Studies on the Immunoregulation of Experimental Allergic Encephalomyelitis with an Emphasis on the Mast Cell

by

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a paralytic autoimmune disease which can be induced in Lewis rats with a single injection of neural tissue or purified myelin basic protein (MBP) emulsified in complete Freund's adjuvant (CFA). This T cell mediated autoimmune disease can be transferred to naive recipients with lymphoid cells derived from actively immunized donors. We have found that MHC-compatibility between transferred lymphocytes and recipient endothelial cells is not a requirement for the elicitation of adoptively transferred disease. Therefore, lymphocyte trafficking into the CNS does not occur though cognate interactions involving antigen presentation by brain capillary endothelial cells to MBP reactive T lymphocytes as they move through circulation. Instead, lymphocyte egress into the CNS is facilitated by the release of inflammatory mediators released by activated mast cells. Pharmacologic agents which deplete mast cells of vasoactive amines, inhibit degranulation, or act as vasoactive amine antagonists, effectively block or significantly alter the expression of EAE. Mast cell activation appears to be mediated by a T cell derived, antigen specific factor similar to what has been described in a variety of other cell mediated immune responses. We have also found that proteases released by activated mast cells are capable of degrading MBP in the myelin sheath, liberating encephalitogenic peptides which can stimulate MBP reactive T cells. Degradation of MBP in the myelin sheath by mast cell proteases may explain how a membrane associated protein, found on oligodendrocytes which do not function as antigen presenting accessory cells, can activate MHC class II restricted lymphocytes. The release of

proteolytic MBP fragments would make MBP available for antigen processing and presentation to MBP reactive lymphocytes which infiltrate the CNS during the course of EAE.

INTRODUCTION AND LITERATURE REVIEW

Autoimmunity, the Breakdown of Self Nonself Recognition

The immune system has the capability to recognize and respond to an almost unlimited number of foreign materials, yet it remains unresponsive to self. Mechanisms allowing the immune system to be tolerant of self antigens are both complex and poorly understood. One proposed mechanism for self tolerance is that autoaggressive T cells are eliminated during thymic development. While elimination of self-reactive cells has been convincingly demonstrated as one mechanism for tolerance (1), other regulatory mechanisms also exist. Under experimental conditions, self antigens can be used to elicit immune responses (2), demonstrating that autoaggressive cells do mature and circulate in normal individuals. Therefore, the immune system must have regulatory elements which prevent self-reactive T cells from responding against their target antigens. Evidence for suppressor cells which regulate the activity of antigen reactive cells has been convincingly demonstrated in models where nonresponsiveness to foreign antigens is induced (3). Similar suppressor cell circuits could prevent autoreactive cells from reacting with self.

Understanding the immunoregulatory events which allow tolerance to self, and responsiveness to non-self is a fundamental goal of contemporary immunology. Research efforts in understanding self/non-self recognition has produced a number of autoimmune disease models which are triggered by immunization with self antigens. These include: experimental allergic neuritis (EAN), an autoimmune response directed against peripheral neural antigen P2 (4); experimental autoimmune

uveoretinitis (EAU), a response directed against retinal protein S-antigen (5); experimental autoimmune thyroiditis (EAT), a response directed toward thyroglobulin (6); collagen arthritis which is initiated to type II collagen (7); and experimental autoimmune encephalomyelitis (EAE) which is induced with neural tissue (8). Perhaps the most widely studied of these autoimmune diseases is EAE, a paralytic autoimmune disease that can be induced in most mammalian species including mice (9), rats (10), guinea pigs (11), rabbits (12), monkeys (13) and most other mammals (14). The ease in which the EAE model can be manipulated has resulted in extensive use of this system in the study of autoimmune diseases and immunoregulation in general. EAE also has a number of similarities with human paralytic diseases including post-infectious encephalomyelitis, post-vaccinal encephalomyelitis and multiple sclerosis (MS) (15). Consequently, this prototypic autoimmune disease has also been used extensively as a model for the study of paralytic diseases in humans (16).

Experimental Allergic Encephalomyelitis (EAE) is a Cell Mediated, Autoimmune Disease.

Experimental allergic encephalomyelitis (EAE) is a paralytic autoimmune disease resulting from an immune response directed toward neuroantigens. The disease can be elicited in most mammals, including man, by an injection of either allogeneic or xenogeneic Central Nervous System (CNS) tissue emulsified in Complete Freund's Adjuvant (CFA) (17). In response to the sensitizing injection of CNS tissue, antigen specific T cells develop in the draining lymph nodes. Once sensitized, these autoaggressive T cells enter the circulation and eventually reach

the CNS where they encounter autologous neuroantigens. The ensuing immune response results in extensive mononuclear cell infiltrates in the post capillary venules and varying levels of demyelination (18). Demyelination is accompanied by edema and fibrin deposition in the CNS, both of which are also suspected of contributing to disease progression (18,19). Damage to the myelin and the associated inflammation results in the clinical manifestations of the disease, which can be described as an overt ascending paralysis.

Since its isolation in the late 1960s, myelin basic protein (MBP) has been viewed as the predominant encephalitogenic protein in the CNS (20). Sensitization with preparations of purified MBP, free of any contaminating neural proteins, results in severe EAE. However, more extensive demyelination is often seen when animals are immunized with whole cord homogenates versus purified MBP, suggesting that immune responses to other neuroantigens may contribute to the disease. Investigators have reported that immunization with the myelin derived protein, lipophilin or proteolipid apoprotein (PLP), can also cause inflammation and damage to the CNS (21). Furthermore, PLP specific T cell lines derived in the SJL mouse, adoptively transfer severe clinical EAE when infused into syngeneic recipients. Histologic examination of recipient spinal cords found lesions that contained perivascular cell infiltrates, fibrin deposition and demyelination (22). And while there is convincing evidence that autoaggressive T lymphocytes mediate EAE (23), the development of antibodies to myelin lipids and glycoproteins may also contribute to the disease process (24,25).

