sequence. The allelic frequency is 90% C and 10% T. Both of these substitutions were noted to be homozygous in the same individual, and absent in the other individuals studied.

Human polymorphisms in Vβ 8.3

Vβ 8.3 coding sequences from thirteen unrelated humans (26 chromosomes) were compared. One polymorphism was observed at position 1816, involves a C <-> G substitution, and results in a His -> Asp amino acid substitution at position 10. The allelic frequency is 62% C and 38% G, and the alleles are in Hardy-Weinberg equilibrium (E(x)= 5 C/C, 6 G/C, 2 G/G; O(x) = 5 C/C, 6 G/C, 2 G/G).

Human polymorphisms in Vβ 8.5

Vβ 8.5 is a pseudogene in humans (Siu 1986). Vβ 8.5 coding region sequences from seven unrelated individuals (14 alleles) were compared, and no polymorphisms were observed.

V. Discussion

Dynamics

The data in this report suggests that the region containing Vβ 8.1 and 8.2 is mechanically active. The proposed dynamics of duplication, gene conversion, and deletion which have apparently occurred during the last 35 MY of primate evolution are illustrated in figure 4.

We have shown that Vβ 8.1 is paralogous to Vβ 8.2 due to duplication of a 3.3 kb region containing the prototypical gene segment. The duplicands are juxtaposed and in the same orientation. Duplicand junctions are identical in species which contain both duplicands, demonstrating that a single duplication event predated their speciation (ca. 25 MYA). The time of occurrence of the single duplication event is suggested by Southern blot analysis and can be estimated from the phylogenetic tree. Single observed gene segments in cebus and squirrel monkeys suggest that they represent pre-duplication organization; if so, the Vβ 8.1/8.2 duplication event occurred since the Old World-New World monkey divergence (ca. 35 MYA). This allows estimation of the timing of the duplication to between 25-35 MYA. Vβ 8.1 vs. Vβ 8.2 non-coding sequence divergence values of 7.5 -10.% (first 700 bases, inside duplication, outside putative conversion) suggest that the duplication event occurred 22-29 MYA (assuming neutral rate = 1.7×10^{-9} (Koop 1989). The Vβ 8.2-containing duplicand has been

deleted in the two chimpanzees studied here at some time since the Homo-Pan divergence (ca. 5 MYA).

The phylogenetic tree shows that both macaque gene segments segregate distinctly from the Vβ 8.1 and 8.2 branches of the hominoid lineage, suggesting that gene conversion events have occurred in both lineages during primate evolution. The directions of the gene conversion events is difficult to determine, however. Since divergence between Vβ 8.1 and 8.2 orthologues is generally greater than human-macaque divergence, the conversion events in the hominoid and macaque lineages may have employed alternate duplicands as the donor. The timing of these putative conversion events can be estimated from the substitution data. As illustrated in figure 5, the presumed converted region involves the 3' 1.3 kb of each duplicand, of which the non-coding portion of this is the 5' 800 base pairs. For this intra-duplicand, intra-conversion region, human Vβ 8.1 cf. 8.2 differ by 7.8%, gorilla Vβ 8.1 cf. 8.2 differ by 9.6%, and macaque Vβ 8.1 cf. 8.2 differ by 5.5%. These data suggest that the gene conversion event in the hominoid lineage occurred between 23-28 MYA, and that the gene conversion event in the macaque lineage occurred approximately 16 MYA.

Variation

We have shown that a relevant Vβ gene segment is evolutionarily conserved overall, but freely substituted in regions which probably code for ligand-binding portions of the TCR protein. We were unable to demonstrate positive selection for sequences which code for CDRs 1 & 2. Consistent with this is the observation that no non-synonymous substitutions were found in comparison of Vβ 8.1 and 8.2 from multiple unrelated human individuals. The only non-synonymous substitution we found was for position 10 in the Vβ 8.3 protein, a position outside the predicted CDRs, this second allele noted in 10 of 26 chromosomes studied.

Compared to non-coding flanking sequences, overall Vβ8.1 and 8.2 non-synonymous substitution rates were slightly conserved. We observed an overall non-synonymous substitution rate of 1-1.5 x 10^{-9} changes/site/year, comparable to that of immunoglobulin (Ig V_H = 1.07 $\pm$ 0.19 x 10^{-9}, Ig gamma1 = 1.46 $\pm$ 0.13 x 10^{-9}). Non-Ig superfamily members tolerate more (e.g., gamma interferon = 2.79 $\pm$ 0.31 x 10^{-9}) or less (α globin = 0.55 $\pm$ 0.11 x 10^{-9}, insulin = 0.13 $\pm$ 0.13 x 10^{-9}) non-synonymous substitution, these differences presumably related to functional constraints on the protein (Li 1991).

Compared to non-coding flanking sequences, CDR 1 and 2 non-synonymous substitution rates were not conserved. We observed a CDR 1 and 2 non-synonymous substitution rate of

$1.5\text{-}3.0 \times 10^{-9}$ changes/site/year for Vβ 8.1 and 8.2, but failed to demonstrate statistically significant positive selection for these regions. By doing so, we have shown that T cell antigen receptor does not use positively selected germline Vβ polymorphism to generate the high degree of TCR variability expected prior to positive and negative selection events. Random combinatorial rearrangement of multiple gene segments with junctional variability, along with random combinatorial pairing of translated products of productive α and β gene rearrangements, are apparently sufficient for the generation of a selectable set of T cell antigen receptor proteins (TCRs) on T cells.

