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Cross -reactive idiotypes: Vaccine targets for autoimmune diseases.

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CROSS-REACTIVE IDIOTYPES: VACCINE TARGETS FOR AUTOIMMUNE DISEASES

by

BRETT D. NOERAGER

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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ABSTRACT OF DISSERTATION
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Multiple sclerosis (MS) and its animal model, experimental allergic encephalomyelitis (EAE), are autoimmune diseases of the central nervous system (CNS) resulting in the destruction of the myelin sheath and impaired nerve impulse conduction. Although traditionally regarded as T cell mediated diseases, it now appears that antibody (Ab) plays an integral role in disease modulation. A myelin basic protein (MBP)-specific monoclonal antibody (mAb), termed F28C4, has been shown to share remarkable functional and structural relatedness and consequently a cross-reactive idiotype (CRId) with T cell receptors (TCR) from certain encephalitogenic T cells. This CRId has been defined using the anti-CRId mAb, F30C7, which blocks both peptide recognition by F28C4 and peptide stimulation of MBP acetyl (Ac) 1-9-specific T cells. In this study we have tested whether the CRId in F28C4 could serve as a surrogate to encephalitogenic T cells or their TCR as a vaccine target in EAE. When we actively immunize animals with mAb F28C4 prior to disease induction we can prevent EAE with 80-100% effectiveness independently of major histocompatibility complex (MHC) haplotype, encephalitogenic epitope, or species. In addition to its use as a vaccine, we also demonstrate the therapeutic potential of this CRId. It appears F28C4 is working through an anti-CRId pathway in manifesting its protective effects as demonstrated by the ability of the anti-CRId mAb F30C7 to protect animals from EAE when passively administered. This probably

occurs through interaction of F30C7 with certain T cells in a clonotypic fashion, thereby causing these cells to become $CD4^+/CD25^-$ regulatory T cells that home to the CNS and down-regulate encephalitogenic T cells in an antigen-non-specific manner. We have further demonstrated that the CRId on this mouse Ab has a human equivalent (i.e. VλVIII). The CRId in human as well as mouse immunoglobulin (Ig) is associated with reactivity to MBP. Collectively, these results suggest that the CRId on mAb F28C4 may be of therapeutic potential for treatment of MS patients. In a more general sense, the use of mAb possessing CRId that induces the generation of $CD4^+/CD25^-$ regulatory T cells may prove to be beneficial in treatment of other autoimmune diseases.

DEDICATION

This dissertation is dedicated to the memory of Dr. John N. Whitaker, a rare physician/scientist who did both with ease and excellence and still had time to enjoy the finer things in life. An outstanding teacher and mentor, he retained a thirst to learn. He is missed.

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LIST OF ABBREVIATIONS

Ab	antibody
Ac	acetyl
Ag	antigen
BJP	Bence Jones protein
BSA	bovine serum albumin
CNS	central nervous system
CDR	complementarity-determining region
CRId	cross-reactive idiotype
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
GP	guinea pig
H	heavy
Id	idiotype, idiotypic
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.p.	intraperitoneal, intraperitoneally

KLH	keyhole limpet hemocyanin
L	light
LNC	lymph node cell
mAb	monoclonal antibody
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
rEAE	relapsing-remitting experimental allergic encephalomyelitis
RNA	ribonucleic acid
SCH	spinal cord homogenate
ssDNA	single-stranded deoxyribonucleic acid
TCR	T cell receptor
V	variable
WM	Waldenstrom's Macroglobulinemia

INTRODUCTION

Specific immunotherapy for autoimmune diseases

Treatment of autoimmune disease involving specifically targeting the errant immune cells would be much more advantageous than more general immunosuppressive agents which also compromise beneficial responses. Several 'antigen (Ag)-specific' approaches have been tested including tolerance induction (Karpus et al., 1996), T cell and T cell receptor (TCR) peptide vaccination (Whitham et al., 1993; Chou et al., 1996), and immune deviation (Ruuls and Sedgewick, 1998). Recent studies showing overlap of disease-associated epitopes that are recognized by T and B cells together with our own data showing functional and structural relatedness of the Ag receptors on certain of the aforementioned cells suggested a novel and relatively specific approach. The central idea is that if there is considerable overlap in the structure and fine specificity of antibody (Ab) and TCR for a disease-associated epitope, then such an Ab could potentially serve as a surrogate vaccine to an encephalitogenic T cell or its TCR. Such Ab vaccines would therefore lead to not only an anti-idiotypic (Id) Ab response but also a cross-reactive anti-clonotypic Ab response against disease-associated T cells. Evidence that supports the feasibility of this approach in the autoimmune disease multiple sclerosis (MS) and its model, experimental allergic encephalomyelitis (EAE), is discussed below.

Immune mechanisms in MS and EAE

MS is a chronic inflammatory demyelinating disease afflicting over 350,000 people in the U.S. alone. Focal infiltration of myelin-specific lymphocytes into the white matter of the central nervous system (CNS) results in a loss of neurologic function. T cells specific for human myelin basic protein (MBP), which comprises 30% of CNS myelin, have been implicated in disease (Bashir and Whitaker, 1998). Residues 82-100 of MBP have been shown to harbor the immunodominant T cell epitope. It was demonstrated that this peptide binds with high affinity to the HLA-DR2 molecule, which has been implicated in disease susceptibility (Martin et al., 1990; Wucherpfennig et al., 1990, 1991). Another encephalitogenic peptide located within this region of MBP, residues 87-99, induces a chronic relapsing form of experimental allergic encephalomyelitis (rEAE) in SJL mice (Gaur et al., 1997).

EAE is an organ-specific autoimmune disease affecting the CNS and is considered to be the prime animal model for MS. EAE is characterized by the infiltration of myelin-specific $CD4^+$ Th1 type cells into the CNS following immunization with a variety of myelin proteins or their encephalitogenic peptides. Although MBP is the most extensively studied encephalitogenic CNS molecule, other myelin components have encephalitogenic properties as well including proteolipid protein, myelin oligodendrocyte glycoprotein, and myelin-associated glycoprotein (MAG). It is clear that T cells play a central role in MS and EAE, whereas the role of B cells has yet to be fully defined. There is increasing evidence to suggest that humoral components may be involved in initiation, perpetuation, or modulation of MS or EAE. It has been shown that in both MS and EAE, B cells penetrate the blood brain barrier into the CNS, transform into plasma cells, and can locally synthesize immunoglobulin (Ig), some of which is anti-MBP

(Martino et al., 1991). The MBP 83-97 peptide was recently shown to be immunodominant for autoantibodies purified from CNS lesions from MS patients. Furthermore, residues crucial for autoantibody binding were located in the same region as that shown to be crucial for T cell recognition, thus suggesting that human MBP-specific autoantibodies can have fine specificity similar to that of autoreactive T cells for the same region of human MBP (Wucherpfennig et al., 1997). Indeed, it has been shown that in MS and marmoset EAE, autoantibodies to CNS myelin are present and bind to disintegrating myelin around axons in lesions (Genain et al., 1999). Moreover, since Ag-specific B cells are competent antigen-presenting cells, such data suggest a role of B cells in MBP-specific T cell activation. We have previously shown that a monoclonal antibody (mAb) and TCR against the same encephalitogenic peptide can share a common cross-reactive idiotype (CRId) (Maier et al., 1994); thus, it is tempting to speculate that this sharing of Ids may extend into the human as well.

The role of humoral responses in modulation of EAE

Although T cells clearly are central players in the pathogenesis of EAE, there is accumulating evidence to suggest B cells are important in modulation of disease. Not only can B cells infiltrate the CNS in both MS and EAE, but they can locally synthesize Ig as well (Ewan and Lachmann, 1979). In chronic rEAE, which more closely resembles MS both clinically and histopathologically, increased IgG levels and oligoclonal Ig bands have been found in serum, cerebrospinal fluid (CSF), and CNS extracts (Mehta et al., 1985). Unlike T cells, however, B cells are present in the CNS lesions of animals with acute EAE but only in small numbers. Active immunization with CNS Ag induces Ab production, but titers do not correlate with the severity of EAE. For example, in the

Lewis rat model Ab is initially detected during the onset of disease and is maximal after recovery (Day and Pitts, 1974). Lewis rats are subsequently resistant to reinduction of EAE following recovery from active disease. $CD4^+$ T cells isolated during the recovery phase can transfer resistance only if co-transferred with MBP-primed B cells (Karpus and Swanborg, 1991). The finding that sera from rats recovered from EAE passively protect against active EAE parallels such B cell modulation of resistance (Macphee et al., 1990). Also, animals that are Ig deficient or have B cell suppression are resistant to actively induced EAE (Willenborg et al., 1986). Preventing the development of mature B cells by disrupting the IgM heavy (H) chain transmembrane region caused animals to exhibit no differences in disease onset or severity of acute EAE but rarely recovered (Wolf et al., 1996). Collectively, these data suggest that humoral responses play an important regulatory role in the pathogenesis of EAE.

The Id network in EAE

One of the hallmarks of EAE is the observation that the usage of TCR genes can be biased or restricted, particularly Vβ8 gene products, in the recognition of MBP or encephalitogenic MBP peptides (Acha-Orbea et al., 1988; Burns et al., 1989). mAb administration against Vβ8 or its peptides is effective in preventing EAE (Hashim et al., 1990; Weinberg et al., 1994). In addition, anti-Id or anti-clonotypic T cells can also prevent EAE, suggesting that an Id network may be involved in the pathogenesis or immunoregulation of EAE (Lider et al., 1988). The previously mentioned expression of CRId on Abs as well as TCR against encephalitogenic epitopes of MBP provides evidence for this.

Within the MBP molecule reside multiple immunogenic and encephalitogenic epitopes. MBP peptide acetyl (Ac) 1-9 is the dominant, I-A^u restricted encephalitogenic epitope for PL/J mice (Zhou and Whitaker, 1990). In PL/J mice and Lewis rats TCR Vα2.3β8.2 similarly restricts the TCR repertoire of the encephalitogenic T cell response to different MBP epitopes (Esch et al., 1992). F28C4, a mAb raised in the PL/J mouse against MBP peptide Ac 1-9, shares a CRId with the PL/J Vβ8.2$^+$ TCR recognizing the same MBP peptide. This CRId has been defined using the anti-Id mAb, F30C7, which can block both peptide recognition by F28C4 (Zhou and Whitaker, 1992) and peptide stimulation of MBP Ac 1-9-specific T cells (Zhou and Whitaker, 1993), suggesting that the CRId resides at or near the combining site of F28C4 and the TCR and therefore is not a public Id. The ability of mAb F30C7 anti-Id to lessen clinical EAE in the adoptive transfer model in PL/J mice, possibly by T cell anergy (Zhou and Whitaker, 1993; Zhou et al., 1994), suggests a role for this CRId and the related Id network in EAE.

The structure and fine specificity of F28C4 appear to be crucial for its paratope and Id characteristics. Sequence homology of 75% exists between complementarity determining region (CDR) 3 of F28C4 H and L chain V regions and the V-D-J junction of the TCR Vβ8.2 from encephalitogenic T cells (Maier et al., 1994). Other Ig CDR3s do not share this homology and arises, in part, because F28C4 uses an unusual L chain V region called Vλx (Dildrop et al., 1987; Sanchez et al., 1987). No Ab with this L chain had any known specificity prior to the demonstration that an Ab with the Vλx L chain could bind MBP.

Characteristics of Vλx

Vλx is a newly described Vλ gene segment that was discovered in 1987 in polyclonally activated B cells (Dildrop et al., 1987; Sanchez et al., 1987). Vλx rearranges with Jλ2-Cλ2 and displays at least three unique features: Its amino acid sequence is only 30-33% homologous to other known Vλ and/or V-kappa (κ) genes; the last codon of the Vλx gene is a TAA termination codon that must be disrupted by Jλ2 to create a function-al codon; and such appropriate joining at the V-J junction results in a third hypervariable region extended by an additional four amino acids. It is interesting to note that homol-ogous sequences have been found in various mammalian species including man, thus suggesting that Vλx apparently existed before the speciation of mammals (Sanchez et al., 1990). Considering the rarity of Vλx usage, less than 0.5% of all Ig in normal mice sera, it was tempting to speculate that the expression of Vλx in response to an encephalito-genic immunogen may not have been a coincidental event but may rather represent an underlying component involved in the immunopathogenesis of CNS myelin damage.

The expression of Vλx in response to an MBP peptide led to the notion that this finding was not just fortuitous but may represent an underlying association of Vλx with MBP reactivity. Consistent with this notion was the observation that the only known Vλx-containing IgM mAbs, each of previously unknown specificity, as well as recombin-ant Vλx alone, were able to bind human MBP (Galin et al., 1996a). Since mAbs are somewhat artificial in their selection and might not occur in a more natural setting, we purified the Vλx-containing Ig from normal BALB/c sera and found that these Abs also bind human MBP. Interestingly, the average affinity of these Abs was higher than that

of the Vλx-containing IgM mAbs and was closer to that of F28C4 (IgG2a, Vλx). Evaluating the H chain isotype distribution of Vλx-bearing Abs in normal mouse sera made it possible to understand the apparently higher MBP reactivity of naturally occurring Vλx Igs relative to that of the Vλx$^-$ IgMs. A biased H chain isotype association with a skewing to IgG2a and b was demonstrated (Galin et al., 1996b). The IgG2a restriction was even more apparent in Vλx-containing Abs that bind MBP. Thus, the interaction of Vλx with MBP seems to be facilitated by an association with γ2a that may reflect restricted H chain V region usage by this isotype. This suggests that, as with MBP reactive TCR in EAE, restricted V region usage may extend to an Ab.

Human VλVIII L chains

Human λ L chains were originally classified on the basis of distinctive amino acid and nucleotide sequence homologies into six V region subgroups (Kabat et al., 1991). Five of the six subgroups, VλI, VλII, VλIII, VλIV, and VλVI, have been described and defined serologically (Solomon and Weiss, 1987). Other human λ-type L chains have subsequently been identified, including VλVIII (Ch'ang et al., 1995; Solomon et al., 1995). Using a panel of mAbs to the individual Vλ L chains made it possible to quan-titate the absolute concentration and percentage of distribution of each Vλ subgroup among λ-type Igs both in normal serum as well as in certain disease processes including multiple myeloma, AL amyloidosis, and Waldenstrom's Macroglobulinemia (WM) (Abe et al., 1994; Ozaki et al., 1994). The expression of the VλII and VλVI gene families in the pathogenesis of the aforementioned diseases is remarkably different from that found in the normal state (Ozaki et al., 1994).

VλVIII was cloned from a monoclonal IgMλ rheumatoid factor-producing cell line (Robbins et al., 1990). Two monoclonal λ-type Bence Jones proteins (BJPs), designated Hag and Biv, were obtained from patients having features typical of multiple myeloma and shown to be VλVIII⁻ (Solomon et al., 1995). A monoclonal anti-VλVIII mAb termed 31-8C7 was obtained by immunizing mice with BJP Hag and was found to be specific for recognition of λVIII-related determinants on intact Igs as well as on free L chains (Abe et al., 1994; Solomon et al., 1995). BJPs Hag and Biv were shown to contain a CDR2 lengthened by an additional four amino acid residues (Ch'ang et al., 1995). VλVIII components contain other serologic and chemical features besides the elongated CDR2 that further distinguish them from other Vλ L chain subgroups, resulting in homology that is nearer to murine Vλx than to other human Vλ or Vκ gene families (Ch'ang et al., 1995). As a consequence of Vλx's antigenic specificity, we have investigated whether VλIII is also reactive with MBP and immunologically related to Vλx.

WM

WM is a plasma cell dyscrasia characterized by the infiltration of plasma cells and lymphocytes into bone marrow and by high levels of monoclonal macroglobulin (IgM) (Waldenstrom, 1944; Dimopoulos and Alexanian, 1994). This disease predominantly affects people aged 60 years and older and accounts for approximately 2% of hematological cancers. Symptoms arise as a result of tumor infiltration, circulating or tissue-bound monoclonal IgM, or a combination of features (Dimopoulos and Alexanian, 1994). Polyneuropathy appears in about 5% of patients with WM (Dellagi et al., 1983). An IgMκ anti-myelin activity usually characterizes the peripheral neuropathy

(Dalakas and Engel, 1981) with approximately one half of these patients secreting Ab against MAG (Dellagi et al., 1983; Latov et al., 1981; Nobile-Orazio et al., 1987). Of these patients with non-MAG-reactive Abs a high proportion have immunoreactivity with other glycolipid Ags such as gangliosides (Shy et al., 1986; Latov et al., 1988).

A recently reported case of WM with polyneuropathy and seropositivity for high levels of an IgM/λ mAb reactive with the 18.5 kDa isoform of MBP (Kira et al., 1997) permitted an investigation of a possible immunogenetic role for this human mAb. Given our previous observations with Vλx (Galin et al., 1996a), we investigated the possibility of a shared Id between Vλx and the patient's IgM/λ. The data reveal a shared Id between Vλx and the patient's macroglobulin manifested by reactivity with MBP, although to two different epitopes.

The F28C4 Id and EAE

The unique association between a particular H chain and L chain forms the Ag-binding site of an Ab. This pairing is also the driving force to generate a unique Id. Thus, a precise match between two V domains in Ab to MBP may be the result of selective pressure to express a CRId important for the regulation of EAE. F28C4, the mAb central to this CRId, has been described above. Raised in the PL/J mouse against MBP Ac 1-9, F28C4 was shown to have similar fine epitope specificity and to share a CRId with TCR against the same peptide (Maier et al., 1994). A role for this CRId and the related Id network in EAE is suggested by the ability of mAb F30C7 anti-Id to lessen clinical disease in the adoptive transfer model of EAE in PL/J mice (Zhou and Whitaker, 1993). Furthermore, this CRId does not appear to be species restricted because F30C7

can immunoprecipitate the TCR from and, apparently, cause anergy in a Lewis rat T cell line against guinea pig MBP peptide 68-88 (Zhou et al., 1994). This is paralleled by the finding that sequence homology was found between the regions containing CDR3 of F28C4 V_L and V_H and the V-D-J junction of certain TCR Vβ8 from encephalitogenic PL/J and Lewis rat T cells. Based on the striking sequence homology between F28C4 and the TCR and the protective effects of F30C7, we speculated that the CRId resulting from a unique and perhaps biased pairing of V domains in F28C4, could, in vivo, induce a regulatory anti-Id response. Based on the structural relatedness of F28C4 and the TCR and the fact that F28C4 harbors a Vλx L chain that confers MBP binding, we have investigated whether F28C4 can be exploited as an Id vaccine for EAE.