The progression of clinical EAE varies with the animal model and the method of sensitization. Adult guinea pigs injected with purified

MBP emulsified in CFA (MBP/CFA) develop an acute and often fatal hindquarter paralysis within two weeks of sensitization (26). In contrast, juvenile guinea pigs (18-21 days of age) immunized with an emulsion of whole spinal cord in CFA develop either a chronic or relapsing form of the disease. Manifestations of chronic EAE are typically seen 12 to 68 weeks post injection (27) often appearing as a mono-phasic disease that may eventually result in death of the animal. Alternatively, animals may undergo relapsing EAE characterized by episodes of disease followed periods of convalescence (28).

In the rat and murine models, immunization with purified MBP results in an acute clinical paralysis that is short lived and usually followed by a complete recovery. In the Lewis rat system, the onset of active disease begins nine to twelve days following sensitization with the loss of motor control in the tail. The ascending hindquarter paralysis progresses to involve the hindquarters, and culminates in complete hindquarter immobilization with incontinence, about fourteen days post sensitization. A remarkably rapid recovery follows this near fatal paralysis, despite the persistence of inflammatory lesions within the CNS (19). Once recovered, convalescing Lewis rats are resistant to spontaneous disease relapses, and unresponsive to additional injections of MBP.

EAE has been demonstrated to be a T cell mediated autoimmune disease. The importance of an intact T cell population was first demonstrated when rats depleted of T cells were found to be unresponsive to injections MBP/CFA (23). These initial observations have been confirmed by using rats which lack a mature T cell population. Both nude rats and rats which have been adult

thymectomized, irradiated and reconstituted with normal lymphoid cells treated with anti-rat thymocyte serum, are resistant to actively induced EAE (29,30).

Additional evidence that EAE is produced by a T cell mediated response has come from adoptive transfer experiments. In the Lewis rat, T cells from MBP/CFA immunized donors can produce clinical EAE when infused into naive, syngeneic recipients. The paralysis seen in adoptive transfer recipients is similar to active EAE, although the disease occurs more rapidly. Clinical EAE is generally apparent four days following cell transfer. Maximum clinical severity is usually reached by day five or six, and most transfer recipients have recovered from adoptive disease by day eight. Successful adoptive transfer has been accomplished with lymph node cells (31,32), spleen cells (33,34), peripheral blood leukocytes (35) and "activated" MBP reactive T cell lines (36), demonstrating that EAE results from a T cell mediated response. In further support, neither serum nor B-cells are capable of transferring disease (37), although some investigators have suggested that serum factors contribute to the demyelination associated with the disease (38,39).

Adoptive Transfer of EAE.

Transfer of EAE from actively immunized donors into naive recipients, has been accomplished with lymphocytes from both the draining and thoracic duct lymph nodes. Functional lymph node cells (LNC), capable of inducing disease, can first be detected in the draining nodes seven days after immunization (40). The presence of transfer active effector cells in the lymph nodes is only temporary, so by the onset of clinical EAE on day 10, this cell population has lost the capacity to

adoptively transfer clinical disease (39,40). Presumably, the loss of transfer potential corresponds with MBP reactive cells moving out of the draining nodes and into circulation where they infiltrate the CNS.

While direct transfer studies can provide valuable information on the induction and pathogenesis of EAE, the system has a number of drawbacks. Donor lymph node cells can only be collected between days 7-9 following immunization, large numbers of cells must be transferred to the recipient (ranging from 1-5 x 10^8 cells) (31,32,39), and there is a high percentage of unsuccessful cell transfers (32). Researchers have reported that even with the proper collection times and adequate cell numbers, only 50% of the recipient animals may undergo adoptively transferred EAE (41). Similar experiments using the spleen as a lymphocyte source have proven to be even less successful. While reports of successful adoptive transfers with spleen cells exist, the large cell numbers and manipulations to the recipient population (42), has lead most researchers to believe that this cell population lacks fully developed MBP reactive cells capable of mediating clinical EAE.

Many of the problems that were encountered with direct cell transfers were overcome with the introduction of *in vitro* tissue culture techniques. Panitch and McFarlin observed that spleen cells from MBP immunized donors placed in culture with the T cell mitogen Concanavalin A (Con-A), gained the capacity to adoptively transfer clinical EAE (34). Following *in vitro* activation, severe clinical disease can be adoptively transferred into nearly 100% of the recipients with a significant reduction in the number of transferred cells. Although 2-5 x 10^7 cells are generally infused into to recipients, as few as 5 x 10^6 culture activated cells have been reported to cause clinical disease (43).

Furthermore, *in vitro* culture extends the time frame in which lymphoid cell populations from the actively immunized donors can be used. Animals 43 days post immunization have been successfully used as cell donors following the culture period (44). The stimulus provided during the *in vitro* culture period is not limited to Con A. Native MBP (45), some MBP fragments (46) and pokeweed mitogen can also drive the *in vitro* activation (47).

While *in vitro* culture clearly enhances the effector function of MBP reactive lymphocytes, the events important in the generation of a transfer active cell population remain obscure. During *in vitro* activation, MBP primed lymphocytes proliferate extensively in response to either specific antigen or T cell mitogens. Phenotypic changes in some lymphocytes accompany the cell proliferation, resulting in many large blastoid cells in the culture. Compared to non-stimulated control cells, culture activated cells express higher levels of CD4, IL-2 receptor, class I & II MHC antigens and the ligand which binds peanut agglutinin (PNA) (48,49).

The adoptive transfer phenomenon could be attributed to the expansion of the MBP reactive T cell population during the culture period. However, several investigators have reported discrepancies between a cell population's proliferative response to MBP and its encephalitogenicity potential (46,50,51). For example, Paterson et al. reported that draining lymph node cells from rats immunized with a guinea pig MBP (GP-MBP) fragment 68-84 (YGSLPQKSQRSQDEN), have a strong proliferative response to GP-MBP 68-84 but fail to respond to a similar peptide, modified bovine MBP (MB-MBP) 68-84 (YGSLPQKAQRPQDEN)(46). However, GP-MBP 68-84 primed lymph node cells, cultured with either GP-MBP or MB-MBP peptides were

capable of adoptively transferring clinical EAE. While these studies demonstrate cell activation in the absence of cell proliferation, Hinrichs et al. have reported the converse situation using the T cell mitogen, PHA (47). Spleen cells activated with PHA were found to proliferate extensively, yet the cell population remained non-encephalitogenic (51). Together, these studies demonstrate that enhanced *in vivo* function following *in vitro* activation cannot be attributed solely to a increase in the number of MBP reactive cells.