The absence of positive selection for TCR CDR 1 and 2 differs from what has been observed for the TCR analogue, immunoglobulin, and for the TCR ligands, the MHC class I and II proteins. The hypervariable regions of rearranged Ig genes are correlated with the crystallographically defined ligand-binding regions (complementarity-determining regions, CDRs). In some immunoglobulin genes, CDR non-synonymous (amino acid-substituting) nucleotide substitution rates exceed synonymous (amino acid-conserving) substitution rates, demonstrating positive selection for high amino acid variation in these ligand-binding loops of the immunoglobulin (Tanaka 1989). The picture that results for Ig, and now TCR, is that of selection-chimeric proteins

which are conserved except at ligand-binding sites, where a spectrum of neutral to positive selection occurs. This occurs in spite of the fact that tens of V gene segments are available for Ig L/H and TCR α/β loci for rearrangement, and that Ig can hypermutate rearranged Ig genes to maximize ligand-binding affinity.

Unlike immunoglobulin, which recognizes a three-dimensional ligand in liquid or solid phase, the T cell receptor is constrained to recognize short peptides, catabolized at sites of intracellular processing, which are non-covalently bound to, and presented by, self major histocompatability complex (MHC) proteins. MHC class I protein structure has been determined crystallographically, and the ligand-binding site has been defined in terms of 57 amino acid residues in the α_1 and α_2 domains (Bjorkman 1987). Nucleotide substitution rates in the sequences coding for these ligand-binding residues have been compared between mouse and human, and it is found that non-synonymous rates exceed synonymous rates (Hughes 1990), arguing that amino acid variability is positively selected for (Maruyama 1981). This empirical observation is consistent with an advantage which would accrue to individuals able to bind more, rather than fewer, of the foreign peptides generated by antigen processing. Because of this focal positive selection, multiple alleles would be expected for a given locus, and a snapshot of human and mouse confirms this high degree of MHC

protein polymorphism for both class I and class II loci (Klein 1986). Amino acid variation in the TCR Vβ (8.1,8.2) CDRs 1 and 2 is neither positively nor negatively selected for, and these Vβ gene segments are consequently minimally polymorphic. The minimal variation in Vβ sequence which we observed does not rule out the existence of such pathogenic Vβ gene segment alleles.

Relevance

Disease risk for a variety of human autoimmune diseases can be stratified by MHC allele, arguing that pathogenic TCR-peptide-MHC combinations exist in these individuals. Similarly, TCR alleles may also confer autoimmune disease risk, and disease association studies suggest this possibility.

Although particular Vβ alleles have not been proven to be etiologic for an autoimmune disease at this time, disease associations with RFLPs within the T cell receptor α and β loci suggest that pathogenic Vβ alleles could increase autoimmune disease risk, much as has been observed for MHC alleles (Klein 1990). Systemic lupus erythematosus, for example, has been associated with a TCR Cα/PstI polymorphism (Tebib 1990), and insulin-dependent diabetes mellitus (McMillan 1990), Graves' disease (Demaine 1987), Hashimoto's thyroiditis (Ito 1989), and membranous nephropathy (Demaine 1988) have been associated with a TCR Cβ/BglII polymorphism

(Robinson 1985).

In particular, gene segments in linkage dysequilibrium with the BamHI/Vβ8.1 RFLP described in this paper (including Vβ 12 (11 kb 5'), Vβ 8.1 (1.0 kb 3'), Vβ 8.2 (4.3 kb 3'), and Vβ 8.3 (also 3') may be relevant to the pathogenesis of a number of human autoimmune diseases. Multiple sclerosis, a T cell-mediated autoimmune disease of the central nervous system, has been associated with the Vβ8/BamHI (described herein) - Vβ11/BamHI RFLP haplotype (Beall 1989) and the Vβ12/BamHI (the same BamHI polymorphism as detected with Vβ8) - Vβ12/HindIII - Cβ/BglII RFLP haplotype (Seboun 1989). The Vβ8-Vβ11 haplotype association may reflect an MS susceptibility gene amongst TCR Vβ, rather than TCR Dβ, Jβ, or Cβ, gene segments (Charmley 1991). Recently, Vβ12 was found in productive TCR rearrangements of T cells from three patients with MS (Lee 1991), and was found to be used in T cells specific for one of the T cell epitopes of human MBP (Wucherpfennig 1990). This same Vβ8/BamHI polymorphism has also been associated with rheumatoid arthritis in HLA $DR4^{+}$ individuals (Funkhouser 1992) and HLA-random individuals (Gao 1988).

Since DNA coding sequence polymorphisms have been identified in Vβ1 (Robinson 1989), Vβ 6.7 (Posnett 1990; Li 1990), and (Vβ2.1, 5.3, 7.2, 8.2, 12.4, and 16) (Plaza 1991), of which several are non-synonymous (Vβ1, Vβ 2.1, Vβ 5.3, 6.7, and Vβ 13.4), it is reasonable to suspect that

the "disease susceptibility genes" linked to Vβ RFLPs in disease association studies are in fact TCR Vβ gene segment alleles. However, particular disease-associated Vβ alleles have not yet been identified for a T cell autoimmune disease, even though reproducible linkage studies imply their existence.