The role of regulatory $CD4^-/CD25^-$ T cells in autoimmune diseases

The immune system has evolved a number of stopgap measures to ensure that T cells recognizing self Ags are deleted before they can cause autoimmune disease. During T cell maturation in the thymus only those cells with a TCR that recognizes self Ag associated with major histocompatibility complex (MHC) with moderate affinity are allowed to develop. T cells with a TCR that either does not recognize or recognizes self Ag associated with MHC with too high affinity are deleted. Peripheral control mechanisms such as anergy also exist to delete those cells which escape thymic negative selection. Occasionally, however, some self reactive T cells escape all of these defense measures. The immune system's last line of defense is a subset of $CD4^-$ T cells that actively down-regulate the activation and proliferation of self reactive T cells in an Ag-non-specific manner (Fowell and Mason, 1993; Powrie, 1995; Takahashi et al., 1998). These

naturally occurring $CD4^{-}/CD25^{-}$ regulatory T cells have been implicated in autoimmune disease through the demonstration that elimination of these cells from the periphery produces various autoimmune diseases while the reconstitution prevents autoimmune development (Sakaguchi et al., 1995; Asano et al., 1996). Based on the capacity of F28C4 to protect animals from EAE when actively immunized, the anti-Id characteristics of mAb F30C7, and the fact that passive immunization with F30C7 protected PL/J mice from adoptive EAE induction, we have further investigated the protective capability of mAb F30C7 and probed the mechanism of its action in order to shed light on the dynamic capabilities of F28C4 as an Id vaccine for EAE.

CROSS-REACTIVE IDIOTYPES ON MYELIN BASIC PROTEIN REACTIVE T CELL RECEPTORS AND ANTIBODIES: VACCINE TARGETS FOR EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

by

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Abstract

Multiple sclerosis and its model experimental allergic encephalomyelitis (EAE) are autoimmune diseases of the central nervous system resulting in the destruction of the myelin sheath surrounding axons. Traditionally regarded as T cell mediated diseases, it now appears that antibody (Ab) may play an integral role in both disease induction and regulation. A myelin basic protein-specific monoclonal antibody, termed F28C4, has been shown to share remarkable functional and structural relatedness and consequently a cross-reactive idiotype (CRId) with T cell receptors (TCRs) from certain encephalitogenic T cells. In this study we have tested whether the CRId in F28C4 could serve as a surrogate to encephalitogenic T cells or their TCR as a vaccine target in EAE. Immunizing multiple strains of inbred mice with F28C4 prior to disease induction prevented disease in 80-100% of animals from each of the different strains. These results demonstrate that the CRId is an effective interstrain vaccine target regardless of encephalitogenic epitope or major histocompatibility complex haplotype. Immunization with F28C4 also protects Lewis rats from disease induction, demonstrating its efficacy in another species. Perhaps more importantly, F28C4 can prevent the onset of EAE in a wild outbred population of rats, thereby suggesting that it can serve as a vaccine even when multiple encephalitogenic epitopes are involved. In addition to its prophylactic effect, F28C4 also demonstrates therapeutic potential in its ability both to prevent disease relapses in a relapsing-remitting model of EAE in SJL mice as well as to prevent the adoptive transfer of disease in Lewis rats. Collectively, these studies suggest a novel CRId target with therapeutic potential in multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The disease is the product of a coordinated immunologic attack by $CD4^+$ T cells, macrophages, complement, and antibody (Ab)-producing B cells leading to the breakdown of the myelin sheath surrounding CNS axons, resulting in impaired saltatory conduction of nerve impulses (Steinman, 1996). MS afflicts over 350,000 people in the United States alone with women being affected twice as often as men (Duquette et al., 1992). The course of the disease is highly variable with the most common form being a relapsing-remitting course in which periods of disease exacerbation are interrupted by episodes of remission. The animal model of MS, experimental allergic encephalomyelitis (EAE), is similar to MS in that it is an autoimmune disease of the CNS mediated by $CD4^+$ T cells and is considered to be the prime animal model for study of the disease (McFarland and McFarlin, 1995). It can be induced experimentally by actively immunizing animals with CNS myelin components such as myelin basic protein (MBP), MBP peptides, or whole spinal cord homogenate (SCH) or adoptively by T cells specific for such components. Like MS, the course of disease may vary depending on the animal model used as well as the inducing encephalitogen.

Although T cells clearly play a role in the pathogenesis of EAE, it is becoming more apparent that B cells may be important in modulation of the disease. In both EAE and MS, B cells infiltrate the CNS, transform into plasma cells, and can locally synthesize immunoglobulin (Ig), some of which is anti-MBP (Ewan and Lachmann, 1979; Catz and Warren, 1986). Indeed, the increased intrathecal production of Ig within the CNS has long been one of the hallmarks and undisputed aspects of MS (Kabat et al., 1948). Active immunization with CNS antigen (Ag) induces Ab production, although no

direct correlation has been observed between disease severity and Ab titers (Paterson et al., 1981). B cell deficient mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 developed disease similar to control mice. However, these same B cell deficient animals when immunized with a recombinant human myelin oligodendrocyte glycoprotein (rMOG) were resistant to EAE induction. Histological examination of the spinal cord in the rMOG-immunized B cell deficient mice revealed both a general lack of inflammation and demyelination (Lyons et al., 1999), suggesting that B cells and Ab are important to EAE pathogenesis when a more complex form of an Ag such as a protein is used for induction of disease.

CNS myelin is comprised of several proteins including MBP, proteolipid protein, and MOG. MBP, a molecule of 170 amino acid residues, comprises 30% of CNS myelin. Within MBP reside several different encephalitogenic epitopes (Day and Potter, 1986). As mentioned above the particular encephalitogen as well as encephalitogenic epitope differ among animal models. For PL/J mice, the dominant encephalitogenic epitope on MBP is peptide acetyl (Ac) 1-9 (Zamvil et al., 1986). A monoclonal antibody (mAb), termed F28C4, raised in the PL/J mouse against MBP peptide Ac 1-9 shares a cross-reactive idiotype (CRId) with the PL/J Vβ8.2^{+} T cell receptor (TCR) recognizing the same MBP peptide (Zhou and Whitaker, 1993). This CRId has been defined using the anti-idiotypic (Id) mAb, F30C7, which can block both peptide recognition by F28C4 and peptide stimulation of MBP Ac 1-9-specific T cells (Zhou and Whitaker, 1992, 1993). These and other findings suggest that the CRId resides at or near the combining site of F28C4 and the TCR and is not a public Id. A role for this CRId and the related Id network in EAE is suggested by the ability of mAb F30C7 anti-Id to lessen clinical EAE in the adoptive transfer model of EAE in PL/J mice, possibly by T cell anergy (Zhou and

Whitaker, 1993; Zhou et al., 1994). The fine epitope specificity of F28C4 and the residues of MBP Ac 1-9 critical for TCR recognition are the same (Maier et al., 1994). Furthermore, sequence homology (75% overall) was found between regions of complementarity-determining region (CDR) 3 of F28C4 heavy (H) and light (L) chain variable (V) regions and the V-D-J junction of the TCR Vβ8. This homology is not shared by other Ig CDR3 regions and arises, in part, because F28C4 uses an unusual Vλ L chain (Dildrop et al., 1987; Sanchez et al., 1987).

Vλx is a Vλ gene segment that was discovered in polyclonally activated B cells (Dildrop et al., 1987; Sanchez et al., 1987). Vλx rearranges with Jλ2-Cλ2, and its amino acid sequence is only 30-33% homologous to any known Vλ or Vκ genes. Homologous sequences have been found in various mammalian species including man, thus suggesting that Vλx apparently existed before the speciation of mammals (Sanchez et al., 1990). Considering the rarity of Vλx usage, less than 0.5% of all Ig in normal murine sera, the expression of Vλx in response to an encephalitogenic immunogen may not have been coincidental but may reflect the underlying repertoire involved in the pathogenesis of EAE. In subsequent investigations of the ability of mAb F28C4 as well as Vλx itself to bind MBP, a panel of Abs including all known Vλx-bearing mAbs (Dildrop et al., 1987; Sanchez et al., 1987, 1990; Zhou and Whitaker, 1992; Chen et al., 1994), all of unknown specificity, and polyclonal Vλx-bearing Ig from normal murine sera were examined (Galin et al., 1996a). Vλx and Vλx-containing Abs were all found to bind MBP (Galin et al., 1996a). This suggested that Vλx and Vλx-containing Abs have an inherent affinity for MBP. Based on the structural relatedness of F28C4 and the

TCR and the fact that F28C4 harbors a Vλx L chain that confers MBP binding, we have investigated whether F28C4 can be exploited as an Id vaccine for EAE.

Materials and methods

Id vaccination protocol

Animals were immunized subcutaneously with 50 μg of F28C4, control Ab including F23C6 (Zhou and Whitaker, 1990) and UPC-10 (Sigma Chemical Co.; St. Louis, MO), Vλx coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co.), KLH alone, or an equal volume of phosphate-buffered saline (PBS). Two weeks later animals received a booster injection of the appropriate immunogen. Four weeks after the booster injection, EAE was induced by challenging the animals with whole MBP, MBP peptides, SCH, or MBP-reactive T cells.

Preparation of mAb F28C4

The preparation and characterization of mAb F28C4 have been previously described (Zhou and Whitaker, 1992). Briefly, Id-bearing mAb to human MBP Ac 1-9 (Fusion 28C4, or F28C4) was developed by hybridoma techniques. Synthetic human MBP peptide Ac 1-9 was conjugated to KLH (Sigma Chemical Co.) with 2% glutaraldehyde and used to immunize three PL/J mice. Each mouse received a total of 0.5 mg of the human MBP peptide in six injections over a period of 3 weeks. Spleen and lymph node cells were then removed and fused with SP 2/0 myeloma cells. Hybridoma cells were screened by enzyme-linked immunosorbent assay (ELISA) for their ability to produce mAb to MBP as well as to human MBP peptide Ac 1-9. Limiting dilution was performed twice. The specific clone, F28C4, was further characterized by ELISA, purified

by affinity chromatography on MBP-Sepharose, and isotyped and was shown to be an IgG2a/λ.

Ags

Whole bovine MBP was purchased from Sigma Chemical Co. Purified whole rat MBP was a kind gift from Dr. J.M. Soos (University of Florida). The human MBP peptides were synthesized in our laboratory on a Biosearch Peptide Synthesizer (Model 9500; Cambridge, MA) and purified by reverse-phase high-performance liquid chromatography or synthesized by Peninsula Laboratories (San Carlos, CA). SCH was obtained by a modification of the protocol of Brown and McFarlin (1981). Lewis rats were euthanized, the spinal cords were removed by insufflation, and the white matter was purified by density gradient centrifugation.

EAE induction

Four weeks following the booster immunization, animals were challenged for disease induction using MBP, MBP peptides, or SCH for active disease induction following previously published protocols (Brown and McFarlin, 1981; Soos et al., 1995; Zhou and Whitaker, 1996; Gaur et al., 1997) or using MBP 68-88-specific T cells for induction of adoptive EAE. The generation of the adoptive transfer model of EAE in Lewis rats has been described previously (Cao et al., 2000). Briefly, to adoptively transfer EAE, the cells from a guinea pig (GP) MBP peptide 68-88-specific T cell line, LR88L1 (Zhou et al., 1994) were stimulated with GP MBP peptide 68-88 (20 μg/ml) for 3 days in the presence of irradiated syngeneic thymocytes as antigen-presenting cells. Activated T cells were isolated by Ficoll density gradient centrifugation and washed

with Hank's solution. Each recipient rat received 4-5x10^6 of newly activated T cells suspended in PBS intraperitoneally (i.p.). Disease signs typically peaked at 5-6 days post-injection. The animals were monitored daily for development and progression of disease. Neurologic deficit was graded according to the following scale: 0, no signs; 1, decreased tail tonicity; 2, weakness of hind limbs; 3, paraparesis; 4, paraplegia, moribund; 5, death.

Production of recombinant Vλx

The polymerase chain reaction (PCR) amplification, cloning, and expression of Vλx have been described previously (Galin et al., 1996a). Briefly, oligonucleotide primers used for cloning Vλx were synthesized on a DuPont Coder 300 DNA Synthesizer (Wilmington, DE) and were purified on Nensorb Prep columns (NEN; Boston, MA). Sequences of primers used for specific amplification of Vλx from F28C4 hybridoma by PCR each contained a restriction site (XhoI and Bpu1102I for the sense and antisense primers, respectively) at the 5' ends to facilitate directional cloning of the PCR products. F28C4 Vλx was amplified using a Vλx-specific sense primer corresponding to nucleotides 229-242 which has the sequence 5'-d(CCG CTC GAG CAA CTT GTG CTC ACT CAG TCA TC) (GenBank Accession No. M34597). The antisense primer is specific for λ2, is complementary to the J-C junction of F28C4 (Maier et al., 1994), and has the sequence 5'-d(GGA CTT GGG CTG AGC TAG GAC AGT GAC). Detailed methods used for reverse transcriptase-PCR amplification of the Ig V regions have been previously published (LeBoeuf et al., 1989). The resulting double-stranded deoxyribonucleic acid sequence was then restriction digested, directionally cloned into the expression vec-

tor, pET-15b (Novagen; Madison, WI), and expressed according to the manufacturer's protocol. Purification of the recombinant protein was facilitated by the addition of a polyhistidine tag to the 5' end of the cloning site. The authenticity of the recombinant protein was verified using Western gel analysis with rabbit anti-Vλx Ab (Galin et al., 1996a).

Papain cleavage of F28C4 into corresponding F_{ab} and F_c fragments

F28C4, an IgG2a isotype mAb, was converted by proteolytic cleavage with papain into its corresponding Ig fragments (Andrew and Titus, 1991). For cleavage of an IgG2a it was first necessary to preactivate the papain with cysteine. Activated papain is incubated over time with F28C4, the reaction stopped with iodoacetamide, and the mixture dialyzed against Protein A buffer. The mixture is then run over a Protein A column to separate the F_{ab} and F_c, and the F_{ab} is then run over a MBP 1-9-specific column. The respective F_{ab} and F_c eluates are collected and used to immunize animals as described for intact F28C4.

Results

F28C4-mediated protection is not major histocompatibility complex (MHC) restricted

As previously mentioned, F28C4, raised in the PL/J mouse against MBP Ac 1-9, was shown to have similar fine epitope specificity and to share a CRId with encephalitogenic TCR from PL/J Vβ8$^+$ T cells against the same peptide. Sequence homology was found between CDR3 of F28C4 V_H and V_L and the V-D-J junction of certain TCR Vβ8 from encephalitogenic PL/J and Lewis rat T cells. Due to this sequence similarity (Maier et al., 1994) and the protection that adoptive transfer of the anti-Id F30C7 confers (Zhou

and Whitaker, 1993), we have tested the efficacy of F28C4 as a CRId vaccine to block the development of EAE.

Since F28C4 was raised in the PL/J (H-2^u) mouse this was the first animal model we tested. Table 1 shows that pretreating animals by active immunization with F28C4 results in protection when disease is actively induced with rat MBP. One of five (20%) mice immunized with F28C4 prior to active disease induction exhibited clinical signs of disease when compared with 86% (13/15) of the controls. The average severity of disease in the F28C4-treated group was 1.0, or nearly half that seen for the controls. The one animal in the F28C4-treated group that did exhibit signs of clinical disease displayed only mild symptoms.

We immunized animals with mAb F23C6, also raised against MBP Ac1-9, as a specificity control because F23C6 has an overlapping but distinct fine specificity relative to F28C4 (Maier et al., 1994). Four of five (80%) animals immunized with F23C6 exhibited clinical signs of disease. Unlike the anti-Id for F28C4, the mAb anti-Id, F25F7, for F23C6 does not recognize the TCR, suggesting the unique nature of the F28C4 Id confers protection rather than the gross MBP Ac 1-9 reactivity of the mAb. To control for the effect, if any, of the H chain contribution to protection, animals were also immunized with H chain isotype-matched control mAb UPC-10 (γ2a, κ) that had no effect on disease incidence (100%) or severity (2.0).

The next model we tested was the NZW (H-2^z) mouse because TCR on $CD4^+$ T cells recognize antigen presented in the context of MHC Class II molecules which differ between animal strains. Although NZW mice differ from PL/J mice in the haplotype they bear, the encephalitogenic epitope of MBP is, like the PL/J mouse, MBP Ac 1-9 (Zamvil et al., 1994). We again examined whether pretreatment with F28C4 could pro-

tect NZW mice from active EAE induction. Table 2 shows that active immunization with F28C4 protects 100% of NZW mice from actively induced disease when compared with the control groups, where 80% (8/10) of animals showed clinical signs of disease. Thus, since the NZW mouse strain recognizes the same encephalitogenic epitope as the PL/J mouse (MBP 1-9) but is of a different MHC haplotype, F28C4 not only serves as an effective intraspecies CRId vaccine but also is not MHC restricted.

Table 1
Prevention of actively induced EAE in PL/J mice by mAb F28C4

Treatment	No. animals sick (%)	Average severity
F28C4	1/5 (20)	1.0
F23C6	4/5 (80)	1.8
UPC-10	5/5 (100)	2.0
PBS	4/5 (80)	2.3

Table 2
Prevention of actively induced EAE in NZW mice by F28C4

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
F23C6	4/5 (80)	2.2
UPC-10	4/5 (80)	1.5

F28C4-mediated protection is not restricted by encephalitogenic epitope

Although the PL/J and NZW mouse strains bear different MHC Class II molecules, the encephalitogenic epitope on MBP, MBP Ac 1-9, that induces disease is the same. We next utilized the SJL mouse model of EAE in order to test the effectiveness of F28C4 further. Not only is the haplotype of SJL ($H\text{-}2^s$) mice different from either NZW or PL/J mice, but the encephalitogenic epitope on MBP used to actively induce disease in SJL mice is MBP peptide 87-99. Table 3 shows that active immunization with F28C4

mAb completely prevents active induction of disease with MBP peptide 87-99, providing further evidence for the effectiveness of F28C4 as a potent intraspecies Id vaccine, regardless of MHC haplotype or encephalitogenic epitope specificity.