An alterative proposal is that *in vitro* activation overrides normal regulatory events, allowing full effector cell function in the transfer recipient. It has been hypothesized that suppressor mechanisms within naive hosts interfere with induction of EAE and prevent successful adoptive transfer when uncultured spleen cells are used (52). Following *in vitro* culture, lymphocytes may be at a level of activation where they are not easily down regulated, thus overriding natural suppressor mechanisms found in the recipient (52). In support of this, rats recovered from an active EAE and resistant to additional disease episodes induced with MBP/CFA, are still susceptible to adoptively transferred EAE. The susceptibility of recovered rats to passive disease (52), suggests that following *in vitro* culture MBP reactive cells differentiate to a point were they are no longer under the control of suppressor cells (53) or other host regulatory mechanisms, which allows them to effectively mediate clinical EAE.

Demonstration that EAE is the Result of a Delayed Type Immune Response.

The use of culture-enhanced adoptive transfer has proved to be a

valuable tool in the study of EAE. Through the use of an adoptive transfer system, researchers have been able to dissect the immune response to MBP and characterize the specific contributions of the T cell independent of other immune mechanisms. One important issue that can be addressed by adoptive transfer experiments pertains to the type of cellular immune response that mediates EAE. There are at least two possible mechanisms through which EAE effector cells could damage the CNS. The first possibility is that EAE effector cells function as cytotoxic T cells (T_c). Activated T_c cells would mediate damage to the CNS by lysing target cells such as oligodendrocytes, which express MBP on their surface (54). An alternative possibility is that EAE effector cells mediate damage to the CNS by eliciting a delayed type hypersensitive (DTH) reaction. Following recognition of antigen, the MBP reactive T cell population could secrete lymphokines which activate accessory cells such as macrophages. Inflammation associated with the activation of accessory cells could damage the CNS and result in EAE.

Initially, the nature of the EAE response was addressed in experiments where various cell populations were depleted prior to cell transfer into recipient animals. In the rat, the W3/25 monoclonal antibody reacts with helper T cells (T_h), which contain the T_{DTH} subset (55), while the monoclonal antibody designated OX-8 is specific for cytotoxic and suppressor T cell populations (56). Complement depletion of the W3/25 positive or T_h cells prevents the transfer of EAE, while removal of cytotoxic and suppressor T cells with OX-8 still permits successful adoptive transfer (57). Further support for a T_{DTH} cell acting as primary effector cell in EAE comes from a number of related studies. The T_h subset has been identified as the predominant lymphocyte

phenotype in CNS lesions of rats with EAE (58). And other researchers have reported a transient depletion of lymphocytes bearing T_h markers in the peripheral blood during the development of clinical paralysis in animals sensitized to MBP (59-61). Collectively, these findings indicate that EAE is mediated by a delayed type hypersensitive "like" reaction.

The Existence of MBP Specific Cell Lines.

An extension of culture activation is the generation of long term, MBP specific T cell lines (62). Cell lines are generally established by removing lymph node cells from actively immunized donors and activating the cells *in vitro* with MBP. Following activation, the cells require T cell factors to continue to proliferate and are transferred to an IL-2 rich growth media. Expansion of the cell lines with growth media results in a loss of effector cell function, as measured by transfer of clinical disease and skin test responses (36). Therefore, the T cell lines must be periodically reactivated by co-culture with syngeneic antigen presenting cells and MBP (62). By repeatedly stimulating then expanding the cell line it has been possible to maintain encephalitogenic, MBP reactive cells in continuous culture for several years.

The use of MBP specific lines have contributed significantly to our understanding of EAE. Cell lines represent an enriched population of MBP reactive lymphocytes, where as few as 10^4 to 10^5 cells mediate severe clinical EAE (36), and an inoculum of 3×10^6 cells is often lethal (63). Recently clones have been derived from cell lines, and grown in sufficient numbers to allow adoptive transfer studies. Some MBP reactive clones have been found to transfer EAE as effectively as cell lines, demonstrating that the capacity to elicit EAE is contained within a

single MBP reactive cell population (64). Analysis of cell surface markers has determined that MBP reactive cell lines and clones are almost exclusively $CD4^+$, placing them in the subcategory of helper/induced T cells (65). These findings provide additional evidence that EAE is the result of a delayed type immune response and not T_c cells attacking cells which express MBP on their surface (66,67,68).

Cell line mediated EAE has also provided valuable insights into immunoregulatory events that occur during recovery from the disease. Recovery from active disease leaves an animal unresponsive to subsequent challenge with MBP/CFA (69), yet recipients of cultured spleen cells develop accelerated EAE when immunized with MBP/CFA (70). Accelerated EAE following adoptive transfer with spleen cells is presumed to be the result of MBP reactive memory cells residing in the host following recovery from disease. What is unclear is why regulatory events triggered during active immunization are not invoked during passive disease. It could be proposed that culture activated cells are at a level of activation not conducive to the development of immune regulation. Alteratively, the short time course of passive EAE may be insufficient for down regulation of the encephalitogenic effector cell. However, these explanations are not satisfying in light of cell line experiments. Recipients of MBP reactive cell lines, are protected from a second disease episode induced by active immunization (63), and in some cases they are protected against adoptive EAE mediated by the same cell line (71).

Additional studies have found that attenuated MBP reactive cells which are unable to mediate adoptive EAE, also confer protection. Vaccination against active EAE has been successfully achieved with cell

lines compromised by irradiation (63,72), mitomycin C (63,72), exposure to extreme hydrostatic pressure (73) or glutaraldehyde fixation (74). The regulatory mechanism(s) responsible for protecting vaccinated animals from additional disease episodes remains unclear, although there is evidence supporting several different models.