This project sought to determine to what degree human T cell antigen receptor protein variation is attributable to DNA sequence variation in V gene segments. Vβ8 gene sequences within humans were compared to gain a sense for the role of polymorphism in creating diversity among higher primates, and hVβ8.1 and 8.2 orthologues in other primates were compared to gain a sense for the role of natural selection in conservation or the promotion of change. No evidence for positive selection of variation in DNA regions coding for ligand-binding domains was observed, and minimal polymorphism amongst unrelated humans was observed. Individual germline Vβ gene segment variation appears to be a minor contributor to the spectrum of T cell antigen receptor protein diversity.

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TABLE LEGENDS

Table 1:

Non-coding vs. coding sequence divergence. Divergence values are given for non-coding flanking sequence and coding sequence for the Vβ 8 subfamily gene segments studied. Non-coding sequence is subdivided into regions which have probably undergone gene conversion events ("700-end") and those which have probably not ("1-700"). Coding sequence analysis is subdivided into changes which result in an amino acid substitution ("non-synonymous") and those which do not ("synonymous") (Saitou and Nei 1986). Divergence is expressed as DNA sequence percentage difference.

Table 2:

Non-coding vs. non-synonymous coding sequence divergence. Non-synonymous coding sequence divergence is estimated for the entire coding sequence and for the putative CDRs. These data are compared to non-coding divergence estimates for non-coding flanking sequence and for the globin β locus. Rates are calculated in units of 10^{-9} changes/site/year. The Wilcoxson Sign Rank test was used to determine statistically significant differences between non-synonymous and non-coding percentage differences.

Table 1: Non-coding vs. Coding Sequence Divergence

Non-Coding Sequence Divergence (%) (1-700/700-end)

		1	2	3	4	5	6	7	8	9	10	11	12	13
Human 8.1a	1		0.44	9.1	0.59	8.1	1.0	10.3	6.3	32.8	—	—	—	—
			0.00	7.8	1.7	7.2	4.8	9.9	8.0	27.2	10.4	57.6	16.9	21.2
Human 8.1b	2	0.0		9.3	1.0	8.3	1.5	10.5	6.8	33.6	—	—	—	—
		0.0		7.8	1.7	7.2	4.8	9.9	8.0	27.2	10.4	57.6	16.9	21.2
Human 8.2	3	1.6	1.6		8.8	3.3	8.6	8.5	10.5	35.4	—	—	—	—
		6.8	6.8		8.0	2.2	10.5	11.8	10.3	30.5	12.3	57.4	16.7	21.4
Chimpanzee 8.1	4	0.8	0.8	2.3		7.8	10.4	10.0	6.0	32.6	—	—	—	—
		2.7	2.7	6.8		7.6	4.5	9.9	8.7	27.1	11.7	61.9	17.0	21.0
Gorilla 8.2	5	5.4	5.4	5.0	5.4		7.6	7.8	10.1	35.1	—	—	—	—
		6.9	6.9	8.5	6.9		9.6	10.7	9.2	30.4	11.0	60.5	15.6	21.2
Gorilla 8.1	6	1.6	1.6	1.6	1.6	5.7		10.3	6.5	32.8	—	—	—	—
		5.4	5.4	6.8	5.5	9.8		12.4	10.9	28.7	16.9	64.3	17.9	24.3
Pig-tailed Macaque 8.2	7	5.1	5.1	4.3	5.1	8.3	4.3		10.1	37.7	—	—	—	—
		13.6	13.6	14.8	10.9	18.7	12.2		5.5	27.6	17.3	63.3	17.8	22.3
Pig-tailed Macaque 8.1	8	5.6	5.6	5.4	5.6	9.0	4.9	3.1		34.4	—	—	—	—
		14.5	14.5	13.7	11.8	19.1	11.7	9.6		28.4	13.0	60.5	16.3	22.0
Squirrel Monkey 8.5	9	17.1	17.1	17.0	16.1	21.6	17.1	16.7	17.7		—	—	—	—
		29.3	29.3	30.8	28.0	28.9	27.7	30.5	27.2		32.5	88.2	26.9	18.6
Squirrel Monkey 8.1	10	8.1	8.1	7.4	8.0	10.7	7.5	7.0	8.4	17.1		—	—	—
		14.0	14.0	10.7	14.1	16.8	14.7	22.8	22.4	36.4		65.2	17.6	24.8
Human 8.3	11	15.3	15.3	15.4	14.5	17.5	14.2	15.9	16.2	22.0	16.4		—	—
		30.6	30.6	30.4	33.5	36.3	30.5	36.7	34.6	34.8	34.8		59.0	61.3
Human 8.4	12	16.4	16.4	15.3	15.6	19.9	15.7	15.3	16.4	14.5	14.5	21.4		—
		35.3	35.3	35.1	32.9	35.4	35.2	33.8	33.0	30.4	41.7	35.4		18.5
Human 8.5	13	17.8	17.8	17.8	17.0	22.2	17.8	17.9	18.4	6.9	18.0	22.9	16.2	
		30.7	30.7	33.3	28.2	30.3	30.6	32.8	34.0	11.3	39.0	38.5	29.7	

Non-Synonymous/Synonymous Coding Sequence Divergence (%)

Table 2:

		Non-Coding Divergence		Non-synonymous Coding Sequence Divergence			
		Globin β Locus	TCR Vβ Locus	TCR Vβ8.1	TCR Vβ8.2	CDRs Vβ8.1	CDRs Vβ8.2
	MYA	%Diff (Rate)	%Diff (Rate)	%Diff (Rate)	%Diff (Rate)	%Diff (Rate)	%Diff (Rate)
Hu-Ch	5	1.61 (1.6)	1.27 (1.3)	0.8 (0.8)	---	1.6 (1.6)	---
Hu-Go	7	1.72 (1.2)	2.96 (2.1)	1.6 (1.1)	5.0 (3.6)	1.6 (1.1)	4.8 (3.4)
Hu-OWM	25	7.18 (1.4)	8.86 (1.8)	5.6 (1.1)	4.3 (0.9)	8.0 (1.6)	9.6 (1.9)
Hu-NWM	35	11.7 (1.7)	11.4 (1.6)	8.1 (1.2)	7.4 (1.0)	18.7 (2.7)	19.3 (2.8)
Ch-Go	7	1.80 (1.3)	3.06 (2.2)	1.6 (1.1)	---	0.0 (0.0)	---
Ch-OWM	25	7.32 (1.5)	7.61 (1.5)	5.6 (1.1)	---	6.4 (1.3)	---
Ch-NWM	35	12.0 (1.7)	11.4 (1.6)	8.0 (1.1)	---	17.1 (2.4)	---
Go-OWM	25	7.77 (1.6)	9.31 (1.9)	4.9 (1.0)	8.3 (1.7)	6.4 (1.3)	10.2 (2.0)
Go-NWM	35	11.9 (1.7)	14.3 (2.0)	7.5 (1.1)	10.7 (1.5)	17.1 (2.4)	19.9 (2.8)
OWM-NWM	35	13.8 (2.0)	15.4 (2.2)	8.4 (1.2)	7.0 (1.0)	12.2 (1.7)	9.6 (1.4)
				[P <0.01)]		[NS]	

FIGURE LEGENDS

Figure 1. Primate genomic southern blot autoradiographs and restriction map for human Vβ 8.1 and 8.2, and their orthologues. Southern blots were probed with ^{32}P-labelled YT35 cDNA and washed in 0.5X SSC at 60 C. Human Vβ 8.1 migrates as a 2.1 kb BamHI fragment and as a 4.8 kb EcoRI fragment. Human Vβ 8.2 migrates as a 3.3 kb BamHI fragment, and as a 2.1 kb EcoRI fragment. Figure 1C shows the proposed restriction map, derived from both Southern blot and sequencing data. The bars over "8.1" and "8.2" estimate the YT35 cDNA probe. Bold lines indicate the availability of sequence data. Brackets indicate the borders of the 3.3 kb duplicands, and parentheses indicate the absence of a duplicand.

Figure 2. Genomic Southern blot autoradiograph of squirrel monkey DNA. This Southern blot was probed with ^{32}P-labelled YT35 cDNA (human Vβ 8.1) and washed under the listed conditions. Black dots indicate the bands which remained after washing at 0.1X SSC, as shown in the phosphor imager data in the lower panel.

Figure 3. Sequence comparison of human Vβ 8.1, human Vβ 8.2, and their orthologues in chimp, gorilla, macaque, and squirrel monkey. Absence of symbols indicates identity of

bases and asterisk (*) indicates deletion of bases. H = human, C = chimp, G = gorilla, R = macaque, and S = squirrel monkey.

Figure 4. Proposed dynamics in the evolution of the Vβ 8.1 and 8.2 region. Open rectangles indicate the Vβ 8.1 and 8.2 gene segments.

Figure 5. Phylogenetic trees for different portions of the compared 2.1 kb sequence. Different phylogenetic trees resulted from parsimony analysis (Fitch, 1971) of the sequence data for the regions (1-700) and 700-end) (see Table 1).