Table 3
Prevention of acute EAE in SJL mice by F28C4

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
PBS	4/5 (80)	3.75

Prevention of relapsing-remitting disease in SJL mice

The aforementioned data were obtained in three models of *acute* EAE. In SJL mice immunizing animals with SCH can induce a *relapsing-remitting* experimental allergic encephalomyelitis (rEAE) that more closely resembles the human disease. We therefore next tested the efficacy of F28C4 as an Id vaccine in preventing rEAE in SJL mice. As shown in Fig. 1, F28C4 not only prevents the initial onset of the disease but also protects animals from developing subsequent relapses. Five of five animals in the control group developed clinical signs of EAE, while only one animal in the F28C4-immunized group developed disease. All of the animals in the control group experienced a clinical score of at least 2.0 during the acute phase of disease, although not all on the same day; however, the severity displayed by the one animal in the treatment group was markedly suppressed. The acute phase of disease lasted for approximately 10 days, at which time all animals recovered from disease. Within 1 week all control animals relapsed, while only one animal in the F28C4-treatment group demonstrated any clinical signs of EAE. Interestingly, when monitored to 200 days (data not shown), all animals

in the control group developed multiple relapses; however, the one F28C4-immunized animal had only two relapses.

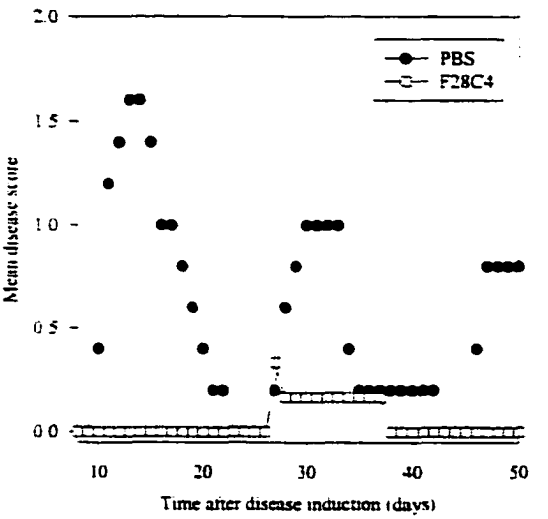

Fig. 1. F28C4 protects SJL mice against EAE actively induced with SCH. Female SJL mice were immunized with 50 μg of F28C4 or control mAbs or an equal volume of PBS followed by a booster injection of 50 μg of F28C4 2 weeks later. Four weeks after the booster animals were challenged with whole SCH. Animals were monitored daily for signs of clinical disease.

F28C4-mediated protection is not species restricted

Closer examination revealed sequence homology also exists between F28C4 V regions and encephalitogenic Vβ8 from the rat. Considering this and since the anti-Id mAb F30C7 for F28C4 can immunoprecipitate the TCR from and, apparently, cause anergy in a Lewis rat T cell line against GP MBP 68-88, we predicted that F28C4 may prevent EAE in the Lewis rat. When immunized with F28C4 Lewis rats are protected from active disease induction with MBP peptide 68-88 (Table 4), leading to the conclusion that F28C4 is not only an effective intraspecies vaccine but is also active across

species. This also provides additional evidence that F28C4 protects animals from disease induction independently of encephalitogenic epitope.

Table 4
Prevention of actively induced EAE in the Lewis rat by F28C4

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
PBS	5/5 (100)	2.3

Prevention of the adoptive transfer model of EAE

While convincing evidence exists suggesting that F28C4 is an effective prophylactic vaccine for EAE, the more important question remains of how effective F28C4 would be for treating disease that is already established. In the adoptive transfer model of EAE, activated encephalitogenic T cells are injected i.p. into subject animals to induce disease. In order to address the question of the efficacy of F28C4 as a therapeutic measure, we induced EAE in Lewis rats by passively transferring MBP 68-88 encephalitogenic T cells (Table 5). While 100% (15/15) of the control animals showed clinical signs of EAE, none (0/5) of the animals immunized with F28C4 exhibited clinical signs of the disease. These results reinforce our previous data indicating that F28C4 is an effective interspecies vaccine by preventing disease in the rat and also that protection can occur after the generation of disease-causing cells. Thus, the F28C4 vaccine may be therapeutic as well as prophylactic.

Preventing induction of disease in an outbred rat model

We have now shown that F28C4 functions as an Id vaccine regardless of intra- or interspecies differences, although all of these studies have been based on well charac-

terized inbred animal models of disease. To test the vaccine in a more rigorous fashion, we employed F28C4 as a vaccine in a wild outbred rat model where EAE can be actively induced either with whole SCH or MBP (Kavelaars et al., 1999). Table 6 shows that 100% (5/5) of animals immunized with F28C4 prior to disease induction with MBP were protected from EAE, whereas 75% of control animals exhibited some signs of clinical disease. Such results are significant because not only are multiple encephalitogenic epitopes involved but the outbred model of disease may more closely resemble that in the human since humans are, of course, an outbred population. Thus, these results may provide the first evidence of the potential of these studies to impact human disease.

Table 5
Prevention of Lewis rat adoptive EAE by F28C4

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
F23C6	5/5 (100)	1.7
UPC-10	5/5 (100)	1.8
PBS	5/5 (100)	1.7

Table 6
F28C4-mediated protection in the outbred model of EAE

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
F23C6	4/5 (80)	0.8
UPC-10	4/5 (80)	1.0
PBS	3/5 (60)	0.5

Intact F28C4 provides optimal protection

Since F28C4 harbors the unusual L chain, Vλx, and given that Vλx on its own can bind MBP, we next determined whether it was the L chain alone that was responsi-

ble for the protection conferred by F28C4 vaccination rather than the intact mAb. We made a recombinant form of this unique V region to be used as a vaccine by cloning the F28C4 Vλx region into an expression system (Galin et al., 1996a). In order to use Vλx as a vaccine it first had to be coupled to KLH due to the small size of Vλx. In these experiments F28C4 was used as a positive control, whereas KLH and PBS served as negative controls. These experiments were conducted both in the PL/J mouse and the Lewis rat, where disease was induced either actively with MBP or passively by the adoptive transfer of an MBP peptide 68-88 T cell line, respectively. In both sets of experiments Vλx alone did not prevent induction of disease but did have an effect in ameliorating the severity of the disease (Tables 7 and 8). Only reducing the severity of disease rather than preventing induction entirely would suggest that Vλx is an important player in protection but is not the sole contributor to the effectiveness of F28C4. Thus, it may be that the unique combination of H and L chain V regions in the F28C4 combining site forms the protective Id that prevents EAE. In fact, we found that when F28C4 was proteolytically fragmented, protection against EAE resided with the F_{ab} but not the F_c (data not shown).

Table 7
Vλx alone reduces the severity of EAE actively induced in the PL/J mouse

Treatment	No. animals sick (%)	Average severity
F28C4	1/5 (20)	1.0
Vλx/KLH	4/5 (80)	1.4
KLH	5/5 (100)	2.0
PBS	4/5 (80)	2.3

Table 8
Vλx alone reduces the severity of adoptively transferred EAE in the Lewis rat

Treatment	No. animals sick (%)	Average severity
F28C4	0/5 (0)	0
Vλx/KLH	4/4 (100)	1.0
KLH	5/5 (100)	2.0
PBS	5/5 (100)	2.3

Discussion

Ideally, treatment of autoimmune diseases should be aimed at reducing the autoimmune response while leaving the rest of the immune system intact. In the treatment of MS, this ideal has not been met. Recent studies showing overlap of disease-associated epitopes that are recognized by T and B cells together with our own data showing functional and structural relatedness of the Ag receptors on certain of the aforementioned cells suggested a novel and relatively specific approach (Maier et al., 1994; Wucherpfennig et al., 1997). The central idea is that if there is considerable overlap in the structure and fine specificity and consequently CRId of Ab and TCR for a disease-associated epitope, as exists between mAb F28C4 and TCR from certain encephalitogenic T cells, then such a CRId could potentially serve as a surrogate vaccine to an encephalitogenic T cell or its TCR. Such Ab vaccines would therefore lead to not only an anti-Id Ab response but also a cross-reactive anti-clonotypic Ab response against disease-associated T cells. We have provided evidence that supports the feasibility of this approach in the autoimmune disease, MS, and its model, EAE.

Tables 1-3 show convincing evidence for the efficacy of F28C4 as an intraspecies vaccine for EAE regardless of MHC haplotype or encephalitogenic epitope. We tested three different strains of laboratory mice–PL/J, NZW, and SJL–each with different MHC haplotypes and with SJL mice differing from the other two strains in that the

encephalitogenic epitope on MBP is also different. In addition to preventing induction of acute EAE, F28C4 is also effective in inhibiting the onset of rEAE in SJL mice (Fig. 1). This experiment also provides substantial evidence for the efficacy of F28C4 to prevent disease induction regardless of encephalitogenic epitope because SCH contains all known encephalitogens.

Tables 4 and 5 demonstrate the ability of F28C4 to be an effective interspecies vaccine in addition to its value as an intraspecies vaccine. We also demonstrated (Table 5) the therapeutic potential of F28C4 in EAE because it prevents adoptive transfer of disease in the Lewis rat. That is, induction of disease can be inhibited even after the generation of disease-causing cells. Further evidence for the therapeutic potential of F28C4 is provided in Table 6 and Fig. 1. As opposed to all of the inbred laboratory animals studied previously in which the MHC haplotype of each individual animal is known, the wild outbred rats are similar to humans in that the MHC haplotypes of the animals are all different. Interestingly, F28C4 is effective in preventing disease induction in this model. Relapsing-remitting MS is the most common form of the disease in humans, and the demonstration that F28C4 can prevent the induction of rEAE in SJL mice provides additional evidence for the efficacy of F28C4 as a potential therapy in MS.

The exact mechanism by which F28C4 protects animals from EAE induction is unknown, although we have garnered some information on the subject. One of the unique features of F28C4 is its possession of a Vλx L chain (Galin et al., 1996a). One of the inherent features of Vλx and Vλx-bearing Abs is their inherent affinity for MBP (Galin et al., 1996a). Our laboratory has also shown that Vλx L chains preferentially associate with γ2a H chains (Galin et al., 1996b). Presumably, something about the asso-

ciation of the Vλx L chain with the γ2a H chain imparts F28C4 with its unique ability to prevent EAE. The contribution of the various parts of the F28C4 Ab molecule to protection from disease induction was investigated in these studies. Recombinant Vλx (Galin et al., 1996a) was effective in reducing the severity of disease when actively induced in PL/J mice (Table 7) and in the adoptive transfer model of Lewis rats (Table 8) without any effect on incidence of disease. We also demonstrated amelioration of disease severity with the F_{ab} fragment of F28C4 but not the F_c portion (data not shown). Collectively, these data suggest the intact Ab is necessary for F28C4 to be an effective vaccine and therapy for EAE and MS.

Our data indicate that F28C4 does not act simply through a pan-Vβ8.2 pathway as is suggested by its ability to protect PL/J mice and Lewis rats from disease. Specifically, F28C4 can protect SJL mice from disease induction, although this mouse strain lacks Vβ8.2. Thus, F28C4 appears to operate through an anti-Id mechanism. Preliminary evidence from our laboratory indicates that cultured lymph node cells from MBP 68-88-sensitized Lewis rats incubated with the anti-Id mAb to F28C4, F30C7, inhibits T cell proliferation as well as increases the production of $CD4^+/CD25^-$ regulatory T cells in vitro (O'Garra et al., 1997). In addition to the ability of passively immunizing animals with F30C7 to prevent adoptive transfer of EAE in PL/J mice, we have preliminary data showing that passively immunizing Lewis rats with F30C7 can also protect Lewis rats from actively induced disease. Collectively, these results suggest that active immunization with F28C4 induces the generation of an anti-Id Ab response in vivo which generates CNS-targeted $CD4^+/CD25^-$ regulatory T cells that nonspecifically down-regulate activated T cells and results in the prevention of disease symptoms.

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INDUCTION OF $CD4^+/CD25^+$ REGULATORY T CELLS WITH MONOCLONAL ANTI-T CELL RECEPTOR ANTIBODIES

by

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Abstract

Multiple sclerosis and its animal model, experimental allergic encephalomyelitis (EAE), are autoimmune diseases of the central nervous system resulting in the destruction of the myelin sheath and impaired nerve impulse conduction. Traditionally regarded as T cell mediated diseases, it now appears that antibody may play an integral role in both disease induction and regulation. A myelin basic protein-specific monoclonal antibody (mAb), termed F28C4, has been shown to share remarkable functional and structural relatedness and consequently a cross-reactive idiotype (CRId) with T cell receptors (TCRs) from certain encephalitogenic T cells. This CRId is capable of serving as a surrogate to encephalitogenic T cells or their TCR as a vaccine target in EAE. This CRId was defined by an anti-idiotypic mAb called F30C7 which has been shown to prevent adoptive EAE induction in PL/J mice. In these studies, we have demonstrated that F30C7 is also capable of reducing active disease induction and severity in Lewis rats by inhibiting proliferation of encephalitogenic T cells. In addition, at high concentrations, F30C7 was shown to inhibit both Th1 and Th2 cytokines completely. On the other hand, at high concentrations, F30C7 induces the generation of a regulatory T cell shown to be $CD4^-/CD25^-$. Therefore, it appears that F30C7 manifests its protective capabilities indirectly by inducing the generation of $CD4^+/CD25^+$ regulatory T cells that down-regulate encephalitogenic T cell production in an antigen (Ag)-non-specific manner. To our knowledge, this represents the first demonstration of the ability to induce the generation of these cells in vitro.

Introduction

The immune system has evolved a number of measures to ensure that T cells recognizing self antigens (Ags) are deleted before they can cause autoimmune disease. During T cell maturation in the thymus, only those cells with T cell receptors (TCRs) that recognize self Ags associated with major histocompatibility complex (MHC) with moderate affinity are allowed to develop. T cells with TCRs that either do not recognize or recognize self Ag associated with MHC with too high affinity are deleted. Peripheral control mechanisms such as anergy also exist to delete those cells which escape thymic negative selection. Occasionally, however, some self reactive T cells escape all of these defense measures. The immune system's last line of defense is a subset of $CD4^+$ T cells that actively down-regulate the activation and proliferation of self reactive T cells in an Ag-non-specific manner (Fowell and Mason, 1993; Powrie, 1995; Takahashi et al., 1998). These naturally occurring $CD4^+/CD25^+$ regulatory T cells have been implicated in autoimmune disease through the demonstration that elimination of these cells from the periphery produces various autoimmune diseases, while their reconstitution prevents autoimmune development (Sakaguchi et al., 1995; Asano et al., 1996).

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) mediated primarily by $CD4^+$ T cells generating a coordinated immunologic attack against protein and lipid components of the myelin sheath such as myelin basic protein (MBP) (Steinman, 1996). Experimental allergic encephalomyelitis (EAE) is similar to MS in that it is an autoimmune disease of the CNS mediated by $CD4^+$ T cells and is considered to be the prime animal model of the disease (McFarland and McFarlin, 1995). Although T cells clearly play a role in the pathogenesis of EAE, it is becoming more apparent that B cells and antibody (Ab) may be important in modula-

tion of the disease. One particular monoclonal antibody (mAb), termed F28C4, raised in the PL/J mouse against MBP peptide acetyl (Ac) 1-9 shares a cross-reactive idiotype (CRId) with the PL/J Vβ8.2^{+} TCR recognizing the same MBP peptide (Zhou and Whitaker, 1993). This CRId has been defined using the anti-idiotypic (Id) mAb, F30C7, which can block both peptide recognition by F28C4 and peptide stimulation of MBP Ac 1-9-specific T cells (Zhou and Whitaker, 1992, 1993). These and other findings suggest that the CRId resides at or near the combining site of F28C4 and the TCR and is not a public Id. A role for this CRId and the related Id network in EAE is suggested by the ability of mAb F30C7 anti-Id to lessen clinical EAE in the adoptive transfer model of EAE in PL/J mice, possibly by T cell anergy (Zhou and Whitaker, 1993; Zhou et al., 1994). The fine epitope specificity of F28C4 and the residues of MBP Ac 1-9 critical for TCR recognition are the same (Maier et al., 1994). Furthermore, sequence homology (75% overall) was found between regions of complementarity-determining region (CDR) 3 of F28C4 heavy (H) and light (L) chain variable (V) regions and the V-D-J junction of the TCR Vβ8. This homology is not shared by other immunoglobulin (Ig) CDR3s and arises, in part, because F28C4 uses an unusual Vλ L chain called Vλx (Dildrop et al., 1987; Sanchez et al., 1987) which confers MBP-binding capabilities on Abs that bear this L chain (Galin et al., 1996).

In previous studies we have investigated the potential of F28C4 as an Id vaccine in EAE. When PL/J mice were actively immunized with F28C4, we were able to prevent disease induction with 80% efficiency. NZW and SJL mice, which vary from PL/J in that they carry different MHC alleles and require different epitopes of MBP to induce disease, were also protected by F28C4 from disease induction with 100% effectiveness. In fact, SJL mice were protected from active induction of a relapsing-remitting disease

course when whole spinal cord homogenate, which contains all known encephalitogens within myelin, was used to induce disease. The potency of F28C4 as an Id vaccine in EAE was further demonstrated by its ability to protect a different species, Lewis rats; F28C4 was also effective in an outbred rat model of EAE (Kavelaars et al., 1999) in which all animals within the population, like humans, are genetically distinct. In these studies we have delved into the explanation of how F28C4, a PL/J mouse mAb against a specific epitope on MBP, can be exploited as a vaccine independently of MHC haplotype, encephalitogenic epitope, or species.