Both D. Wekerle and I. Cohn, have proposed that activity of autoaggressive, MBP reactive lymphocytes (75,76), is regulated by an anti-idiotypic network of $CD4^-$ $CD8^+$ cytotoxic/suppressor cells. In Wekerle's report (75), line mediated EAE was induced in normal Lewis rats with an encephalitogenic dosage of S1 cells, a MBP-specific T cell line. Following recovery from clinical disease, splenic T cells from these rats were found to proliferate in response to irradiated S1 cells, but not to other syngeneic T cell lines specific for MBP or control antigens. Anti-S1 T cell lines were derived from spleens of recovered rats by repeated stimulations with irradiated S1 cells followed by IL-2 expansion. Characterization of anti-S1 cell lines found that they express the $CD8^+CD4^-$ cytotoxic/suppressor phenotype. The cells proliferate in response to irradiated S1 cells but not the autoantigen MBP, although proliferative response to S1 cells was not significantly inhibited by monoclonal antibodies directed to either class I or class II MHC antigens. In functional studies, anti-S1 cells were found to selectively lyse encephalitogenic S1 cells *in vitro* and neutralize their ability to mediate adoptive EAE *in vivo*.

While the S1 cell line elicits the generation of cytotoxic, $CD8^+CD4^-$ cells which protect the host from EAE induced by subsequent challenge with S1 cells, other encephalitogenic cells do not. Wekerle et al. found that injection of their S19 clone into Lewis rats failed to protect the

animals against a second challenge with S19 (71). However, the combination of S19 cells and MBP was found to effectively vaccinate the rats against additional S19 challenges. These results suggest that different mechanisms may be involved in the regulation of encephalitogenic T cells. In some instances both autoreactive T cells and their specific antigen may be needed to activate an effective anti-idiotypic network.

In the Lewis rat, the primary encephalitogenic activity of MBP is contained within amino acid resides 68-88 (77). The small size of the active region predicts a limited number of antigenic determinants, and consequently limited idiotypic diversity in encephalitogenic T cell clones. If the T cell receptor (TCR) gene usage in MBP reactive T cell clones is limited to a few or even one idiotype, then regulation of encephalitogenic cells might easily be accomplished through an anti-idiotypic response. Using a panel of Lewis rat derived, MBP reactive T cell clones, Burns et al. found that 7 of the 10 clones used Vα510 as the α chain variable region, while 10 of the 10 clones used Vβ510 as the β chain variable regions (78). The rat Vα510 and Vβ510 genes show close homology with the mouse Vα2 and Vβ8 families, which are found extensively in encephalitogenic murine T cell lines (79).

To understand the basis of anti-idiotypic regulation, Vandenbark et al. immunized Lewis rats with a hypervariable region of the TCR Vβ8 molecule (80). A 21-amino acid synthetic peptide which included the second complementary determining region, was predicted to be immunogenic for T cells and chosen for the study. Immunization of Lewis rats with the peptide resulted in complete protection against actively induced EAE. T cell lines, responsive to the TCR Vβ8 peptide

were then selected from the draining lymph nodes of the vaccinated rats using repeated stimulations with TCR Vβ8 peptide. Phenotypic characterization of the anti-idiotypic cell lines found high levels of CD4 and much lower levels of CD8. However, the cell's response to the Vβ8 peptide was found to be MHC class I restricted. In functional assays, Vβ8 responsive cells were found to alter the proliferative response of encephalitogenic T cells to MBP *in vitro*, and confer passive protection against actively induced EAE *in vivo*.

The mechanism in which anti-Vβ8 cells regulate encephalitogenic lymphocytes remains unclear. In contrast to the anti-idiotypic cells derived by Wekerle et al (75), anti-Vβ8 were not cytolytic for MBP reactive cells. To explain the activity of anti-Vβ8 cells, it has proposed that stimulated $CD4^+$ T cells internalize their TCR and MHC class II molecules via endosomes (80,81). Once in the endocytic pathway, the TCR, MHC and even antigen are degraded and some of the resulting fragments associate with MHC class I molecules. Fragments of the TCR, MHC or antigen in association with MHC class I molecules are then presented to $CD8^+$ cells by the $CD4^+$ cells. Activated $CD8^+$ suppressor cells then down regulate the activity of the $CD4^+$ cell. Immunization with anti-Vβ8 may produce similar regulatory events. The immunizing peptide may associate with MBP reactive $CD4^+$ cells and activate a suppressor cell population that regulates the activity of Vβ8 expressing T cell population which includes most encephalitogenic T cells.

In contrast to highly specific regulation by anti-idiotype networks, Cohen has proposed that activation antigens found on all activated CD4+ cells may be targets for immunoregulation during EAE (82). The basis for this hypotheses is that only activated MBP reactive T cells effectively

vaccinate against EAE. If EAE was regulated exclusively by anti-idiotypic responses directed against the TCR then vaccination with either activated or non-activated cell populations should be protective. Yet vaccination with 1×10^4 activated cells provides a greater level of protection that 5×10^7 non-activated cells. Furthermore, a mild degree of non-specific protection is seen following vaccination. Rats immunized with the A2b clone which was specific for a nine amino acid sequence of the 65 Kd heat shock protein of *Mycobacterium tuberculosis* showed some resistance to EAE when challenge with a MBP reactive T cell clone. This response, apparently directed toward a T cell activation antigen, has been termed an anti-ergotypic response.

Both anti-idiotypic and anti-ergotypic responses provide plausible mechanisms to explain the regulation of MBP responsive cells. However, to date there has not been a convincing demonstration that either anti-idiotypic and anti-ergotypic responses develop in animals as they recover from clinical EAE.

Basic Protein and the Myelin Sheath.

Myelin forms an insulating sheath on some nerve axons in the central and peripheral nervous systems (PNS). Myelin is formed by cell processes from oligodendrocytes in the CNS and Schwann cells in the PNS which wrap around adjacent axons. The cytoplasm is then extruded, leaving tightly packed, concentric layers of the oligodendrocyte membrane around the axon. Although not clearly demonstrated, MBP is thought to be important in maintaining the multi-laminar structure of myelin.