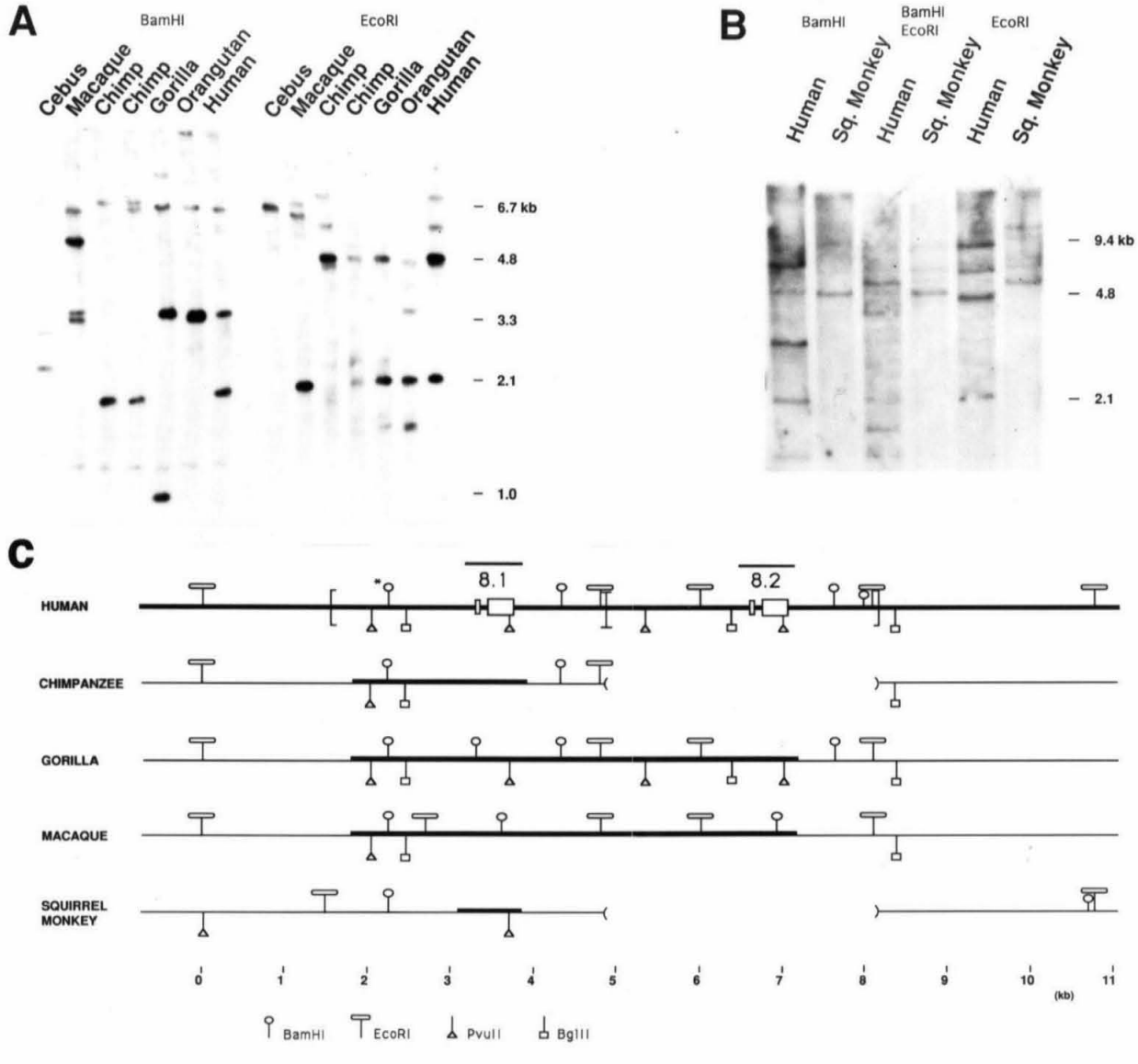

A
BamHI
EcoRI
Cebus
Macaque
Chimp
Chimp
Gorilla
Orangutan
Human
Cebus
Macaque
Chimp
Chimp
Gorilla
Orangutan
Human
6.7 kb
4.8
3.3
2.1
1.0
B
BamHI
BamHI
EcoRI
EcoRI
Human
Sq. Monkey
Human
Sq. Monkey
Human
Sq. Monkey
9.4 kb
4.8
2.1
C
8.1
8.2
HUMAN
CHIMPANZEE
GORILLA
MACAQUE
SQUIRREL MONKEY
0
1
2
3
4
5
6
7
8
9
10
11
(kb)
BamHI
EcoRI
PvuII
BglII