Materials and methods

Anti-Id mAb F30C7

The preparation and characterization of the anti-Id mAb to F28C4, F30C7, has been described previously (Zhou and Whitaker, 1992). Briefly, the mAb anti-Id (Fusion 30 or F30) was developed by fusing SP2/0 myeloma cells with the spleen cells and draining lymph nodes of three PL/J mice immunized with the complementary peptide to MBP 1-9 (Blalock et al., 1989), denoted PBM 9-1. After 2 weeks in culture, hybridoma supernatants were doubly screened by enzyme-linked immunosorbent assay (ELISA) to Id-bearing mAb F28C4. Cells secreting anti-Id were further screened for lack of reactivity against keyhole limpet hemocyanin, bovine serum albumin (BSA), and pooled murine myeloma IgG2a and IgG3. Specific clone F30C7B4 (F30C7) secreting mAb anti-Id was obtained by twice limiting dilution.

Immunization of Lewis rats with mAb F30C7 and active EAE induction

Prior to treatment with F30C7, EAE was actively induced by the subcutaneous injection of 100 μg guinea pig (GP) MBP peptide 68-88 and 200 μg *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratory; Detroit, MI). Female Lewis rats were immunized intraperitoneally (i.p.) on the day of disease induction and again 5 days later with 4.5 mg F30C7, 2.25 mg F30C7, or an equal volume of phosphate-buffered saline (PBS). The animals were monitored daily for development and progression of disease. Neurologic deficit was graded according to the following scale: 0, no signs; 1, decreased tail tonicity; 2, weakness of hind limbs; 3, paraparesis; 4, paraplegia, moribund; 5, death.

Proliferation assay

To determine the effect of mAb F30C7 anti-Id on the proliferation of MBP 68-88-sensitized lymph node cells (LNCs), $3x10^5$ cells/well were cultured in a 96-well plate with 50 μg/ml MBP 68-88 peptide and 500 μg/ml F30C7, 200 μg/ml F30C7, or control Ab. After 54 h in culture, 1 μCi of [^{3}H]-thymidine was added to each well. Cells were harvested 18 h later, and incorporation of [^{3}H]-thymidine was measured by scintillation counting techniques.

Fluorescence-activated cell-sorting (FACS) analysis

LNCs ($5.5x10^6$) from MBP 68-88-sensitized female Lewis rats were cultured with 50 μg/ml MBP 68-88 and varying concentrations of mAb F30C7 anti-Id or murine IgG (Sigma Chemical Co.; St. Louis, MO) as a control Ab. Cells were collected and stained with fluorescein-labeled anti-rat CD25 (PharMingen; San Diego, CA) and phycoerythrin-labeled anti-rat CD4 (PharMingen) Ab at several time points including 0 h,

12 h, 24 h, 36 h, 48 h, 72 h, and 5.5 days. Stained cells were washed with 1% BSA in PBS and resuspended in 1% paraformaldehyde fixative. Cells were counted on a FACS counter at the University of Alabama at Birmingham's Flow Cytometry Core Facility.

Cytokine ELISA

Cell supernatants from the rat LNCs cultured for the FACS analysis were collected and analyzed for production of various rat cytokines including interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)-γ (Biosource, Intl.; Camarillo, CA). ELISA procedures were performed per manufacturer's protocols and color development was quantitated as absorbance at 450 nm on a Tecan WinSeLecT Microplate Reader (Research Triangle Park, NC).

Results

F30C7 protects Lewis rats from active EAE induction

F30C7 has been shown to immunoprecipitate TCR from encephalitogenic T cells and inhibit proliferation of LNCs used to transfer EAE leading to protection of PL/J mice from disease induction (Zhou and Whitaker, 1993). We have accumulated considerable data showing that F28C4 is an effective vaccine in several permutations of the rodent models of EAE. Given that active immunization with F28C4 protects not only PL/J mice but also Lewis rats, we wanted to test the idea that the anti-Id F30C7 could protect both species when passively immunized. We first induced disease by actively immunizing Lewis rats with MBP 68-88 and then treating either with two different doses of F30C7 anti-Id mAb or with PBS both on the day of active disease induction and a booster on day 5 post-induction. Fig. 1 shows that animals in the PBS group first

showed clinical signs of disease on day 8 and were fully recovered by day 18, while F30C7-treated animals developed a milder form of disease which lasted for a shorter duration. Table 1 shows that 100% of both the PBS and low dose F30C7 showed clinical signs of disease, while the high dose group of F30C7 had a slightly lower incidence of disease (3/5). Also, the average disease duration of the 4.5 mg dose of F30C7 was shorter by nearly 2 days when compared with the control group. Likewise, the average severity of disease in the high dose F30C7 group was one half (0.8) that in the control group (1.6).

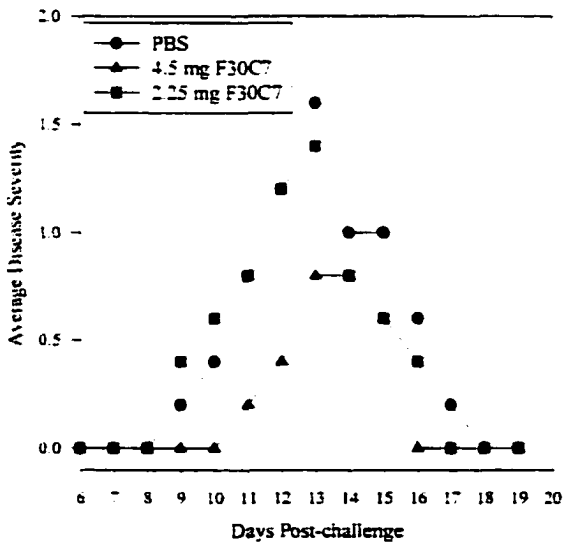

Fig. 1. F30C7 anti-Id mAb treatment modulates EAE induction in Lewis rats. Female Lewis rats were immunized i.p. with F30C7 mAb or an equal volume of PBS on the day of disease induction and again 5 days later. Disease was actively induced with GP MBP peptide 68-88. Animals were monitored daily for clinical signs of disease.

F30C7 inhibits T cell proliferation

F30C7 has previously been shown to inhibit the proliferation of PL/J mice LNCs (Zhou and Whitaker, 1993). In these studies, there was reduced incidence, severity, and

duration of disease, indicating that F30C7 is inhibiting some aspect of the disease process. To test whether F30C7 inhibits LNC proliferation in the Lewis rat, we sensitized Lewis rats to MBP 68-88, harvested the lymph nodes, and cultured the LNCs with F30C7 and MBP 68-88. A [^{3}H]-thymidine incorporation assay showed that F30C7 completely inhibited the proliferation of MBP 68-88-sensitized LNCs when compared with culture with the control mouse IgG Ab (Fig. 2).

Table 1
Passive immunization with F30C7 protects Lewis rats from actively induced EAE

Treatment	No. animals sick (%)	Average duration	Average severity
PBS	5/5 (100)	5.8	1.6
2.25 mg F30C7	5/5 (100)	4.6	1.6
4.5 mg F30C7	3/5 (60)	4.0	0.8

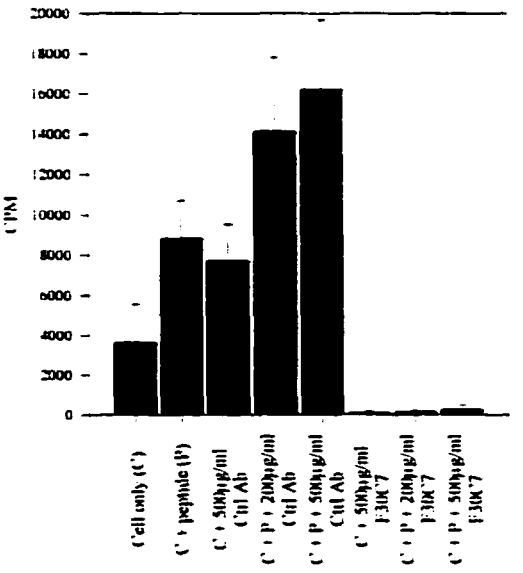

Fig. 2. F30C7 inhibits LNC proliferation. Female Lewis rats were immunized with 50 μg MBP 68-88. LNCs were collected 9 days after sensitization to MBP 68-88 and cultured on 96-well flat-bottom plates with anti-Id mAb F30C7 or control mouse IgG. After 54 h in culture, [^{3}H]-thymidine was added to culture wells; 18 h later plates were read on a scintillation counter.

Cytokine profile of LNCs cultured with F30C7

Considerable efforts have been devoted to the study of the role of Th1 and Th2 cytokines in MS, EAE, and other autoimmune diseases. IL-2 and IFN-γ are typically considered to be cytokines produced by Th1 cells and deemed to be immunostimulatory, whereas Th2 cells are generally regarded as immunoprotective and secrete cytokines such as IL-4 and IL-10. Considering that F30C7 when passively administered to Lewis rats protects them from active EAE induction, presumably through inhibiting the proliferation of encephalitogenic T cells, we investigated whether F30C7 is influencing the production of various Th1 and Th2 cytokines. In Fig. 3 we show that a high dose of F30C7 completely abolishes any production of IL-10 at all time points studied, while the low dose F30C7 and control Ab had similar production profiles with the generation of IL-10 increasing over time, peaking at 72 h, and beginning to decrease after 5 days. Fig. 4 shows that F30C7 completely inhibits production of IFN-γ, while generation of IFN-γ increased in the other two groups over the first 36 h and then held steady through 5 days. Cell supernatants were also analyzed for the production of IL-2 and IL-4, but no measurable levels of these cytokines were observed (data not shown).

In vitro production of $CD4^-/CD25^-$ regulatory T cells

The notion that Th1 cells are pathogenic while Th2 cells are regulatory with regard to EAE may be too simplistic. As evidenced by the observations in Figs. 3 and 4, there is no parallel in the induction of IL-10 and IL-4 and suppression of IL-2 and IFN-γ. Therefore, there must be another explanation for why F30C7 inhibits T cell proliferation and reduces disease incidence and severity. Regulatory T cells appear to be related to yet distinct from the traditional Th2 cells. In order to test whether regulatory T cells

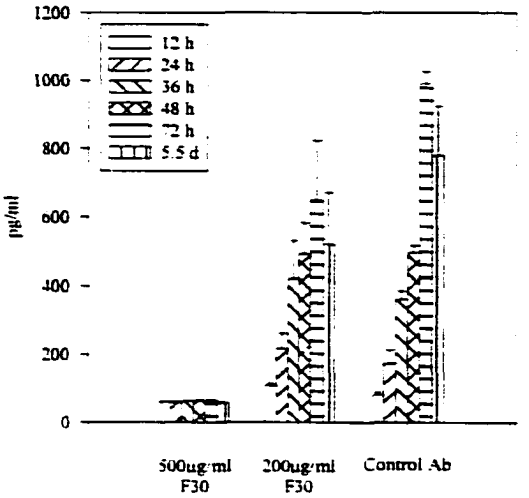

Fig. 3. High dose F30C7 inhibits IL-10 production by Lewis rat LNCs. LNCs ($5.5x10^6$) from MBP 68-88-sensitized Lewis rats were cultured with 50 μg/ml MBP 68-88 and varying concentrations of mAb F30C7 anti-Id or mouse IgG. Cell supernatants were collected and analyzed for production of IL-10 (Biosource, Intl.; Camarillo, CA). ELISA protocols were provided in each kit, and color development was quantitated as absorbance at 450 nm.

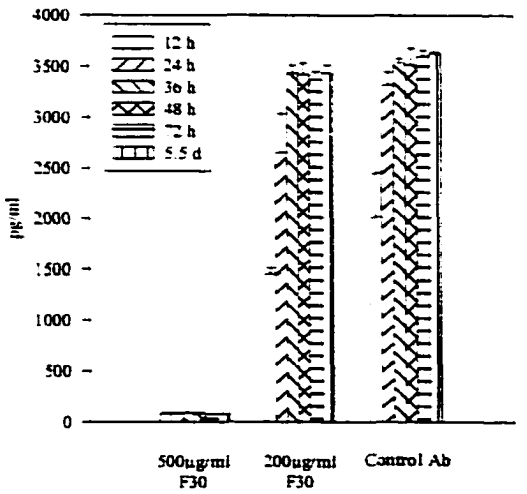

Fig. 4. High dose F30C7 inhibits IFN-γ production by Lewis rat LNCs. LNCs ($5.5x10^6$) from MBP 68-88-sensitized Lewis rats were cultured with 50 μg/ml MBP 68-88 and varying concentrations of mAb F30C7 anti-Id or control Ab. Cell supernatants were collected and analyzed for production of IFN-γ (Biosource, Intl.; Camarillo, CA). ELISA protocols were provided in each kit, and color development was quantitated as absorbance at 450 nm.

play a role in suppression of T cell proliferation and protection from clinical signs of disease, FACS studies were performed to test whether MBP 68-88-sensitized LNCs from Lewis rats cultured with either F30C7 or control Ab generated the production of $CD4^+/CD25^-$ regulatory T cells. Fig. 5 shows that cells cultured with 500 μg/ml F30C7 anti-Id mAb produced higher percentages of this regulatory T cell population after 36 h in culture when compared with levels produced by LNCs cultured with either 200 μg/ml F30C7 or control Ab. Fig. 6 shows the generation of this regulatory T cell response over time.

Discussion

Based on the structural and functional relatedness of F28C4 and TCR from certain encephalitogenic T cells for a specific Ag (Maier et al., 1994) and the fact that F28C4 harbors a Vλx L chain that confers binding to MBP (Galin et al., 1996), we have previously tested whether mAb F28C4 could serve as a vaccine in the rodent models of EAE. Subsequently, we have accumulated evidence verifying the efficacy of this vaccine to prevent disease induction with 80-100% efficiency, thus accumulating data pointing to the therapeutic potential of F28C4. This led to the hypothesis that vaccination with mAb F28C4 induces the generation of an in vivo anti-Id Ab response which interferes with disease pathogenesis, leading to prevention of disease.

In Fig. 1 we have extended studies begun by Zhou and Whitaker (1993) in which they reduced adoptive disease incidence and significantly lowered average disease severity in PL/J mice. Through the passive immunization of Lewis rats with 4.5 mg of the anti- Id mAb F30C7, we were successful in reducing incidence and severity of actively induced EAE. Due to the lack of response with 2.25 mg F30C7 and the marginal re-

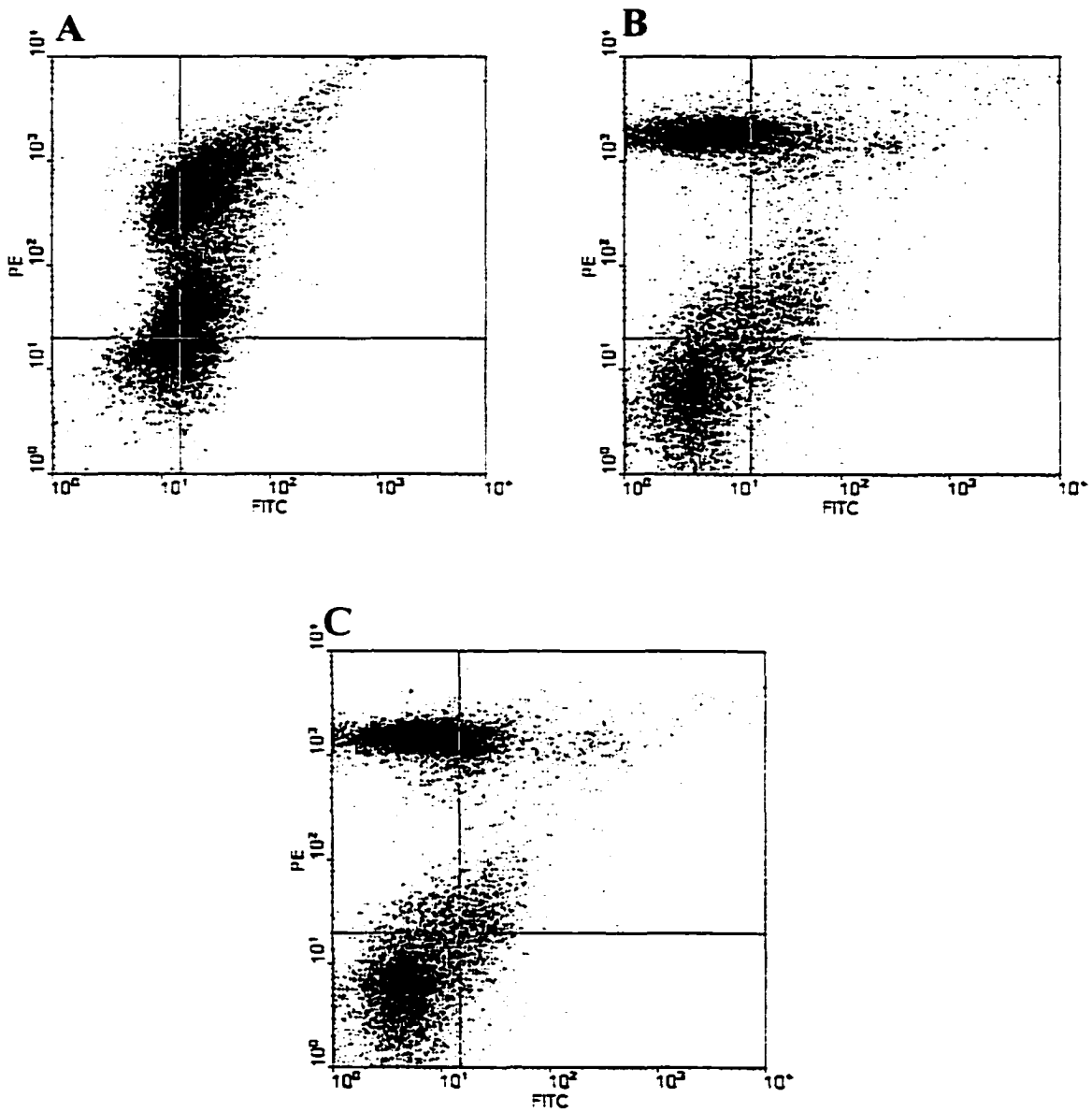

Fig. 5. F30C7 anti-Id mAb induces the generation of $CD4^+/CD25^+$ regulatory T cell after 36 h in culture. MBP 68-88-sensitized Lewis rat LNCs were cultured with 50 μg/ml MBP 68-88 and (A) 500 μg/ml F30C7, (B) 200 μg/ml F30C7, or (C) control mouse IgG. Cells were collected and stained with fluorescein-labeled anti-rat CD25 and phycoerythrin-labeled anti-rat CD4 Ab after 36 h. Stained cells were washed with 1% BSA in PBS, resuspended in 1% paraformaldehyde fixative, and counted on a FACS counter at the University of Alabama at Birmingham's Flow Cytometry Core Facility.