In mice and rats, MBP occurs as a series of small, highly basic polypeptides that collectively account for 30% of the protein in the myelin sheath (83). In adult mice, four forms of MBP with molecular weights of 21.5 Kd, 18.5 Kd, 17 Kd and 14 Kd occur in the myelin at a relative frequency of 1:10:3.5:35 (84). Amino acid sequencing has determined that the four MBP molecules are all closely related. For example, 14 Kd MBP is a truncated form of the larger 18.5 Kd molecule, the result of a 44 amino acid deletion beginning immediately following the tryptophan at position 113 and extending to within 15 residues from the C terminus (85). Furthermore, a deletion spanning 30 amino acids in the N-terminus of the molecule distinguishes the 21.5 Kd and 17 Kd MBPs from the more prevalent 18.5 Kd and 14 Kd forms of the protein (86). While deletions occurring through post-translational processing could be responsible for generating the different MBP forms, experimental results do not support this proposal (87). Therefore, current opinion is that all four peptides are encoded by a single gene, and alternative splicing of the mRNA gives rise to the different forms of the protein (88).

The reason for MBP heterogeneity in mice and rats remains unclear. The ratio of 18.5 Kd to 14 Kd MBP can vary depending on whether the protein is extracted from whole brains or purified myelin. The differences in MBP ratios seen in various myelin preparations suggests that different cells in the CNS may express different levels of 18.5 Kd and 14 Kd MBP, although the implications of this remain unclear. Alterations in the normal 18.5 Kd to 14 Kd MBP ratio are also seen in developing brains. In contrast to adults, 18.5 Kd MBP is more prevalent than 14 Kd MBP in neonatal rats. However, despite conjecture as to how different forms of MBP may be important in development and

maintenance of myelin structure, not all species express multiple forms. Isolation of MBP from the CNS of guinea pig, rabbit, ox, and monkey has detected only a single form of MBP with a size and charge equivalent to the 18.5 Kd MBP found in rats and mice (92). This suggests that only the 18.5 Kd form of the MBP molecule is required to maintain the multi-laminar structure of myelin..

Localization of MBP is thought to occur along the major dense line, which represents the opposing cytoplasmic faces of the myelin membrane when viewed by electron microscopy. One model for the molecular organization of myelin proposes that MBP interacts directly with the surface of the adjacent membrane (93). The interspersion of basic and hydrophobic amino acid resides would allow MBP to interact with both the acidic head groups and hydrophobic regions of membrane phospholipids. Alteratively, MBP could bridge the major dense line by forming a dimer with a MBP molecule on the opposing cytoplasmic face. In support of this model, exposure of myelin to difluorodinitrobenzene a covalent cross linking agent, produces MBP dimers devoid of other polymeric forms (94,95). Biochemical analysis of the MBP dimers has determined that cross linking occurs between two lysine resides in the N-terminal and C-terminal regions of the adjacent MBP molecules. These findings suggest that associating MBP molecules are oriented in a tail-to-head arrangement as they span the cytoplasm and help maintain the structure of the myelin sheath. A second protein, PLP is also believed to play a role in maintaining the structural features of myelin. PLP is thought to be located on the extracellular face of the myelin membrane where it helps maintain the myelin structure by holding the cytoplasmic faces of myelin in close proximity (96).

Integrity of the myelin sheath is required for normal nerve conduction by many axons. The myelin sheath does not completely envelop nerve axons, but is interrupted by gaps known as nodes of Ranvier. By acting as insulating wrapping on the axon, myelin facilitates saltatory conduction by forcing electrical currents to jump between the nodes of Ranvier (97). Compared to continuous conduction used by non-myelinated axons, saltatory conduction produces a significant increase in the rate of signal transmission (97). Therefore, damage to the myelin sheath impairs nerve conduction and can ultimately lead to the loss of coordination, paralysis and even death.

Myelin Damage During Experimental Allergic Encephalomyelitis.

While EAE is clearly mediated by autoreactive T cells, the means by which these cells cause clinical paralysis is not readily apparent. One proposal is that clinical EAE is the result of classic delayed type immune response taking place within the CNS. During the induction of EAE, $CD4^+$ T cells which are responsive to neuroantigens infiltrate the CNS, recognize antigen and respond with the production of various lymphokines. Lymphokines recruit circulating mononuclear cells into the region, resulting in conspicuous lesions along many of the post capillary venules in the CNS. Additional signals activate infiltrating mononuclear cells to mediate non-specific damage to the myelin sheath through the release of hydrolytic enzymes (68,98) and the production of free radicals (98). This results in damage to the myelin sheath which can be so extensive that in some models the myelin sheath is completely stripped off axons adjacent to cellular infiltrates (98,100).

Although encephalitogenic T cells can easily mediate a delayed type response, as demonstrated by skin test reactivity (36), there is compelling evidence indicating that neurologic impairment does not result from simply the recruitment and subsequent activation of mononuclear cells. In animals with EAE, a close temporal association between mononuclear cellular infiltrates in the CNS and clinical disease is not always apparent. At the onset of clinically apparent EAE in the Lewis rat, which occurs approximately ten days following sensitization with MBP, lesions are readily identified in the CNS (101). However, extensive perivascular infiltration can still be detected 58 days post immunization, nearly six weeks after most rats have recovered from clinical disease (102). Discordance between clinical disease and cellular infiltration of CNS are also seen when Lewis rats are immunized with moderately encephalitogenic bovine MBP. It has been reported that less than fifty percent of rats develop clinical disease when immunized with bovine MBP, while cellular infiltrates can be detected in the CNS of all the sensitized animals (19).

Sedgwick et al. may have provided the most convincing evidence that EAE is the result of inflammatory events not associated with the mononuclear cells infiltrates in the CNS (103). It is generally believed that mononuclear cells recruited into cell mediated immune responses, including EAE, are cells that have recently been released from the bone marrow. By exposing would-be recipients to high levels of ionizing radiation, rapidly dividing bone marrow stem cells are irreversibly damaged, blocking normal hematopoiesis. Classic delayed-type immune reactions cannot be adoptively transferred into these irradiated recipients because of the absence of new mononuclear cells in the recipients (104).