Figure 1

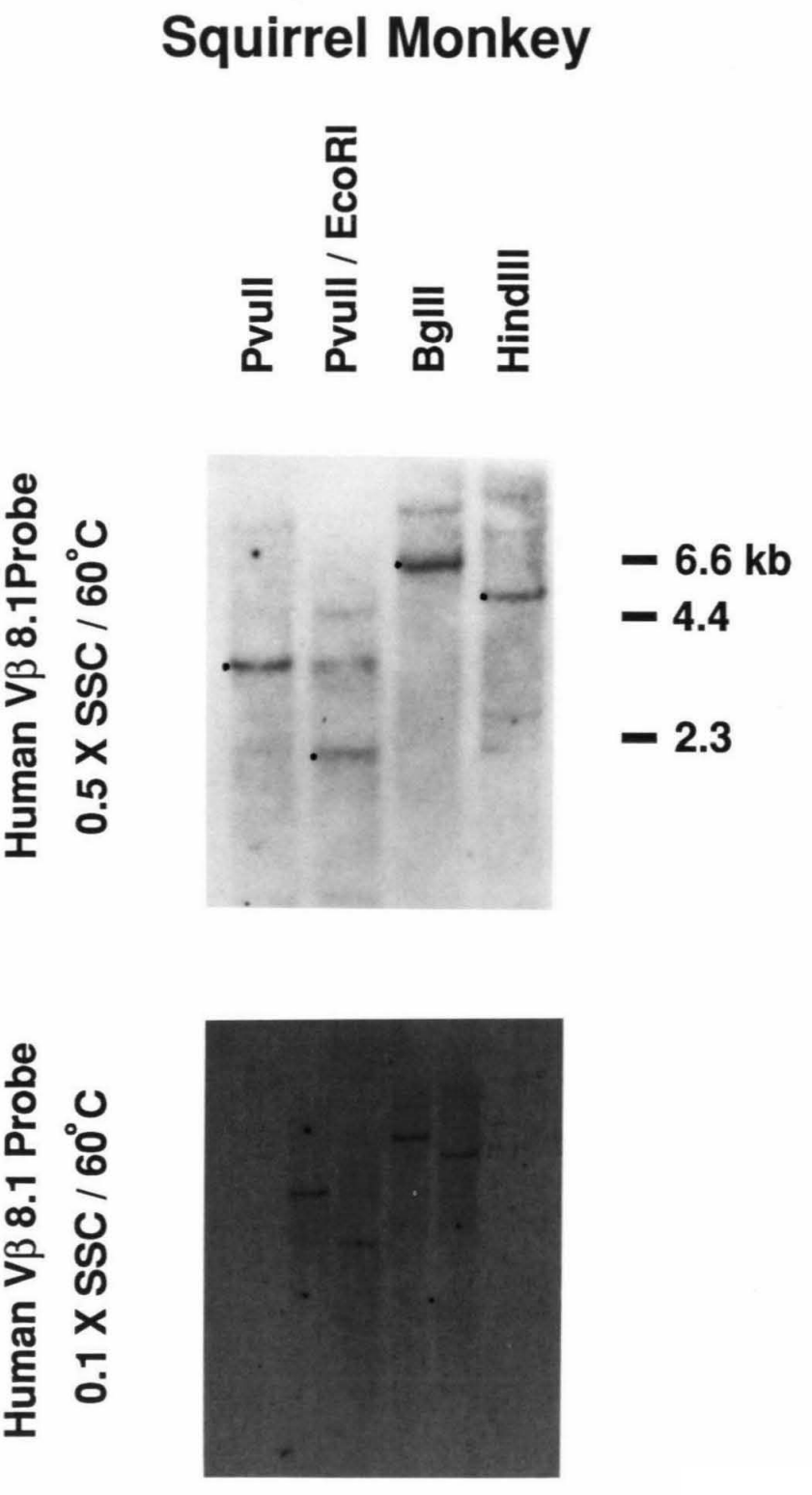

Squirrel Monkey
PvuII
PvuII / EcoRI
BglII
HindIII
Human Vβ 8.1Probe
0.5 X SSC / 60°C
6.6 kb
4.4
2.3
Human Vβ 8.1 Probe
0.1 X SSC / 60°C