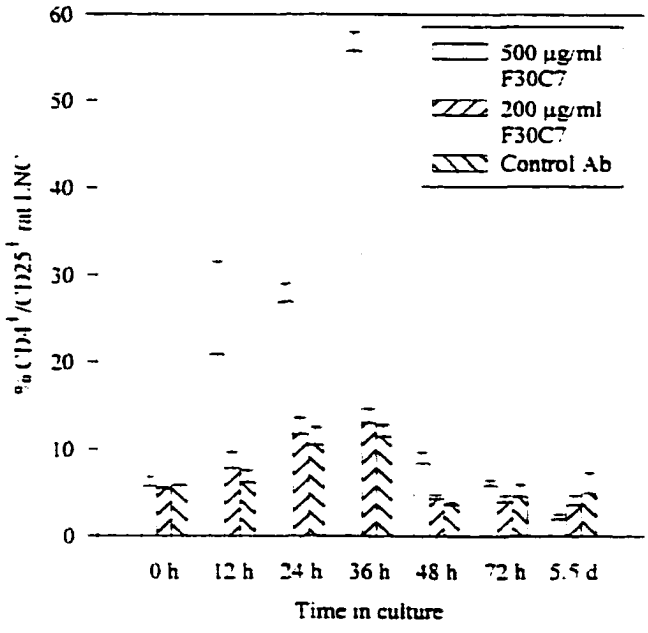

Fig. 6. F30C7 induction of $CD4^+/CD25^+$ regulatory T cells over time. MBP 68-88-sensitized Lewis rat LNCs were cultured with 50 µg/ml MBP 68-88 and 500 µg/ml F30C7, 200 µg/ml F30C7, or control mouse IgG. Cells were collected and stained with enzyme-labeled anti-rat CD4 and CD25 Ab at several time points including 0 h, 12 h, 24 h, 36 h, 48 h, 72 h, and 5.5 days. Stained cells were washed with 1% BSA in PBS, re-suspended in 1% paraformaldehyde fixative, and counted on a FACS counter at the University of Alabama at Birmingham's Flow Cytometry Core Facility.

sponse with 4.5 mg F30C7, we speculate that at higher doses we will be able to optimize this protocol and further reduce EAE incidence and severity. In addition, it appears that anti-Id F30C7 decreases the proliferation of autoreactive T cells in order to manifest its effects on ameliorating disease (Fig. 2).

EAE has traditionally been considered to be a disease initiated by Th1 cells secreting proinflammatory cytokines. Indeed, T cells lines and clones used to adoptively transfer EAE produce proinflammatory cytokines such as IL-2 and IFN-γ (Zamvil and Steinman, 1990; Liblau et al., 1995). This is in contrast to the recovery phase of disease, in which the Th2 cytokines such as IL-4 and IL-10 have been shown to predominate (Kennedy et al., 1992; Khoury et al., 1992). Cross talk exists between Th1 and Th2 cells in which cytokines secreted by one cell type actively inhibit the production of cytokines by the other cell type. More recent studies have contradicted current dogma somewhat with respect to the previous strict definition regarding Th1 cytokines as proinflammatory and Th2 cytokines as anti-inflammatory (reviewed in O'Garra et al., 1997). New $CD4^-$ T cell subsets are now emerging with the introduction of Th3 and Tr1 subsets which predominantly secrete transforming growth factor-β and IL-10, respectively, and resemble Th2 cells in that they have been shown to be effective in treating autoimmune or T cell mediated inflammatory disease in animal models (Chen et al., 1994; Groux et al., 1997).

In Figs. 3 and 4 the production of IL-10 and IFN-γ was studied, respectively, in Lewis rat LNCs cultured with F30C7 or control Ab. The predominant observation in these figures is that 500 μg/ml F30C7 anti-Id completely inhibits the production of both cytokines when compared with either 200 μg/ml F30C7 or control Ab. Interestingly, the cytokine production profile in both of these experiments looked similar in the 200 μg/ml

F30C7 and control Ab groups. That is, IL-10 production in these two groups increased over time, peaking at 72 h, and then began to decline; however, IFN-γ production increased quickly over the first 24 h and then held steady throughout the duration of the experiment. If we compare the ratios of IFN-γ production to IL-10 production, we observe an interesting phenomenon. That is, the ratio of IFN-γ:IL-10 in the control Ab cultured LNCs is twice that of the 200 μg/ml F30C7 group at the earliest time point; over time these ratios slowly equilibrate with the overall ratio of IFN-γ:IL-10 decreasing as well. In addition, we looked at production of IL-2 and IL-4 and did not observe production of either of these cytokines. Together, these data suggest that F30C7, particularly at higher concentrations, which appears to also be necessary for maximal protection of animals in vivo from disease symptoms, does not strictly adhere to the traditional observations of Th1 and Th2 cytokines and EAE pathogenesis.

Considering these results, the best explanation lies in data shown in Figs. 5 and 6. $CD4^-/CD25^-$ regulatory T cells actively down-regulate the activation and proliferation of self reactive T cells that cause autoimmune disease. By culturing rat LNCs sensitized to MBP 68-88 with 500 μg/ml F30C7 anti-Id, we have induced the in vitro generation of $CD4^-/CD25^-$ regulatory T cells. These findings, taken with the fact that 500 μg/ml F30C7 inhibits proliferation of activated T cells (Fig. 2), led us to speculate that at higher concentrations F30C7 will target into the CNS of immunized animals, where it generates production of a regulatory T cell in an Ag-non-specific manner. Therefore, it is tempting to speculate that, in this model, F30C7 at lower concentrations can modulate subclinical EAE through the ratio of Th1:Th2 cytokine production but at higher concentrations generates production of regulatory T cells to modulate active disease. Collec-

tively, these results suggest that when we actively vaccinate animals with the Id-bearing F28C4 mAb, we can induce the generation of an F30C7-like anti-Id response that initiates the production of a CNS-targeted $CD4^-/CD25^-$ regulatory T cell that down-regulates encephalitogenic T cells in an Ag-non-specific manner, thereby protecting animals from EAE induction. This is the first study to demonstrate the ability to induce the generation of a regulatory $CD4^-/CD25^-$ T cell in vitro to affect autoimmune pathogenesis. Implications for other autoimmune diseases are further demonstrated by similar results obtained in the animal model of myasthenia gravis, experimental autoimmune myasthenia gravis.

Acknowledgements

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AN IGM ANTI-MBP AB IN A CASE OF WALDENSTROM'S MACROGLOBULINEMIA WITH POLYNEUROPATHY EXPRESSING AN IDIOTYPE REACTIVE WITH AN MBP EPITOPE IMMUNODOMINANT IN MS AND EAE

by

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Abstract

In a previously described case of Waldenstrom's Macroglobulinemia, complicated by polyneuropathy, the IgM/λ monoclonal antibody (mAb) was highly reactive with myelin basic protein (MBP). Given our demonstration that Vλx, a recently described murine λ variable region gene product, can itself bind MBP as well as confer MBP reactivity to an antibody (Ab), the possibility of a shared idiotypy between murine Vλx and this human IgM/λ anti-MBP was investigated. We characterized the epitope specificity of the macroglobulinemia patient's MBP-reactive IgM/λ using indirect enzyme-linked immunosorbent assay (ELISA) procedures with MBP, a citrullinated isomer of MBP termed C8, or peptide fragments of MBP as the coating antigens and monospecific Ab to Vλx as the secondary Ab. The patient's MBP-reactive IgM/λ was recognized by Ab specific for Vλx and, like murine mAb containing Vλx bound human MBP but not MBP-C8 nor other common autoantigens such as deoxyribonucleic acid, thyroglobulin, or actin. The anti-MBP reactivity was selective for MBP peptide 90-170 and preferentially recognized MBP peptide 84-96. Thus, the patient's macroglobulin and perhaps certain other human Ab with a 'Vλx idiotype' bind to MBP peptide residues 84-96, an immunodominant peptide in multiple sclerosis patients. Such binding may be involved in the pathogenesis of neural damage in patients with neuroimmunologic disorders related to plasma cell dyscrasias or autoimmunity.

Introduction

A monoclonal Ab (mAb) termed F28C4, raised in the PL/J mouse against myelin basic protein (MBP) peptide acetyl (Ac) 1-9, shares a cross-reactive idiotope (CRId)

with PL/J Vβ8$^-$ T cell receptors (TCR) against the same peptide (Zhou and Whitaker, 1993). This CRId has been defined using an anti-idiotypic (Id) mAb (F30C7) which can block both peptide recognition by F28C4 (Zhou and Whitaker, 1992) and peptide stimulation of MBP Ac 1-9-specific T cells (Zhou and Whitaker, 1993), implying that the Id resides at or near the combining site of F28C4 and the TCR. Moreover, the CRId is probably very relevant to the animal model of multiple sclerosis (MS), experimental allergic encephalomyelitis (EAE), since F30C7 lessens clinical disease in the adoptive transfer model of EAE in PL/J mice (Zhou and Whitaker, 1993). The fine epitope specificity of F28C4 and the residues of MBP Ac 1-9 critical for TCR recognition are the same (Maier et al., 1994). Furthermore, sequence homology (75% overall) was found between regions of complementarity-determining region (CDR) 3 of F28C4 heavy (H) and light (L) chain variable (V) regions and the V-D-J junction of the TCR Vβ8. This homology is not shared by other immunoglobulin (Ig) CDR3s and arises, in part, because F28C4 uses an unusual Vλ L chain (Dildrop et al., 1987; Sanchez et al., 1987).

Vλx is a Vλ gene segment that was discovered in polyclonally activated B cells (Dildrop et al., 1987; Sanchez et al., 1987). Vλx rearranges with Jλ2-Cλ2, and its amino acid sequence is only 30-33% homologous to any known Vλ or Vκ genes. Homologous sequences found in various mammalian species, including man, suggest that Vλx apparently existed before the speciation of mammals (Sanchez et al., 1990). Considering the rarity of Vλx usage, less than 0.5% of all Ig in normal murine sera, the expression of Vλx in response to an encephalitogenic immunogen may not have been coincidental but reflect the underlying repertoire involved in the pathogenesis of EAE. In subsequent investigations of the ability of mAb F28C4 as well as Vλx itself to bind MBP, a panel of

antibodies (Abs) including all known Vλx-bearing mAbs (Dildrop et al., 1987; Sanchez et al., 1987, 1990; Zhou and Whitaker, 1992; Chen et al., 1994), all of unknown specificity, and polyclonal Vλx-bearing Ig from normal murine sera were examined (Galin et al., 1996). Vλx and Vλx-containing Abs were all found to bind MBP (Galin et al., 1996). This suggested that Vλx and Vλx-containing Abs have an inherent affinity for MBP.

Waldenstrom's Macroglobulinemia (WM) is a plasma cell dyscrasia characterized by the infiltration of plasma cells and lymphocytes into bone marrow and high levels of monoclonal macroglobulin (IgM) (Dimopoulos and Alexanian, 1994; Waldenstrom, 1944). This disease predominantly affects people aged 60 years and older and accounts for approximately 2% of hematologic cancers. Symptoms arise as a result of tumor infiltration, circulating or tissue-bound monoclonal IgM, or a combination of features (Dimopoulos and Alexanian, 1994). Polyneuropathy appears in about 5% of patients with WM (Dellagi et al., 1983). An IgM-kappa (κ) anti-myelin activity usually characterizes the peripheral neuropathy (Dalakas and Engel, 1981) with approximately one half of these patients secreting Ab against myelin-associated glycoprotein (MAG) (Latov et al., 1981; Dellagi et al., 1983; Nobile-Orazio et al., 1987). Of these patients with non-MAG-reactive Abs a high proportion have immunoreactivity with other glycolipid antigens (Ags) such as gangliosides (Shy et al., 1986; Latov et al., 1988).

A recently reported case of WM with polyneuropathy and seropositivity for high levels of an IgM/λ mAb reactive with the 18.5 kDa isoform of MBP (Kira et al., 1997) permitted an investigation of a possible immunogenetic role for this human mAb. Given our previous observations with Vλx (Galin et al., 1996), we investigated the possibility

of a shared Id between Vλx and the patient's IgM/λ. The data reveal a shared Id between Vλx and the patient's macroglobulin manifested by reactivity with MBP, though to two different epitopes.

Materials and methods

Antigens

Human MBP and its citrullinated isomer, termed C8, were prepared by previously described techniques (Whitaker et al., 1992). Briefly, human MBP (1-170) was isolated from human brain by delipidation, acid extraction at pH 3, and carboxymethylcellulose chromatography at pH 10.6. MBP-C8 was purified from the fraction of delipidated brain extract that had not been retained on carboxymethylcellulose at pH 9.6 or 10.6. The human MBP peptides were synthesized in our laboratory on a Biosearch Peptide Synthesizer (Model 9500; Cambridge, MA) and purified by reverse-phase high-performance liquid chromatography or synthesized by Peninsula Laboratories (San Carlos, CA).

Digestion of basic protein with brain cathepsin D

Digestion of MBP with brain Cathepsin D resulting in large peptide fragments has been previously described (Whitaker and Seyer, 1979). For analytical polyacrylamide disc gel electrophoretic studies, 300–400 μg of MBP or MBP peptide were dissolved in 1 ml of 0.05 M ammonium acetate, pH 3.5, with or without varying amounts of enzyme and incubated at 37°C in a shaking water bath. When incubation mixtures contained pepstatin, this proteolytic inhibitor was mixed with 0.05 M ammonium ace-

tate, pH 3.5, and added in a volume of 50 µl. Incubation mixtures were frozen, lyophilized, and dissolved in 100 µl of sample buffer for disc gel studies.

For preparation of MBP peptides to be isolated by chromatographic procedures, MBP was dissolved at a concentration of 5 mg/ml in 0.05 M ammonium acetate, pH 3.5, and incubated with enzyme at 37°C. Following the incubation period, samples were frozen and lyophilized prior to CM-cellulose chromatography.

Preparation of mAb F28C4

The preparation and characterization of mAb F28C4 has been previously described (Zhou and Whitaker, 1992). Briefly, Id-bearing mAb to human MBP Ac 1-9 (Fusion 28C4, or F28C4) was developed by hybridoma techniques. Synthesized human MBP peptide Ac 1-9 was conjugated to keyhole limpet hemocyanin (KLH) (Sigma Chemical Co.; St. Louis, MO) with 2% glutaraldehyde and used to immunize three PL/J mice. Each mouse received a total of 0.5 mg of the human MBP peptide in six injections over a period of three weeks. Spleen and lymph node cells were then removed and fused with SP 2/0 myeloma cells. Hybridoma cells were screened by enzyme-linked immunosorbent assay (ELISA) for their ability to produce mAb to MBP as well as to human MBP peptide Ac 1-9. Limiting dilution was performed twice. The specific clone, F28C4, was further characterized by ELISA, purified by affinity chromatography on MBP-Sepharose, and isotyped and shown to be an IgG2a/λ.

Polyclonal monospecific Ab to Vλx

A New Zealand white rabbit (Myrtle Laboratories; Thompson Station, TN) was injected subcutaneously at four sites every two weeks over a 6 week period with purified

F28C4 (50 μg/injection), first in Freund's complete adjuvant and twice in incomplete adjuvant as described previously (Galin et al., 1996). Serum was obtained before the first injection and 2 weeks after each booster to monitor anti-F28C4 titers using ELISA procedures. F28C4 is of the γ2a isotype and, as with all Vλx-bearing Abs, is a λ2 isotype. In order to make the antiserum specific, it was exhaustively absorbed on a polyclonal IgG2a Sepharose 4B affinity column to deplete H chain reactivity. Next, the Jλ2Cλ2 reactivity was removed using a MOPC 315 (IgA, λ2) Sepharose 4B affinity column. The monospecificity of the resulting anti-Vλx Ab was shown by Western analysis (Galin et al., 1996).

ELISA

Microtiter wells (96-well ELISA plates; Corning Glass Works; Corning, NY) were coated with human MBP 1-170 (10.0 μg/ml in phosphate-buffered saline (PBS)), MBP-C8 (10.0 μg/ml in PBS), MBP-related peptides (either 10.0 μg/ml or 100 μg/ml in PBS), or other self Ags (concentrations equimolar to MBP) at 4°C for 16 h and plates were blocked with 2% casein in PBS. Washings were performed with PBS containing 0.05% Tween 20. Abs were diluted in PBS containing 1% bovine serum albumin (BSA) and allowed to bind for 2 h at room temperature. Ab binding was detected with rabbit anti-Vλx Ab followed by an alkaline phosphatase conjugated anti-rabbit IgG Ab (Southern Biotechnology; Birmingham, AL). Reactions were developed using *p*-nitrophenyl phosphate (Sigma Chemical Co.) and color development was quantitated as absorbance at 405 nm on a Tecan WinSeLecT Microplate Reader (Research Triangle Park, NC). Background optical density was the mean of wells where coating antigen was omitted.

Controls were also performed on Ag-coated wells with all ELISA components except for the primary Ab. These were always negative.

Macroglobulinemia patient and normal control sera

Serum from a patient with WM as well as from a normal control individual who was age, sex, and ethnically matched were previously described (Kira et al., 1997). Serum was diluted in PBS containing 1% BSA for ELISA procedures.

Quantification of the patient's IgM/λ

In order to estimate the amount of Vλx Id-containing IgM in the patient's serum, ELISA assays were done to compare the patient's serum to an F28C4 standard curve. A two-fold dilution scheme from 1000 to 7.8 ng/ml and from 1:400 to 1:51,200 for F28C4 and the patient's serum, respectively, were incorporated. ELISA procedures were then followed as outlined above using monospecific rabbit anti-Vλx Ab. The average of duplicate wells was plotted and lines of regression fitted to each plot. The slopes of the dose responses for the patient's serum and F28C4 were parallel.