However, transfer of MBP reactive cells into irradiated recipients still results in clinical paralysis. Histologic sections of the recipient spinal cords reveals the absence of extensive mononuclear cell infiltrates, although $CD4^+$ IL-2-R^+ cells are present. Inconsistences between clinical disease and cellular infiltration suggest that inflammatory events, not associated with mononuclear cell infiltration, are important in the induction of clinical EAE.

The correlation between damage to the myelin sheath and clinical paralysis also remains obscure. Severity of demyelination can vary in different animal models and with the type of neuroantigen preparation used for sensitization. Immunization with crude spinal cord homogenates produces extensive demyelination in most animal species including guinea pigs, rabbits, and Lewis rats (105,106). In contrast, demyelination is not always seen when purified MBP is used to induce EAE. While guinea pigs immunized with purified MBP develop plaques with obvious demyelination, rats and rabbits do not (106,107). The lack of demyelination in Lewis rats has led some investigators to suggest that neurological impairment in acute EAE is not the result of damage to the myelin sheath. Furthermore, recovery from acute EAE is thought to occurs too rapidly to be attributed to remyelination of damaged axons (108). Therefore, it has been suggested that edema and fibrin deposition may account for the neurologic impairment which is seen in Lewis rats with EAE (18).

At the onset of EAE, a generalized breakdown in the blood-brain-barrier (BBB) occurs. Increased pressure from fluid accumulation in the physically limited extracellular space of the CNS can perturb normal neurophysiological function (109). Edema also produces electrolyte

imbalances which affect normal nerve conduction and may contribute to the clinical manifestations of EAE (110). Increased vascular permeability of vessels within the CNS may also allow fibrinogen and other circulating clotting factors to easily diffuse into the CNS. Activation of the clotting cascade converts soluble fibrinogen into insoluble fibrin which accumulates within and around the microvasculature of the CNS (111).

In both actively induced and passively transferred EAE, there is a statistically significant correlation between fibrin deposition in the CNS and signs of neurological impairment in individual rats (19). The role of fibrin in the pathogenesis of EAE has been further examined with pharmacologic agents that have specific antifibrinolytic activities. Inhibitors of plasminogen activator and other neutral proteinases such as trans-4-(aminomethy)cyclohexanecarboxylic acid (AMCA), ε-amino-caproic acid (EACA) and p-nitrophenylguanidinobenzoate (NPGB), protect actively immunized rats against the expression of clinical EAE (112). However, spinal cord sections from rats treated with either AMAC or EACA, still had extensive inflammatory lesions in the CNS, again demonstrating a dissociation between clinical and histological EAE.

How fibrin deposition in the CNS contributes to clinical paralysis remains unclear. Neutral proteinases, including plasminogen activator, are required to initiate the clotting cascade and their presence in the CNS could mediate damage to the myelin sheath (113). Alternatively, fibrin deposition could increase edema in the CNS and mediate clinical paralysis indirectly. Deposition of fibrin activates a latent fibrinolytic activity in nearby endothelial cells (114). Degradation of the deposited fibrin releases small biologically active peptides which increase vascular permeability and produce more edema. This series of events would allow

additional fibrin deposition and increase inflammation and edema in a self perpetuating manner.

While overt demyelination is not seen in the Lewis rats immunized with purified MBP, damage to the myelin sheath does occur. Brosnan et. al. have described a marked dilation of the myelin sheath resulting from expansion of the periaxonal space, which occurs in the spinal cords of rats with EAE (115). Other researchers have observed a disruption of the multi-laminar structure of the myelin sheath, which may be attributable to a loss of MBP from oligodendrocyte membranes during EAE (116). It has also been reported that some demyelination occurs in the Lewis rat, and that most investigators fail to detect it because of inadequate histological techniques and incomplete examination of the CNS (106). In his report, Pender demonstrated that demyelination occurs in the lumbar, sacral and coccygeal dorsal and ventral spinal roots and to a lesser extent in the spinal cord, including the dorsal and ventral spinal root exit zones (117). Furthermore, electrophysiologic studies on Lewis rats with EAE found reduced conduction velocities between the lumbar ventral roots and the sciatic nerve, and a conduction block in the ventral root exit zone of the lumbar spinal cord (117). Therefore, this modest demyelination is sufficient to impair nerve conduction, and may account for clinical paralysis in rats with EAE.

Recognition of MBP by Responsive T Cells.

Initiation of delayed type responses requires T cell recognition of specific antigen in association with MHC class II antigens on the surface of an antigen presenting cell. During the induction of delayed type responses, the $CD4^+$ molecule on the surface of T_h cells has a vital

function in lymphocyte activation (118). The current feeling is that CD4 molecules interact with a conserved region of the MHC to stabilize the interaction between the TCR on the T cell and the MHC complex on the antigen presenting cell (119). To demonstrate that the induction of EAE is mediated through encephalitogenic T_h cells, investigators have attempted to alter the expression of clinical EAE with antibodies directed towards the $CD4^+$ antigen on the surface of T_h cells (120,121). These antibodies interfere with the interactions that take place between T_h cells and antigen presenting cells, and should alter the course of EAE if the disease is mediated by T_h cells.

When the monoclonal antibody W3/25, specific for rat CD4, was given to Lewis rats, the course of clinical disease was significantly altered. Rats given antibody treatments on either day 12 or 13, recovered from disease in an average of two days, while untreated controls required four days to fully recover (120). Similar findings have been reported in murine EAE using the anti-L_3T_4, the mouse equivalent of CD4. Immunized mice, treatment with anti-L_3T_4 prior to clinical disease did not develop EAE, while administration of the antibody to mice with clinically apparent EAE, resulted in a rapid recovery from the disease (121). It has been theorized that anti-L_3T_4 treatments prevent EAE by blocking MBP recognition by reactive cells during the initial sensitization in active disease, and by interfering with the recognition of MBP in the CNS during the course of adoptively transferred EAE.