Figure 2

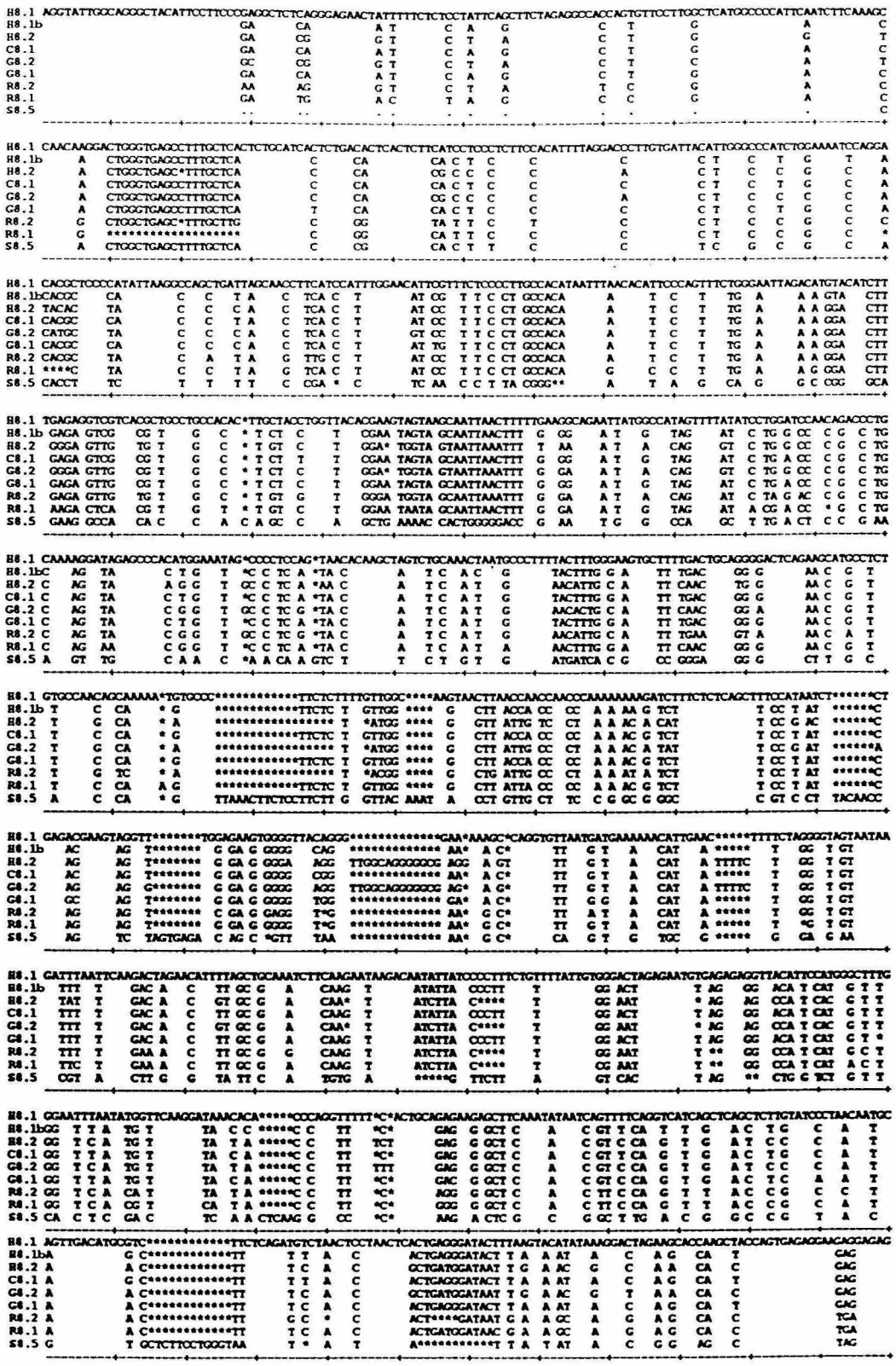
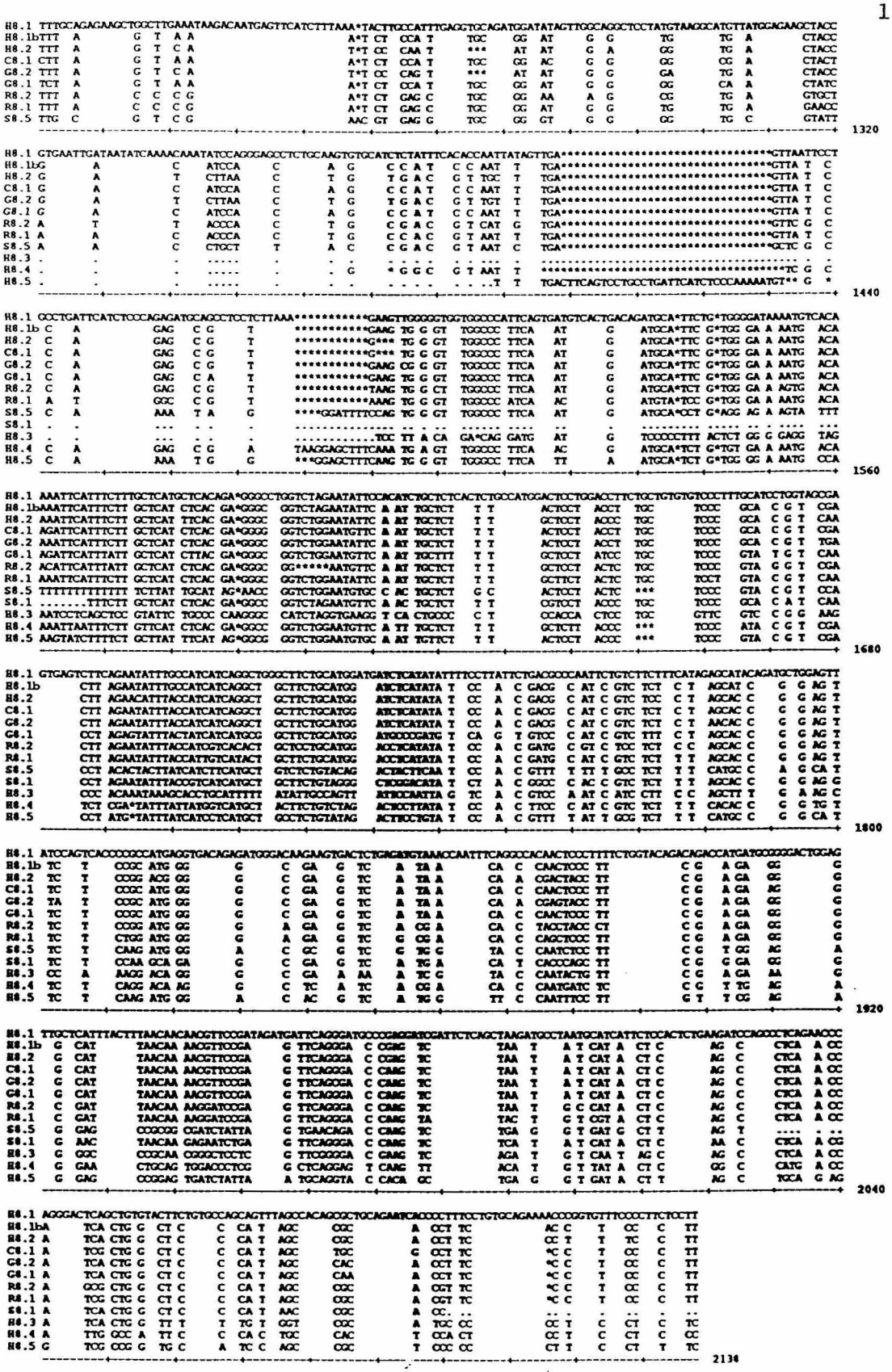