By extrapolation it was determined that the concentration of IgM/λ macroglobulin with a Vλx Id in the patient's serum is 12.5 mg/ml. Since the total IgM concentration in the macroglobulinemia patient's serum is 17.8 mg/ml (Kira et al., 1997) the IgM/λ macroglobulin with a Vλx Id accounts for at least 70% of the patient's total serum IgM.

Results

Reactivity of antibody against Vλx with the patient's serum

To investigate whether the patient's MBP-reactive IgM/λ contained an Id related to Vλx, the patient's serum was added to microtiter wells coated with human MBP (1-170), and the anti-Vλx Ab was used to detect binding. Fig. 1 shows strong reactivity against the macroglobulinemia patient's serum while the control patient's serum was not reactive. This would suggest that the IgM/λ contained in the patient's serum shares an Id in common with Vλx.

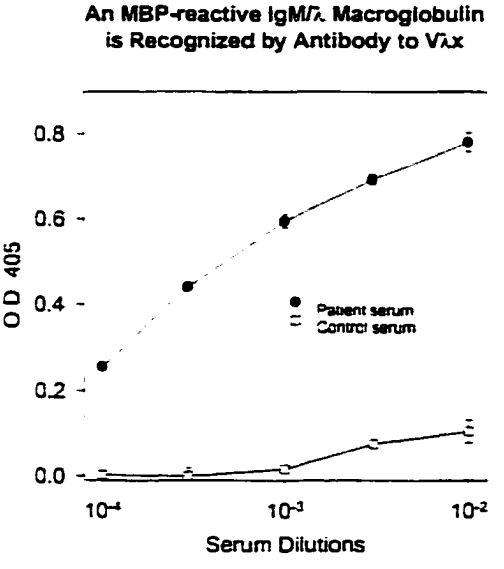

Fig. 1. The MBP-reactive IgM/λ macroglobulin is recognized by antibody to Vλx. Whole human MBP (1-170) was coated onto microtiter wells at a concentration of 10 μg/ml at 4°C for 16 h. The patient's serum or serum from a normal control were diluted in PBS containing 1% BSA and allowed to bind at RT for 2 h. Reactivity of the sera was then monitored by Ab to Vλx. Binding of the Ab to Vλx was then detected by an alkaline phosphatase labeled goat anti-rabbit Ab and measured at 405 nm on an ELISA reader.

In addition, Fig. 1 shows that the IgM/λ interaction with MBP is relatively high affinity. The 0.6 OD_{405} value at 1:1000 dilution of the patient's serum represents an approximate concentration of $1x10^{-8}$ M for the patient's IgM/λ that has a Vλx Id. This compares favorably to the affinity of naturally occurring murine Vλx-containing IgG and is higher than most murine Vλx-containing IgM (Galin et al., 1996).

Polyreactivity of the patient's macroglobulin

IgM paraproteins are often polyreactive due to the multivalency of the IgM. To determine whether the patient's macroglobulin was polyreactive we looked for reactivity of the macroglobulin against other common autoantigens. In Fig. 2 we show the IgM/λ macroglobulin bound human MBP (1-170) but did not bind other common autoantigens including actin, single-stranded deoxyribonucleic acid (ssDNA), or thyroglobulin. This result suggests the patient's Vλx Id-containing IgM, like murine Vλx-containing IgM (Galin et al., 1996), is not polyreactive but rather has a measure of specificity for MBP.

Coating ELISA plates with MBP (1-170) vs. MBP-C8

Human MBP exists in five major isoforms that result from differential splicing of the MBP gene. Post-translational modification of MBP results in a microheterogeneous population of five differentially charged isomers. Among the modified isomers, one termed C8 has undergone deimidation of at least six arginyl residues (positions 25, 31, 122, 130, 159, and 170) generating citrulline at these positions (Wood and Moscarello, 1989). Because of the altered residues, reactivity with C8 was tested in an attempt to restrict where in MBP the IgM/λ macroglobulin is binding. In Fig. 3 the patient's macro-

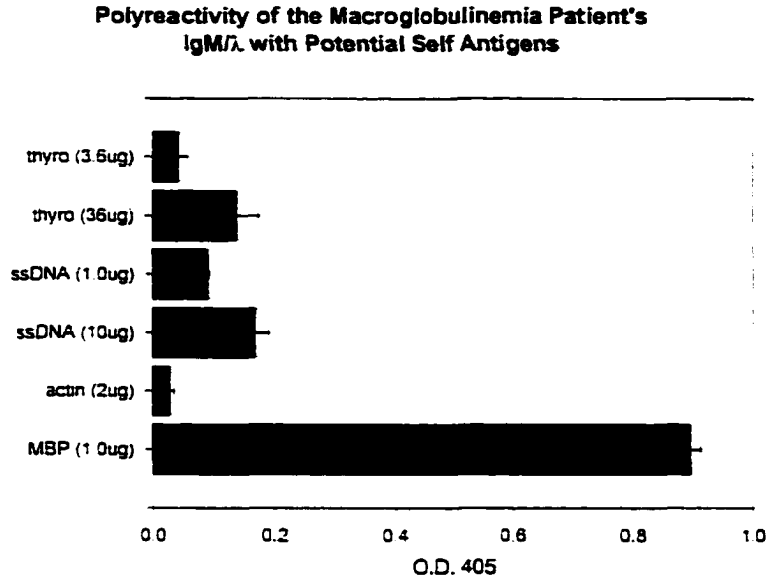

Fig. 2. Polyreactivity of the macroglobulinemia patient's IgM/λ with potential self Ags. ELISA microtiter wells were coated with equimolar concentrations (540 nM) of MBP (1-170), actin, ssDNA, and thyroglobulin. Both thyroglobulin and ssDNA were also coated at 10-fold lower concentrations. Plates were incubated at 4°C for 16 h. The patient's serum was diluted 1:300 in PBS containing 1% BSA and allowed to bind for 2 h at RT. Reactivity of the Ab to Vλx was then detected by an alkaline phosphatase labeled goat anti-rabbit Ig Ab and measured at 405 nm on an ELISA reader.

globulin clearly reacts with MBP (1-170) but not to MBP-C8 whereas reactivity of the control patient to both MBP (1-170) and MBP-C8 was negative. Thus, citrullination may disrupt or mask specific epitopes on MBP, resulting in the lack of binding. Considering that the C-terminal region of MBP is predominantly modified suggests that it might be the site of interaction.

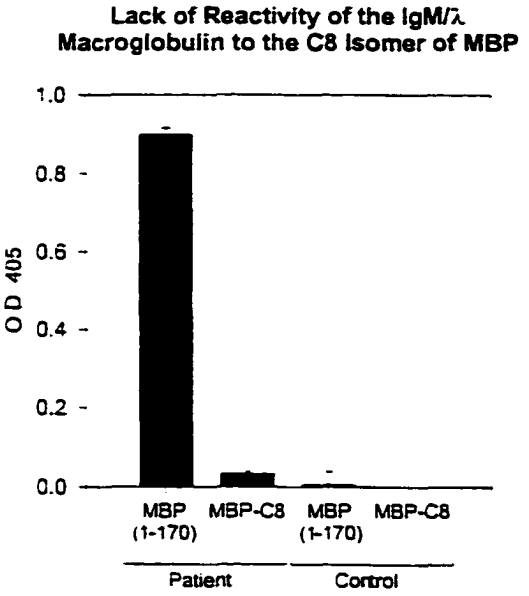

Fig. 3. Lack of reactivity of the IgM/λ macroglobulin to the C8 isomer of MBP. Either human MBP or MBP-C8 was coated on ELISA plates at a concentration of 10 μg/ml. The patient and control serum were diluted 1:100 in PBS containing 1% BSA and allowed to bind for 2 h at RT. Reactivity of the sera was then monitored by Ab to Vλx. Binding of the Ab to Vλx was then detected by an alkaline phosphatase labeled goat anti-rabbit Ig Ab and measured at 405 nm on an ELISA reader.

Using peptides and MBP fragments to determine the epitope on MBP being bound

In order to further localize possible epitopes within human MBP for the patient's MBP-reactive macroglobulin, we assayed binding to synthetic peptide fragments of human MBP. Because all of the known murine Vλx-containing Abs have been shown to

preferentially react with MBP residues 25-34 except for mAb F28C4, which was raised in the PL/J mouse specifically to MBP peptide Ac 1-9 (Zhou and Whitaker, 1993), we expected to see reactivity of the patient's IgM/λ to residues 25-34 of MBP. Reactivity of the macroglobulinemia patient's IgM/λ to MBP peptide residues 25-34 was absent (Fig. 4). While unexpected this would seem to confirm that arginines altered in the C- rather than N-terminus are disrupting the epitope. Consistent with this idea, there was strong binding to the carboxyl-terminal half of the molecule. When microtiter plates were coated with MBP peptide residues 90-170, strong reactivity of the IgM/λ macroglobulin was observed (Fig. 4). There was no reactivity with MBP peptides 1-42 and 43-88. No reactivity was observed against MBP peptide 87-99 or residues 116-170 (Fig. 4) prompting us to synthesize a set of peptides encompassing MBP residues 90-118 and test these for reactivity in order to define the epitope on MBP against which the patient's macroglobulin was reactive.

In Fig. 5 we show the patient's IgM/λ macroglobulin reacts very well with MBP peptide 90-118, but as amino acid residues are removed from the amino-terminal region of the peptide, the reactivity declines. The patient's macroglobulin reacts to a slightly greater extent to MBP peptide 84-96 than it does to MBP 90-118 (Fig. 5), which we might expect since N-terminal residues in the region encompassed by MBP peptide 90-118 seem to have a greater contribution to binding by the IgM/λ macroglobulin than do carboxy-terminal residues.

Discussion

Neuropathy is a relatively uncommon feature of WM yet results in widespread neural damage for which a mechanism remains to be defined (Abramsky, 1980). For

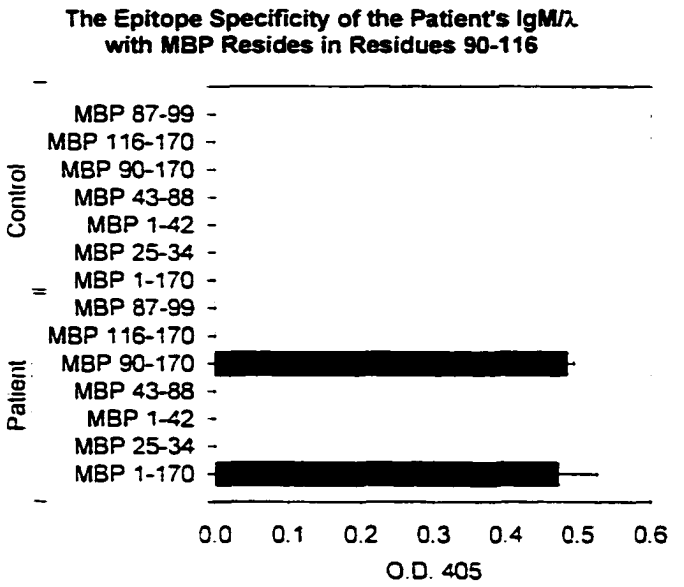

Fig. 4. The epitope specificity of the Patient's IgM/λ with MBP resides in residues 90-116. Whole human MBP (1-170) or MBP peptides 90-170, 116-170, 87-99, 25-34, 1-42, and 43-88 were coated on ELISA microtiter wells at a concentration of 10 μg/ml in PBS containing 1% BSA at 4°C for 16 h. Serum from the macroglobulinemia patient and the normal control were diluted 1:100 and allowed to bind for 2 h at RT. Reactivity of the sera was then monitored by Ab to Vλx. Binding of the Ab to Vλx was then detected by an alkaline phosphatase labeled goat anti-rabbit Ig Ab and measured at 405 nm on an ELISA reader.

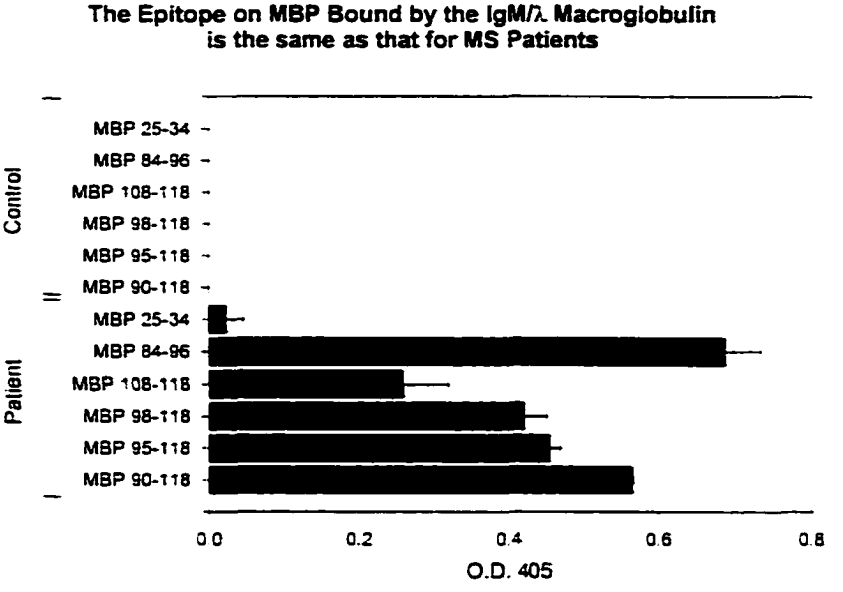

Fig. 5. The epitope on MBP bound by the IgM/λ macroglobulin is the same as that for MS patients. MBP peptides 25-34, 84-96, 90-118, 95-118, 98-118, and 108-118 were coated on ELISA plates at a concentration of 100 μg/ml. Serum from the macroglobulinemia patient and the normal control were diluted 1:300 in PBS containing 1% BSA and allowed to bind for 2 h at RT. Anti-Vλx Ab was used to monitor reactivity. Binding of the Ab to Vλx was then detected by an alkaline phosphatase labeled goat anti-rabbit Ig Ab and measured at 405 nm on an ELISA reader.

those patients with a neuropathy it is usually a peripheral neuropathy due to IgM Abs with a κ light chain (Dalakas and Engel, 1981) reacting with carbohydrate moieties of MAG (Latov et al., 1981; Dellagi et al., 1983; Nobile-Orazio et al., 1987) or gangliosides (Shy et al., 1986; Latov et al., 1988). Very little information exists in regard to direct central nervous system involvement in WM. The patient referred to in the present study (Kira et al., 1997) was diagnosed with a cranial polyneuropathy with extensive radiculoneuropathy. This is the only reported case of an IgM paraproteinemia reactive with MBP.

The impetus for this study was to further characterize this MBP-reactive IgM/λ. When this macroglobulin was reacted with Ab to Vλx, a rarely used murine λ variable region gene product, strong binding was observed by ELISA. This indicates a Vλx-like Id resides on the patient's MBP-reactive macroglobulin. This further suggests that certain human Abs could contain a Vλx Id and, as with the murine Vλx-containing Abs, the presence of a Vλx Id seems to correlate with MBP specificity.

Since the presence of a Vλx Id in murine Vλx-bearing Abs correlates with MBP binding preferentially to MBP peptide 25-34 (Galin et al., 1996), we hypothesized that the presence of a Vλx Id on an Ab indicates MBP reactivity, specifically to residues 25-34 of the molecule. When the patient's macroglobulin failed to react with MBP-C8, we concluded deimidation of the arginine residues forming citrulline, probably at residues 25 and/or 31, disrupts the epitope on MBP bound by the IgM/λ macroglobulin. But upon reacting the patient's IgM/λ macroglobulin with several N-terminal peptide fragments of MBP including MBP peptide 25-34 no binding was observed.

Citrullination of the MBP molecule completely blocks binding of the IgM/λ macroglobulin to the C8 isomer of MBP. Arginine residues located in both the N- and C-terminal regions of MBP are sites of citrullination. We first tested the N-terminal peptide 25-34 for reactivity because it contained two citrullination sites at positions 25 and 31. Considering the N-terminal peptide was not bound we concluded the epitope being recognized by the patient's macroglobulin must reside in the C-terminal region of the molecule as this region of the molecule has more modification sites. Indeed, we found MBP fragment 90-170, which encompasses the four remaining citrullination sites of MBP, was bound by the patient's IgM/λ macroglobulin while reactivity to MBP 116-170 was not observed, we concluded the epitope is most likely between residues 90-116 of MBP.

We made a set of four peptides encompassing residues 90-118 in order to determine the exact epitope on MBP being bound by the patient's IgM/λ macroglobulin. We learned that the extent of reactivity is greatest when the intact 90-118 peptide is tested, and when N-terminal amino acid residues are removed reactivity declines. Finally, we tested MBP peptide 84-96 and found binding by the IgM/λ macroglobulin. We examined reactivity to this peptide because it is an immunodominant epitope bound by autoantibodies from MS patients (Wucherpfennig et al., 1997). It thus appears that in the present patient and perhaps the small number of other WM patients neural damage may be mediated via Abs with a Vλx-like Id binding to peptide residues 84-96.

Collectively, these results show that, as with murine Vλx-bearing Abs, the presence of a Vλx Id seems to correlate with MBP specificity and perhaps more importantly, such an Id can be found on human Abs. We speculate that such MBP reactivity may be

involved in the pathogenesis of neural damage in patients with primary macroglobulinemia. It is tempting to speculate that there may be a link between the pathogenesis of neuroimmunologic disease and IgM/Vλx Abs especially given the idiotope-epitope binding characteristics. Studies are currently underway to further investigate this commonality.