T cell recognition of MBP and subsequent clinical disease also requires MBP to be presented in the context of MHC class II antigens. Evidence for this recognition in EAE has been established by the use of monoclonal antibodies directed toward class II antigens. In a chronic

relapsing model, Steiman et al. found that treating SJL/J (H-2^s) mice with anti-I-A^s antibodies at the time of immunization, prevented paralysis in many of the mice (122). Clinical disease was evident in only 3 of 28 mice that received anti-I-A^s, compared to 19 of 28 mice that received noncross-reactive anti-I-A^k antibody. When anti-I-A^s antibody was administered to mice undergoing acute clinical disease, dramatic improvements were seen during the following 24-48 hours with all animals recovering by 72 hours (121). In the Lewis rat model, monoclonal antibodies to both I-A and I-E, designated OX-6 and OX-17, respectively, have been tested for their effects on clinical disease (123). Activation of MBP reactive T cell lines in the presence of OX-6 completely blocked adoptive transfer of clinical EAE, while OX-17 had little effect. *In vivo* administration of OX-6 was also found to reduce or completely abrogate clinical disease in recipients of activated cells, while OX-17 again had little effect. These findings demonstrate the requirement for MBP presentation in association with I-A for clinical paralysis, in both adoptively transferred and actively induced EAE.

Recognition of MBP in the context of MHC class II antigens implies that the antigen is taken up by appropriate accessory cell, processed, and presented on the cell surface where it can be seen by responsive lymphocytes. However, MBP is a integral membrane protein found on opposing cytoplasmic faces of the myelin sheath. It is hard to explain why phagocytic accessory cells would attack healthy myelin and present neuroantigens to encephalitogenic T cells. One proposal is that MBP is constantly shed from the myelin sheath at concentrations sufficient to allow it to be processed and presented to responsive lymphocytes during EAE. If MBP fragments are present in normal healthy individuals, what

prevents spontaneous induction of EAE, as T cells become sensitized to MBP? Further evidence contradicting the constant availability of MBP has come from transfer studies placing MBP reactive cells directly into the CNS (124). If MBP is normally shed and processed by antigen presenting cells, then activated MBP reactive T cells injected intrathecally should mediate clinical EAE. However, injecting MBP reactive cells directly into CNS does not result in clinical EAE. Perhaps even more surprising is that MBP reactive T cells introduced intrathecally do not remain in the CNS but migrate back into circulation, suggesting that the T cells had failed to recognize their target antigen.

An alternative possibility is that damage mediated to the myelin makes MBP available to responsive T cells. Currently, there is experimental evidence that during EAE there is disruption of the myelin sheath, and the loss of MBP from the membrane. Under the electron microscope, light myelin subfractions obtained from normal Lewis rats by sucrose gradients, appeared as large, often multilayered whorles of membranes (116). In contrast, the light myelin subfraction from rats with EAE lacked the classic multi-laminar structure and appeared as small, single or double walled vesicles. SDS-PAGE was then used to compare the protein profiles of the control and EAE myelin subfractions. Quantitative analysis of myelin proteins by densitometer scans revealed a loss of MBP in rats affected with EAE, while the quantity of a second myelin protein, PLP was normal (116). This injury to the myelin sheath could result in the release of encephalitogenic MBP fragments into the surrounding region.

Digestion of myelin proteins by proteolytic enzymes is a mechanism that would explain the disruption of the myelin sheath and

make MBP accessible for recognition by responsive lymphocytes. Biochemical assays have determined that the levels of lysosomal enzymes including acid proteinase and B-glucuronidase increase during EAE and seem to correlate with the intensity of clinical disease (125). The source of these enzymes remains unclear. If these lysosomal enzymes contribute to the disease pathology, their source cannot be mononuclear cells, since clinical EAE can occur in the absence of mononuclear cell infiltration (103).

Proteases from other sources could also damage myelin during EAE. Lymphocyte derived proteolytic enzymes capable of degrading myelin, have been described (126). Activation of the clotting cascade and deposition of fibrin also results in the activation of proteases (113), which could mediate damage to the CNS and liberate MBP fragments from the myelin sheath. Another important source of myelinolytic proteases may be mast cells (MC) located in the CNS. Weiner et al. reported that the proteases released by stimulated MC had high myelinolytic activity (127). Incubation of freshly prepared myelinated axons from the CNS with supernatants from degranulated MC resulted in significant degradation of certain myelin proteins including PLP and MBP. Local degranulation of MC in the CNS (128), could contribute to demyelinating lesions seen in EAE. Furthermore, degradation of MBP by MC proteases could result in the release of encephalitogenic fragments which could stimulate MBP reactive T cells as they infiltrate the CNS.

Identification of the Encephalitogenic Region of MBP.

Purified MBP fragments obtained from proteolytic digestions have allowed investigators to identify encephalitogenic portions of the MBP

molecule. Encephalitogenic regions of MBP have been found to vary considerably with both the animal model and the species derivation of the MBP. In the Lewis rat, the region of GP-MBP located between amino acid resides 68 and 89 was thought to contain the entire encephalitogenic portion of the protein (132), although a second encephalitogenic region has recently been identified (133). Guinea pigs do not respond to the 68-89 fragment of GP-MBP, but react with the portion of the molecule centered around a tryptophan reside at position 115 (129). The PL/J mouse strain responds to a sequence within residues 1-37 (130), while SJL/J mice respond to two regions of the MBP molecule, contained within the 1-37 and 89-169 peptides (130,131).