Figure 3



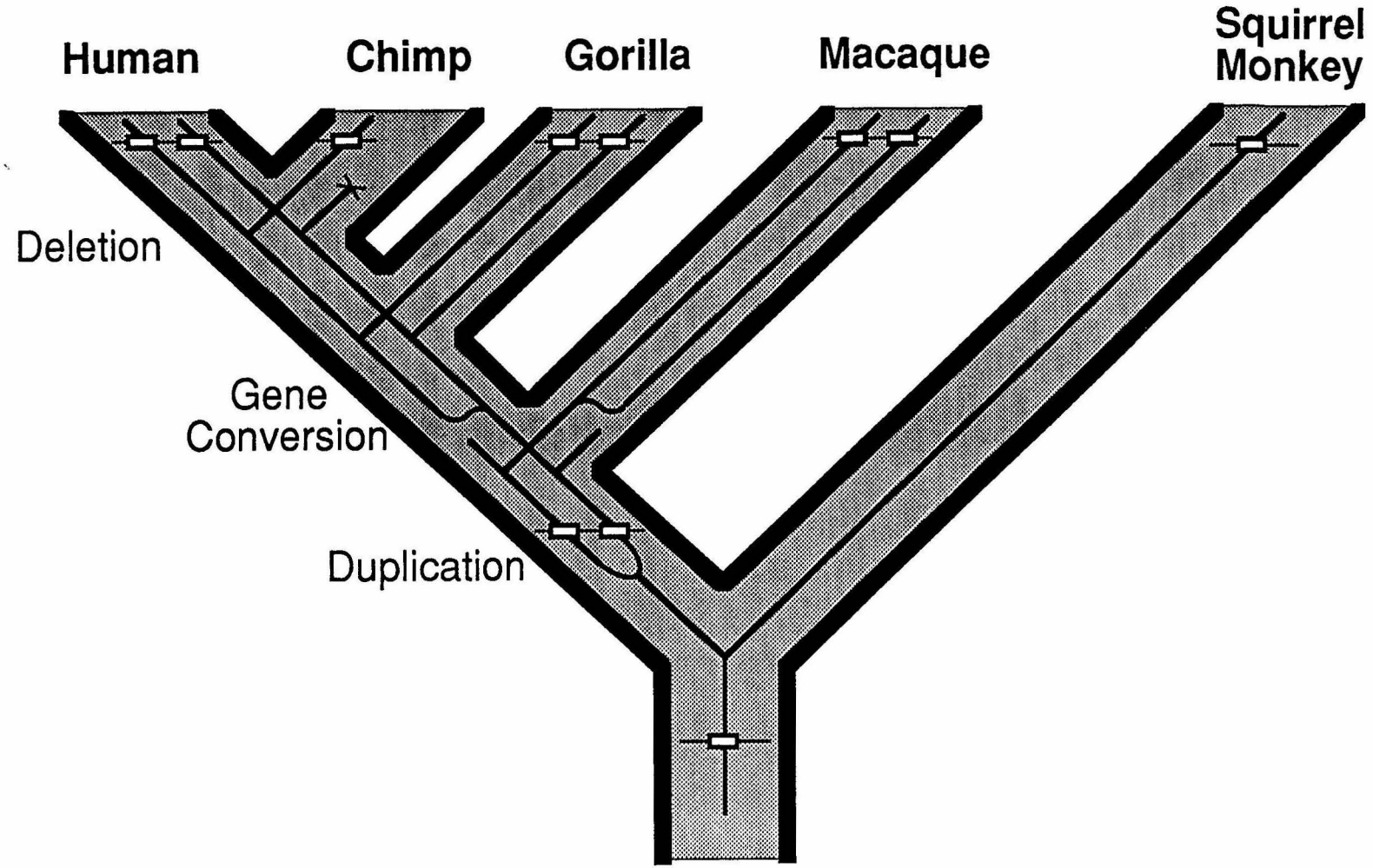

Human
Chimp
Gorilla
Macaque
Squirrel Monkey
Deletion
Gene Conversion
Duplication

Figure 4

Vβ 8 gene region

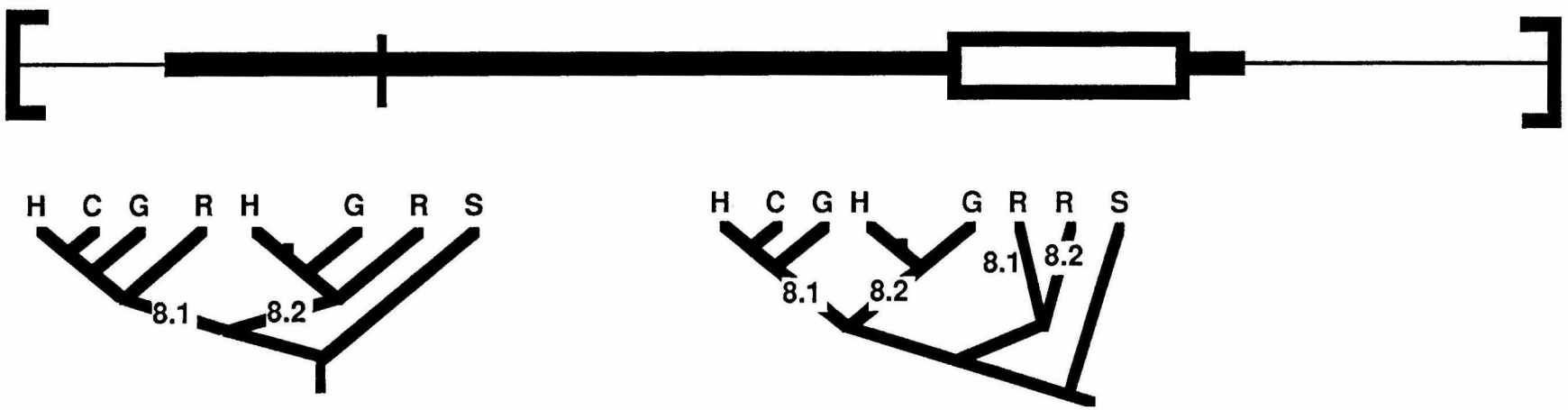


Figure 5