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HUMAN Vλ VIII-CONTAINING ANTIBODIES ARE REACTIVE WITH MYELIN BASIC PROTEIN

by

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Abstract

A novel human λ variable region (Vλ), termed VλVIII, has been shown to possess certain similarities including sequence homology with an unusual murine Vλ gene product, Vλx. We have previously shown that Vλx both binds human myelin basic protein (MBP) and can confer MBP reactivity to an antibody (Ab). As a consequence of the sequence similarity with Vλx, we tested whether VλVIII and VλVIII-containing IgG could bind MBP and were antigenically related to Vλx. Using enzyme-linked immunosorbent assay procedures, we found that a polyclonal monospecific Ab to Vλx was cross-reactive with an intact IgG/VλVIII Ab and, to a lesser extent, with two VλVIII Bence Jones Proteins (BJPs). Conversely, we observed cross-reactivity between an anti-VλVIII monoclonal antibody (mAb) and a Vλx-bearing mAb, termed F28C4. Like Vλx, which can confer MBP reactivity to an Ab, the monoclonal IgG/VλVIII from a plasmacytoma patient as well as naturally occurring IgG/VλVIII had a high degree of reactivity against MBP. However, unlike Vλx the reactivity of the BJPs to MBP was minimal. The reactivity of the IgG/VλVIII to MBP seems specific since binding to a panel of other potential autoantigens was negative. Collectively, these results suggest that VλVIII is the human homolog of murine Vλx. Considering its reactivity with MBP it is tempting to speculate that VλVIII expression may be associated with multiple sclerosis and/or other neurological diseases.

Introduction

Kabat et al. (1991) originally classified human lambda (λ) light (L) chains into six λ variable (V) region subgroups or gene families on the basis of distinctive amino

acid and nucleic acid sequence homologies. Five of these six chemically defined subgroups–VλI, VλII, VλIII, VλIV, and VλVI–have also been confirmed serologically (Solomon and Weiss, 1987). VλVIII, first recognized by Robbins et al. (1990) in a monoclonal IgMλ (termed HAF10) rheumatoid factor-producing cell line, is an additional and apparently minor subgroup which has been defined both chemically and serologically. Immunizing mice with Bence Jones proteins (BJPs) representing each of the aforementioned human Vλ gene families including VλVIII has resulted in a panel of Vλ subgroup-specific monoclonal antibodies (mAbs) (Abe et al., 1994). This panel of mAbs has been used to determine both the absolute concentration and percentage of distribution of each Vλ subgroup among λ-type immunoglobulins (Igs) in normal serum as well as in certain disease states such as multiple myeloma, AL amyloidosis, and Waldenstrom's macro-globulinemia (WM) or in response to immunizations (Abe et al., 1994; Ozaki et al., 1994).

Two BJPs designated Hag and Biv were obtained from multiple myeloma patients (Solomon et al., 1994). Both Hag and Biv were not recognized by any of the polyclonal or monoclonal anti-VλI, II, III, IV, or VI subgroup-specific antibodies (Abs) and were shown to possess an unusually long complementarity-determining region (CDR) 2 that contained 11 residues rather than the 7 typically found for λI, λII, λIII, λIV, and λVI L chains. BJP Hag was used to immunize mice to obtain a monoclonal anti-VλVIII subgroup-specific mAb (termed 31-8C7) which recognized Hag, Biv, and HAF10 but not L chains from the other Vλ subgroups. The amino acid sequences of Hag and Biv were closely related to that deduced from the complementary deoxyribonucleic acid specifying the prototype VλVIII protein HAF10 (82% homology between proteins Hag

and Biv, 77% homology between Hag and HAF10, and 79% homology between Biv and HAF10), whereas these VλVIII proteins exhibited only 38-45% amino acid homology with the other Vλ subgroups. The extent of homology between the VλVIII germline gene and other known human gene families ranged from 56-66% and 36-58% at the nucleotide and protein levels, respectively (Ch'ang et al., 1995). Interestingly, the VλVIII germline gene was more closely related to a pair of rabbit Vλ germline genes–RVλ2 and RVλ3–and to the mouse Vλ gene–Vλx–than to the other human Vλ gene families. All of these similarities and sequence homologies make it appear likely that the VλVIII gene evolved independently of the other human Vλ genes (Hayzer, 1990).

Originally discovered in 1987 in polyclonally activated mouse B cells, Vλx is a newly described Vλ gene segment (Dildrop et al., 1987; Sanchez et al., 1987). Vλx comes about as a result of rearrangement with Jλ2-Cλ2, and its amino acid sequence is only 30-33% homologous to other known Vλ or V-kappa (κ) genes. Homologous sequences have been found in various mammalian species including man as noted above, thus suggesting that Vλx apparently existed before the speciation of mammals (Sanchez et al., 1990). Vλx and VλVIII show more homology to each other than to other murine Vλ and Vκ genes or human Vλ subgroups, respectively. Both gene products are rarely expressed in normal sera: Vλx is in less than 0.5% of all Ig in normal mouse sera, and approximately 3% of normal human Ig contains VλVIII.

F28C4, a mAb raised in the PL/J mouse against myelin basic protein (MBP) peptide acetyl (Ac) 1-9, was found to harbor this unusual λ L chain (Zhou and Whitaker, 1993; Maier et al., 1994). Prior to the demonstration that F28C4 could bind MBP, no Ab with this particular L chain V region had any known specificity. In subsequent investi-

gations Vλx alone (Galin et al., 1996) and other Vλx-containing Abs of previously unknown specificity (Dildrop et al., 1987; Sanchez et al., 1987, 1990; Chen et al., 1994) as well as polyclonal Vλx Ig from normal murine sera were all found to bind MBP but not other common autoantigens (Galin et al., 1996). These results demonstrate that Vλx and Vλx-containing Abs have an inherent affinity for MBP. As a consequence of Vλx's antigenic specificity, we have investigated whether VλVIII is also reactive with MBP and immunologically related to Vλx.

Materials and methods

Human MBP

Human MBP, the C1 isoform, was prepared by previously described techniques (Whitaker et al., 1992). Briefly, human MBP C1 (1-170) was isolated from human brain by delipidation, acid extraction at pH 3, and carboxymethylcellulose chromatography at pH 10.6. The human MBP peptides were synthesized in our laboratory on a Biosearch Peptide Synthesizer (Model 9500; Cambridge, MA) and purified by reverse-phase high-performance liquid chromatography or synthesized by Peninsula Laboratories (San Carlos, CA).

Preparation of mAb F28C4

The preparation and characterization of mAb F28C4 have been previously described (Zhou and Whitaker, 1992). Briefly, synthetic human MBP peptide Ac 1-9 was conjugated to keyhole limpet hemocyanin (Sigma Chemical Co.; St. Louis, MO) with 2% glutaraldehyde and used to immunize PL/J mice. Each mouse received a total of 0.5

mg of the human MBP peptide in six injections over a period of 3 weeks. Spleen and lymph node cells were then removed and fused with SP 2/0 myeloma cells. Hybridoma cells were screened by enzyme-linked immunosorbent assay (ELISA) for their ability to produce mAb to MBP as well as to human MBP peptide Ac 1-9. Limiting dilution was performed twice. The specific clone, F28C4, was further characterized by ELISA, purified by affinity chromatography on MBP-Sepharose, and isotyped and was shown to be an IgG2a/λ.

Polyclonal monospecific Ab to Vλx

A New Zealand white rabbit (Myrtle Laboratories; Thompson Station, TN) was injected subcutaneously at four sites every 2 weeks over a 6-week period with purified F28C4 (50 μg/injection), first in Freund's complete adjuvant and twice in incomplete adjuvant as described previously (Galin et al., 1996). Serum was obtained before the first injection and 2 weeks after each booster to monitor anti-F28C4 titers using ELISA procedures. F28C4 is of the γ2a isotype and, as with all Vλx-bearing Abs, is a λ2 isotype. In order to make the antiserum specific, it was exhaustively absorbed on a polyclonal IgG2a Sepharose 4B affinity column to deplete heavy (H) chain reactivity. Next, the Jλ2Cλ2 reactivity was removed using a MOPC 315 (IgA, λ2) Sepharose 4B affinity column. Monospecific Ab to Vλx was collected after binding to and elution from a F28C4 affinity column. The monospecificity of the resulting anti-Vλx Ab was shown by Western analysis (Galin et al., 1996).

ELISA

Microtiter wells (96-well ELISA plates) (Corning Glass Works; Corning, NY) were coated with human Vλ VIII proteins, mAb F28C4, human MBP C1 (1-170) (10.0 μg/ml in phosphate-buffered saline (PBS)), various other self antigens (Ags) (concentrations equimolar to MBP), or human IgG (Sigma Chemical Co.) at 4°C for 16 h and plates were blocked with 2% casein in PBS. Washings were performed with PBS containing 0.05% Tween 20. Abs were diluted in PBS containing 1% bovine serum albumin (BSA) and allowed to bind for 2 h at room temperature. Ab binding was detected with either rabbit anti-Vλx Ab followed by an alkaline phosphatase conjugated anti-rabbit IgG Ab (Southern Biotechnology; Birmingham, AL) or mouse anti-Vλ VIII Ab followed by an alkaline phosphatase labeled goat anti-mouse IgG Ab (Southern Biotechnology). Reactions were developed using *p*-nitrophenyl phosphate (Sigma Chemical Co.), and color development was quantitated as absorbance at 405 nm on a Tecan WinSeLecT Microplate Reader (Research Triangle Park, NC). Background optical density was the mean of wells where coating Ag was omitted. Controls were also performed with Ag-coated wells and all ELISA components except the primary Ab. These were always negative.

Anti-Vλ VIII mAb

An anti-Vλ VIII mAb, mAb 31-8C7, was obtained by injection of the λ BJP Hag. This mAb, when tested against a reference group of >100 serologically and chemically classified monoclonal Igλ proteins, was found to react with only two, which, by sequence, were classified as Igλ VIII (Levy et al., 1988; Robbins et al., 1990). Hybridoma

cells were injected intraperitoneally into pristane-primed mice and the mAbs isolated from the ascites fluid by 45% ammonium sulfate precipitation followed by ion-exchange chromatography using a Q-Sepharose Fast Flow column (FPLC system) (Pharmacia; Piscataway, NJ). The purity of the mAbs was determined by agarose gel electrophoresis. mAb 31-8C7 was stored at –20°C in PBS, pH 7.4, containing 50% glycerol.

Human VλVIII proteins

BJPs, termed Hag and Biv, were obtained from patients with multiple myeloma. By amino acid sequencing and reaction with mAb 31-8C7, the BJPs were shown to be VλVIII. An intact IgG/VλVIII Ab was obtained from a plasmacytoma patient (Wee IgG/VλVIII).

Results

Immunologic cross-reactivity between murine Vλx and human VλVIII

We have previously shown that a human IgM/λ possessed a Vλx-like idiotype (Id) (Noerager et al., 2001). This observation along with the demonstration of sequence homology between Vλx and VλVIII suggested the possibility of immunologic cross-reactivity between these two λ L chain V regions. To address this possibility we first investigated potential cross-reactivity between our anti-Vλx Ab and the human VλVIII BJP from two multiple myeloma patients as well as the IgG/VλVIII from a plasma-cytoma patient. In Fig. 1 ELISA plates were coated with IgG/VλVIII Ab or the two BJPs and tested for reactivity to both anti-Vλx and anti-VλVIII Abs. The binding of the anti-Vλx Ab to IgG/VλVIII was of a magnitude similar to that of the anti-VλVIII Ab. In

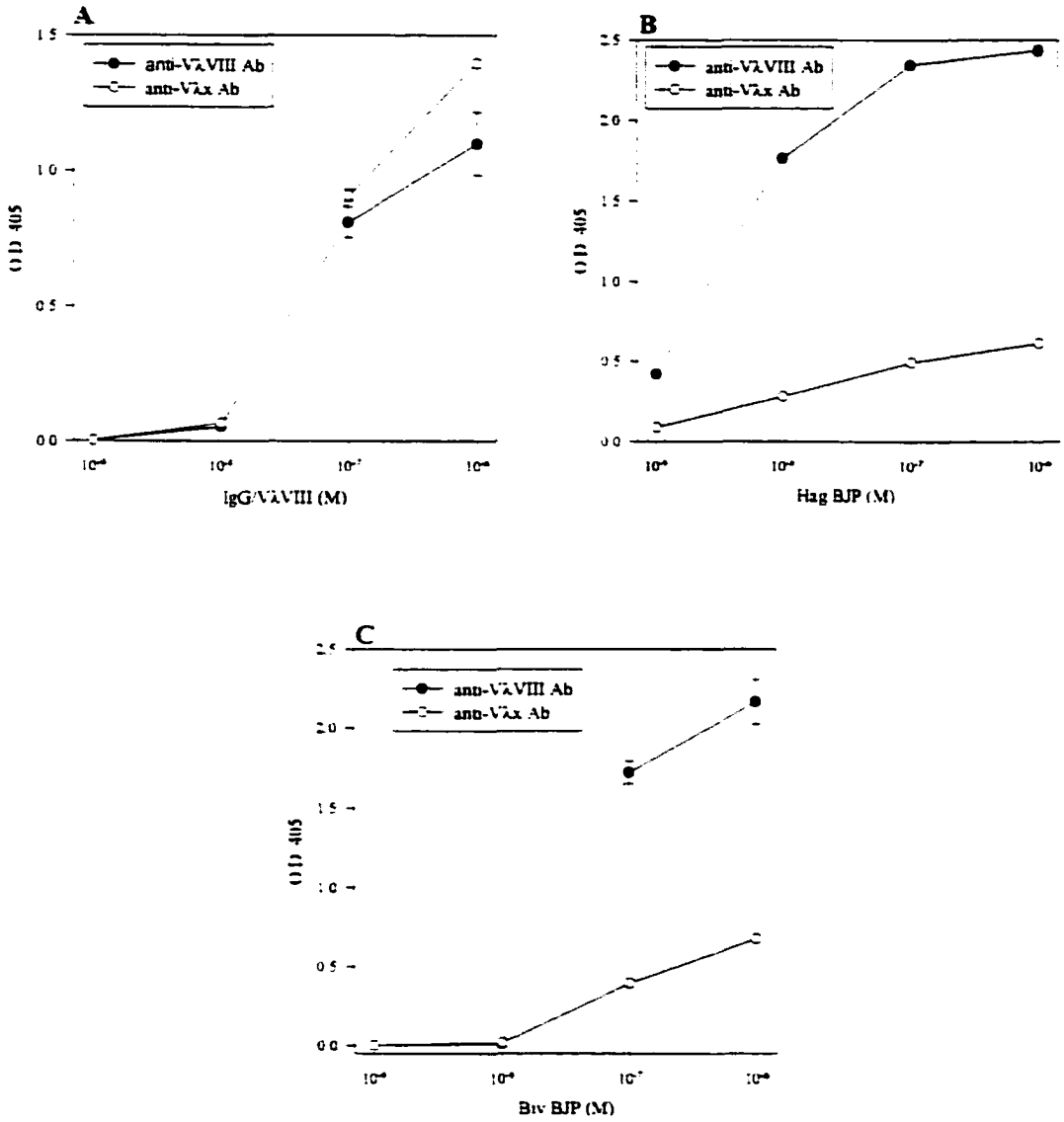

Fig. 1. Cross-reactivity between anti-Vλx Ab and human VλVIII. (A) IgG/VλVIII Ab, (B) Hag BJP. or (C) Biv BJP was coated onto microtiter wells at concentrations ranging from 10^{-6} M to 10^{-9} M at 4°C for 16 h. Abs to either Vλx (1:100) or VλVIII (0.1 μg/ml) were then allowed to react with the human VλVIII antigens for 2 h at room temperature. Binding of the anti-Vλx and anti-VλVIII Abs was then detected by alkaline phosphatase labeled goat anti-rabbit IgG or goat anti-mouse IgG, respectively. Absorbance was measured at 405 nm on an ELISA reader.

fact, the reactivity as monitored by Ab to Vλx was actually slightly higher than that of the Ab to VλVIII. Figs. 1B and C show that the VλVIII BJPs, Hag and Biv, respectively, exhibit strong reactivity with anti-λVIII Ab as well as lesser but nonetheless substantial binding by Ab to Vλx. Fig. 2 shows the converse. That is, the Vλx-containing mAb, F28C4, was bound by anti-VλVIII Ab. These results demonstrate a strong immunologic relatedness of murine Vλx and human VλVIII.

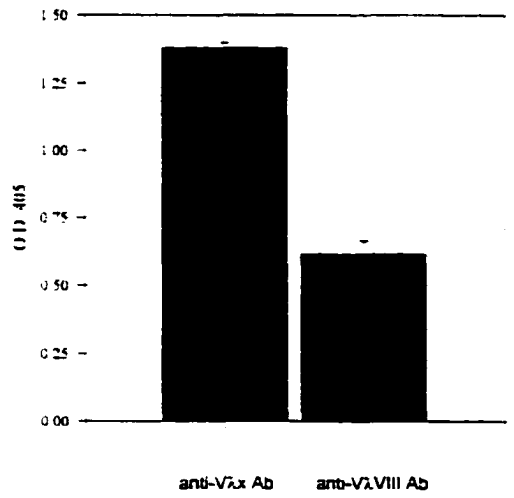

Fig. 2. Cross-reactivity between anti-VλVIII Ab and F28C4 (IgG2a/Vλx). F28C4 was coated at 4°C overnight onto 96-well ELISA plates at concentrations ranging from 10^{-6} M to 10^{-9} M. Plates were then washed and Abs to either Vλx (1:100) or VλVIII (0.1 μg/ml) were allowed to react with mAb F28C4 for 2 h at room temperature. Binding of anti-Vλx and anti-VλVIII Abs was then detected by alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG1 (anti-mouse IgG1 was used in this case to negate any cross-reactivity of the secondary Ab with F28C4,γ2a), respectively. Absorbance was then measured at 405 nm on an ELISA reader.

IgG/VλVIII binds human MBP

In a previous study we have shown that a panel of Abs including all known Vλx-bearing mAbs (all but one of previously unknown specificity) as well as polyclonal Vλx containing Ig from normal murine sera bound human MBP (Galin et al., 1996). In this same study it was also demonstrated that recombinant murine Vλx alone had a high degree of reactivity against MBP. These findings together with the present observations of strong immunologic relatedness suggested that like Vλx, human VλVIII//IgG might have inherent affinity for MBP. In fact, we found that the plasmacytoma-derived IgG/VλVIII was highly reactive with human MBP (Fig. 3) when monitored with Ab to Vλx or VλVIII. Unlike recombinant murine Vλx, the human VλVIII BJPs exhibited little inherent affinity for human MBP when monitored with Ab to either Vλx or VλVIII (data not shown). This suggests that the H chain of the human IgG/VλVIII is essential for binding MBP.

Lack of polyreactivity of IgG/VλVIII

The aforementioned panel of Vλx-containing mAbs has been shown to have a measure of specificity for human MBP (Galin et al., 1996). Likewise, a human IgM/λ WM with a Vλx Id was also found to react specifically with MBP (Noerager et al., 2001). As a consequence, we examined whether the reactivity of this human IgG/VλVIII was specific for MBP. As shown in Fig. 3 we found that the IgG/VλVIII was not polyreactive with other potential autoantigens such as actin, single-stranded deoxyribonucleic acid (ssDNA), and thyroglobulin but rather has specificity for MBP.

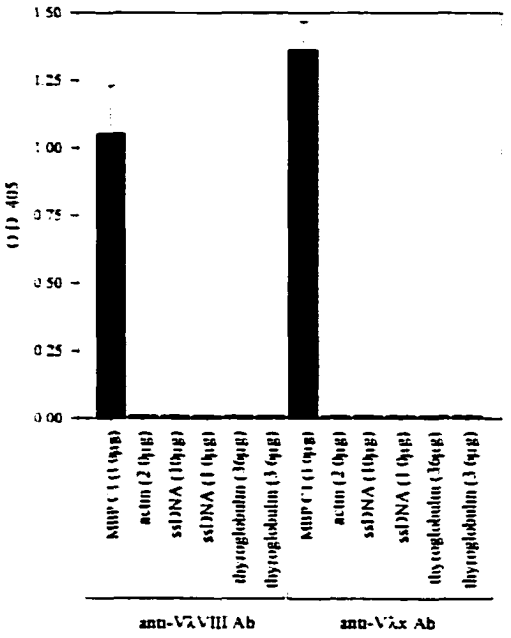

Fig. 3. Polyreactivity of the IgG/VλVIII Ab to MBP and other common autoantigens. ELISA microtiter wells were coated with equimolar concentrations (540 nM) of human MBP, actin, ssDNA, and thyroglobulin. Both thyroglobulin and ssDNA were also coated at 10-fold lower concentrations. Plates were incubated at 4°C for 16 h. The IgG/VλVIII Ab was then diluted in PBS containing 1% BSA to a concentration of 10^{-6} M and allowed to bind for 2 h at room temperature. Reactivity of the IgG/VλVIII Ab to MBP and the other potential self Ags was monitored by Ab to either Vλx (1:100) and VλVIII (0.1 μg/ml) and then detected by an alkaline phosphatase conjugated goat anti-mouse or goat anti-rabbit Ab, respectively. Absorbance was measured at 405 nm on an ELISA reader.

Naturally occurring VλVIII-containing IgG binds MBP

In order to determine whether, like Vλx (Galin et al., 1996), naturally occurring VλVIII-containing Ab could bind human MBP, we tested pooled human IgG for such reactivity. By comparison with the appropriate standard curves, we found that while similar to previous reports that VλVIII-containing IgG constitutes 3.6% of total human IgG, 25% of naturally occurring VλVIII positive IgG molecules have affinity for MBP. Thus, the IgG/VλVIII fraction is enriched almost seven-fold for Ab with affinity for MBP (Table 1). These results strongly suggest that the presence of a VλVIII L chain increases the probability that an IgG will bind MBP. This possibility is borne out by the finding that 10 μg/mg or 1% of total IgG binds human MBP (Table 1). Considering that 9 μg/mg of total IgG reactive with MBP is IgG/VλVIII demonstrates that about 9 of 10 IgG with inherent affinity for MBP have VλVIII L chains.

Table 1
Naturally occurring VλVIII-containing IgG binds MBP

Immunoglobulin	μg/mg Total IgG	% Total
Total IgG/VλVIII	36 +/- 3.0	3.6
MBP-bound IgG/VλVIII	9 +/- 1.6	25
MBP-bound IgG	10 +/- 1.66	1.0

Discussion

An important feature shared by virtually all murine Vλx-bearing Abs is that they exhibit an intrinsic affinity for human MBP and are not polyreactive with other common autoantigens such as actin, thyroglobulin, or ssDNA (Galin et al., 1996). Furthermore, one Vλx-containing Ab, F28C4, exhibits 75% sequence homology in CDR3 of its L and

H chain V regions with the VDJ junction of the T cell receptor (TCR) Vβ8.2 from certain encephalitogenic T cells (Maier et al., 1994). This results in F28C4 and these TCRs having a fine specificity and mode of binding similar to those of the epitope MBP 1-9 as well as sharing a cross-reactive idiotope (CRId) (Zhou et al., 1994). Perhaps most importantly, mAb to the CRId can lessen the severity of experimental allergic encephalomyelitis (Zhou and Whitaker, 1993), the animal model of multiple sclerosis (MS).

As a result of these observations it seemed important to determine whether there were human homologs of Vλx-containing Ab. Considering that VλVIII and Vλx share more sequence homology with each other than with other human or murine Vλ or Vκ regions (Ch'ang et al., 1995), VλVIII-containing Ab seemed a prime candidate. This seems to be the case since we found immunologic cross-reactivity between these two V regions using Ab to Vλx as well as VλVIII. The finding that, like Vλx, VλVIII-containing IgGs have inherent affinity for human MBP provides particularly strong evidence that VλVIII is the human equivalent to murine Vλx.

These findings are particularly intriguing when we consider the recent finding that an IgM/λ macroglobulin from a WM patient had a Vλx Id, bound human MBP at an immunodominant epitope for MS, and apparently caused a polyneuropathy (Noerager et al., 2001). Indeed, the predominance of the expression of particular L chain subgroups has been noted in certain disease states or in response to immunization, which contrasts with their distribution in normal individuals (Abe et al., 1994; Ozaki et al., 1994). For example, the expression of the VλII subgroup accounts for ~3% of the Igλ protein population in normal serum but is expressed by ~40% of λ-type IgG, IgA, and BJPs obtained from patients with multiple myeloma and AL amyloidosis and ~60% of IgM proteins from patients with WM (Solomon and Weiss, 1987). Furthermore, there is

from patients with WM (Solomon and Weiss, 1987). Furthermore, there is evidence that the pairing of particular L and H chains in systemic lupus erythematosus and autoimmune thyroid disease is not a stochastic process (Radic et al., 1991; Portolano et al., 1993).

These findings together with the present observations make it tempting to speculate that there might be skewing from the normal distribution of VλVIII in patients with MS or other neurological diseases. In preliminary studies we have found that the cerebrospinal fluid from 4/6 MS patients and 1/4 controls contained Abs which bound human MBP and were recognized by anti-Vλx Ab. Considering the recent recognition of a probable role of humoral immunity in MS (Rudick, 1987; Whitaker and Benveniste, 1996; Wekerle, 1999), these results would seem to warrant further study with Ab to VλVIII.

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SUMMARY

Developing treatments for autoimmune diseases such as MS can be a difficult prospect due to the fact that the causal agent or agents is oftentimes unknown. Therefore, intervening with much specificity in the immunopathogenic process can be challenging. Ideally, treatment of autoimmune diseases should be aimed at reducing the autoimmune response while leaving the rest of the immune system intact. In the treatment of MS, this ideal has not yet been met. Recent studies showing overlap of disease-associated epitopes that are recognized by T and B cells together with our own data showing functional and structural relatedness of the Ag receptors on certain of the aforementioned cells suggested a novel and relatively specific approach to modulating disease (Maier et al., 1994; Wucherpfennig et al., 1997). The central idea is that if there is considerable overlap in the structure and fine specificity and, consequently, CRId of Ab and TCR for a disease-associated epitope, as exists between mAb F28C4 and TCR from certain encephalitogenic T cells, then such a CRId could potentially serve as a surrogate vaccine to an encephalitogenic T cell or its TCR. Such Ab vaccines would therefore lead to not only an anti-Id Ab response but also a cross-reactive anti-clonotypic Ab response against disease-associated T cells. We have provided data supporting the validity of this approach in EAE, the animal model of MS.

When vaccinated with the CRId on mAb F28C4 as an Ag, animals mount an anti-Id response that protects them from disease induction with 80-100% efficiency regardless of species, MHC haplotype, or encephalitogenic epitope. Although epidemi-

ologic studies of MS have determined certain populations are more susceptible to the disease than others, determining with any certainty who will get the disease is impossible at this time due to the combination of genetic as well as unknown environmental factors that contribute to disease pathogenesis. Therefore, developing methods of treating active disease is perhaps more beneficial at this time. In addition to substantial data on the value of F28C4 as a vaccine in EAE, we have gathered evidence on its therapeutic potential for MS.

We have shown that animals immunized with F28C4 prior to disease induction using activated encephalitogenic T cells are protected from disease induction with >80% efficiency, thus indicating that protection conferred by F28C4 occurs even after the activation of T cells. In addition, the ability of F28C4 to protect SJL mice from rEAE (relapsing-remitting disease being the most common form of MS in humans) induced with whole spinal cord homogenate holds great promise for its value in treating many patients afflicted with MS. Further evidence of F28C4's therapeutic potential lies in its ability to reduce incidence and ameliorate severity in an outbred rat population (Kavelaars et al., 1999) in which every individual, like humans, is genetically distinct. One way in the future of directly testing the therapeutic ability of F28C4 is to induce rEAE in SJL mice and then immunize animals with F28C4 after the initiation of the primary phase of disease in order to prevent disease relapses.

One of the hallmarks of EAE is the observation that the usage of TCR genes can be biased or restricted, particularly Vβ8 gene products, in the recognition of MBP or encephalitogenic MBP peptides (Acha-Orbea et al., 1988; Burns et al., 1989). As mentioned previously, F28C4, raised in the PL/J mouse against MBP peptide Ac 1-9, shares a CRId with the PL/J Vβ8.2^{-} TCR recognizing the same MBP peptide (Zhou and

Whitaker, 1993). Our data indicate that F28C4 does not act simply through a pan-Vβ8.2 pathway as is suggested by its ability to protect PL/J mice and Lewis rats from disease. Specifically, F28C4 can protect SJL mice from disease induction, although this mouse strain lacks Vβ8.2. Thus, F28C4 appears to operate through an anti-Id mechanism as is suggested by the ability of mAb F30C7 anti-Id to lessen clinical EAE in the adoptive transfer model of EAE in PL/J mice, possibly by T cell anergy (Zhou and Whitaker, 1993; Zhou et al., 1994).

We have advanced these findings by passively immunizing Lewis rats with the anti-CRId mAb F30C7 and then actively inducing disease with MBP peptide 68-88 and demonstrating this mAb's ability to reduce disease incidence and severity through inhibiting proliferation of encephalitogenic T cells. Furthermore, rather than modulating the production of Th1 proinflammatory cytokines or Th2 anti-inflammatory cytokines, F30C7 confers its effects through a novel mechanism that to our knowledge has not been demonstrated elsewhere. When MBP 68-88-sensitized LNC are cultured in F30C7, the anti-CRId mAb apparently induces the in vitro generation of $CD4^{-}/CD25^{-}$ regulatory T cells (O'Garra et al., 1997) that down-regulate production of encephalitogenic T cells in an Ag-independent manner. Collectively, these results suggest that active immunization with F28C4 induces the generation of an anti-CRId Ab response in vivo which generates CNS-targeted $CD4^{-}/CD25^{-}$ regulatory T cells that non-specifically down-regulate activated T cells and results in the prevention of disease symptoms.

Additional experiments are aimed at optimizing ways in which F30C7 anti-CRId mAb can improve clinical disease scores. The first way in which we propose to address this is to immunize animals passively with a higher dose of F30C7 to see whether this immunization can further lower disease incidence and severity. Additionally, we intend

to investigate whether culturing encephalitogenic T cell lines in combination with $CD4^+/CD25^+$ regulatory T cells generated by co-culture of sensitized lymph node cells (LNCs) with F30C7 can protect animals from EAE induction. Finally, we are interested in whether culturing F30C7-treated LNCs in differing ratios with naïve cells can result in inhibition of proliferation and subsequently inhibit disease.

A predominant reason F28C4 is an effective vaccine in EAE is that this Ab bears the unusual Vλx L chain (Maier et al., 1994). An important feature shared by virtually all murine Vλx-bearing Abs, including F28C4, is that they exhibit an intrinsic affinity for human MBP and are not polyreactive with other common autoantigens such as actin, thyroglobulin, or single-stranded deoxyribonucleic acid (Galin et al., 1996a). As a result of these observations it seemed important to determine whether there were human homologs of Vλx-containing Ab. The first demonstration that there might be a human homolog of murine Vλx occurred when Kira et al. (1997) treated a patient with WM complicated by polyneuropathy whose serum contained an MBP-reactive IgM/λ macroglobulin. After obtaining a sample of this patient's serum, we were able to demonstrate that the sample possessed certain intriguing similarities with murine Vλx. Specifically, the serum possessed a Vλx Id that recognized an epitope on MBP immunodominant in MS patients and was not polyreactive against other common autoantigens (Noerager et al., 2001). Considering this result, we have further investigated the possibility that a human homolog of Vλx exists.

Considering that VλVIII (Robbins et al., 1990), a relatively new member of the human λ L chain V region subgroups originally classified by Kabat et al. (1991), and Vλx share more sequence homology with each other than with other human or murine

Vλ or Vκ regions (Ch'ang et al., 1995), VλVIII containing Ab seemed a prime candidate to be the human homolog of murine Vλx. As a consequence of Vλx's antigenic specificity, we have investigated whether VλVIII is also reactive with MBP and immunologically related to Vλx, which seems to be the case since we found immunologic cross-reactivity between these two V regions using Ab to Vλx as well as VλVIII. The finding that, like Vλx, VλVIII-containing IgGs have inherent affinity for human MBP provides particularly strong evidence that VλVIII is the human equivalent to murine Vλx. Considering that the plasmacytoma patient from whom this IgG/VλVIII Ab was obtained also exhibited neurologic symptoms of disease, we are in the process of determining whether other human VλVIII-containing Abs exhibit immunologic cross-reactivity with Vλx.

These findings make it tempting to speculate that there might be skewing from the normal distribution of VλVIII in patients with MS or other neurological diseases. In preliminary studies we have found that the CSF from 4/6 MS patients and 1/4 controls contained Abs which bound human MBP and were recognized by anti-Vλx Ab. Considering the recent recognition of a probable role of humoral immunity in MS (Rudick, 1987; Whitaker and Benveniste, 1996; Wekerle, 1999), these results would seem to warrant further study with Ab to VλVIII.

In these studies we have demonstrated that a mAb, F28C4, possessing a CRId with TCR from certain encephalitogenic T cells can prevent with nearly 100% effectiveness both the active and adoptive inductions of EAE regardless of species, MHC haplotype, or encephalitogenic epitope. Furthermore, we have demonstrated that the intact Ab is necessary for optimal protection from disease. In addition to its value as a

vaccine, F28C4 also possesses therapeutic potential. Further implications for the therapeutic potential of F28C4 lies in the demonstration that the L chain borne by F28C4, Vλx, has a human homolog, VλVIII. It appears that F28C4 is manifesting its effects through the induction of an F30C7-like anti-Id response that inhibits proliferation of encephalitogenic T cells through the induction of a $CD4^-/CD25^-$ regulatory T cell response. Collectively, these results suggest that a novel and specific way of modulating autoimmune disease is with mAb possessing CRId inducing the generation of $CD4^-/CD25^-$ regulatory T cells to down-regulate the autoimmune process while leaving the rest of the immune system intact.

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APPENDIX A
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF
ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: May 10, 2001

TO: J. Edwin Blalock, Ph.D.
MCLM-896 0005
FAX 934-1446

FROM: Clinton J. Grubbs, Ph.D., Chairman
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on May 10, 2001.

Title of Application: A Therapeutic Vaccine For Myasthenia Gravis

Fund Source: **Muscular Dystrophy Assoc.**

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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APPENDIX B

INSTITUTIONAL REVIEW BOARD APPROVAL FORM

OMB No. 0925-0418
Approved for use through 01/31/2001

Protection of Human Subjects
Assurance Identification/Certification/Declaration
(Common Federal Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Department and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the common rule. See section 101(b) the common rule for exemptions. Institutions submitting applications or proposals for support must submit an assurance of compliance that covers the research institutions or proposal unless otherwise advised by the Department or Agency. Institutions which do not have such an assurance must submit an certification or appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the or a written request from the Department or Agency. certification of IRB review and approval with each application or assurance and certification of IRB review and approval within 30 days

1. Request Type
☐ ORIGINAL ☐ FOLLOW-UP ☐ EXEMPTION

2. Type of Mechanism
☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP ☐ COOPERATIVE AGREEMENT ☐ OTHER:

3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.

4. Title of Application or Activity
Chiasl Sulfate and Multiple Sclerosis

5. Name of Principal Investigator Program Director, Fellow or Other
BLALOCK, J EDWIN

6. Assurance Status of this Project (*Respond to one of the following*)

☒ This Assurance, on file with Department of Health and human Services, covers this activity
Assurance identification no. M-7149 IRB identification no. _____

☐ This Assurance, on file with (*agency/dept*) _____ covers this activity
Assurance identification no. _____ IRB identification no _____ (*if applicable*)

☐ No assurance has been filed for this project. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

☐ Exemption Status: human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph _____

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

☒ This activity has been reviewed and approved by the IRB in accordance with the common rule and any other governing regulations or suppliers on (date) 5/11/01 by: ☐ Full IRB Review or ☒ Expedited Review

☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certifications will be submitted.

8. Comments
Please note: UAB IRB Protocol Number is X000324010
Protocol subject to Annual continuing review.

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed and certification will be provided.

10. Name and Address of Institution
University of Alabama at Birmingham
701 20th Street South
Birmingham, AL 35294

11. Phone No. (*with area code*)
(205) 934-3789

12. Fax No. (*with area code*)
(205) 934-1301

13. Name of Official
Marilyn Doss, M.A.

14. Title
Vice Chair, IRB

15. Signature

16. Date

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Brett D. Noerager

Graduate Program Physiology and Biophysics

Title of Dissertation Cross-Reactive Idiotypes: Vaccine Targets for Autoimmune Diseases

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name	Signature
J. Edwin Blalock, **Chair**	
Frederick S. Galin	
Richard B. Marchase	
Douglas A. Weigent	
Thane Wibbels	

Director of Graduate Program

Dean, UAB Graduate School

Date 6/27/02