In the Lewis rat model of EAE, GP-MBP is normally used to induce active EAE and drive antigen specific responses *in vitro*. However, rat MBP (Rt-MBP) has successfully been used to induce the active disease in Lewis rats although it is ten times less encephalitogenic than GP-MBP (134). The reduced encephalitogenicity of the Rt-MBP, can be traced to a single amino acid substitution at position 79, where serine in GP-MBP has been replaced by threonine in Rt-MBP (134,135). It has been hypothesized that the reduced encephalitogenicity of Rt-MBP may result from a lower T cell affinity for MBP when threonine occupies position 79. As a result of its higher affinity, GP-MBP stimulates a larger T cell population at suboptimal concentrations of MBP, although work done with rat T cell lines fails to support this proposal (36,136). Lymphocyte proliferation experiments measuring [^{3}H]-thymidine uptake have determined that regardless of whether GP-MBP or Rt-MBP was used during antigen presentation, the proliferative response were the same (137). Reduced affinity may, however, account for the reduced response

of these same cell lines to human MBP (H-MBP). In addition to the threonine substitution of serine, H-MBP differs from the GP-MBP by a glycine-histidine insertion replacing glutamine at position 75. Bovine MBP (B-MBP), which shows even greater sequence differences in the 68-88 fragment, completely fails to activate these Lewis rat derived cell lines (137). This finding is consistent with observations that the 68-88 fragment of B-MBP is not encephalitogenic in the Lewis rat.

Factors Controlling Susceptibility to EAE.

The induction of a cellular immune response to a given antigen, obviously requires a population of T cells with receptors capable of recognizing antigen. However, the capacity to respond to a given antigen is also controlled by immune response genes, which reside within the MHC. In order to trigger responsive T cells, antigen must be able to associate with molecules of the MHC complex, allowing it to be expressed on the surface of antigen presenting cells.

While susceptibility to EAE is in part under the control of the MHC complex, other factors are also involved. Analysis of inbred mice strains has determined that haplotypes, H-2^s and H-2^q conferred susceptibility to EAE (138-140). However, not all H-2 congenic mice bearing either the H-2^s or H-2^q haplotypes are susceptible to EAE, indicating background or non-MHC encoded products also influence disease susceptibility. It has been determined that in addition to the appropriate H-2 haplotype, the gene(s) for vasoactive amine sensitization (VAAS) are equally important in determining susceptibility to EAE. Mouse strains with the H-2^s or H-2^q haplotype that undergo VAAS following the administration

of *Bordetella pertussis* were found to be susceptible to EAE, while strains not sensitive to VAAS were resistant to EAE (141).

The correlation between VAAS sensitivity with *B. pertussis* and susceptibly to EAE is of considerable interest. Early investigators found that EAE was difficult to induce in murine models, until it was found that treatment with *B. pertussis* accentuated disease in susceptible strains (142). *B. pertussis* has a number of *in vivo* biological activities which include facilitating the induction of DTH, and enhancing and prolonging the inflammation at the site of antigen challenge (143-146).

In murine EAE, sensitization with *B. pertussis* has been found to increase permeability of the blood-brain-barrier (147), presumably by increasing the sensitivity of the microvasculature to the effects of vasoactive amines (VAA) like serotonin and histamine (144,145). By increasing sensitivity to VAA, alteration in vascular permeability which allow the migration of MBP sensitive T cells into the CNS occur more readily. Sensitivity to vasoactive amines may also be important in the induction of EAE in the rat. While EAE can be induced in susceptible Lewis rats with a single injection of MBP in CFA, Brown Norway rats fail to develop disease when treated in an identical manner. Resistance to clinical EAE can be overridden by treating BN rats with *B. pertussis* following sensitization with BP/CFA (146).

Studies with congenic rat strains have also demonstrated that a non-MHC encoded gene is partially responsible for resistance to EAE in BN rats (148). BN.B1 (Lew MHC on BN background) rats are not susceptible to actively induced EAE, although MBP specific T cells from Lewis donors transfer severe clinical EAE into these rats. When BN.B1 rats are immunized with MBP, lymphocytes from these rats responded to

native MBP and 68-88 fragment. However, MBP reactive cells from BN.B1 rats transferred only modest clinical disease when infused into either Lewis or BN.B1 rats. While issue of VAA sensitivity is not addressed, it is conceivable that non-MHC encoded gene similar to those encoding VAA sensitivity in mice are responsible for the resistance to EAE seen in BN rats. This is supported by the findings that *B. pertussis* breaks resistance in BN rats.

The Role of Endothelial Cells in the Induction of EAE.

In both actively induced and passively transferred EAE, MBP reactive lymphocytes must traffic from the circulation into the CNS to produce EAE. In the active form of the disease, lymphocytes develop in the draining lymph node and then enter the circulation, while in adoptively transferred EAE the cells are placed directly into circulation by i.v. injection. Nevertheless, in both instances MBP reactive lymphocytes are on the lumen side of the vascular endothelium while MBP is found on the adjacent side. What then is the mechanism which permits MBP responsive cells to recognize and respond to MBP within the CNS?

Some investigators have proposed that the vascular endothelium may be an active component of the immune response. *In vitro* experiments with purified endothelial cells have demonstrated that under some conditions these cells can express MHC class II molecules and present antigen to responsive lymphocytes (149). These observations have lead researchers to propose that vascular endothelial cells in the CNS may play a relevant role in the induction of EAE (150,151). *In vivo*, vascular endothelial cells could pinocytose MBP shed from the CNS, then

process and present it on their lumen side in association with MHC class II molecules. On encountering the Ia positive endothelial cells presenting MBP, circulating encephalitogenic T cells would be activated and subsequently release mediators which increase vascular permeability and facilitate their extravasation into the CNS (152). Additional support for endothelial cells acting as integral accessory cells during the induction of EAE, has come from histologic studies. Ia positive cerebral vasculature endothelial cells were found in mice exhibiting clinical EAE but not in normal syngeneic mice (149), while capillary endothelial cells in immunized guinea pigs turn Ia positive just prior to the onset of clinical disease (150).

A study using adoptive transfer into semi-syngeneic chimeric rats, argues against endothelial cell antigen presentation during the induction of EAE (153). Chimeric animals were constructed by reconstituting lethally irradiated Brown Norway (BN) rats with a bone marrow transplanted from semi-syngeneic (BN X LEW)F_1 donors. In these recipients, the F_1 graft replaces the original BN bone marrow damaged by irradiation. As the new bone marrow takes over hematopoiesis in the (BN X LEW)F_1->BN chimeras, newly formed cells including antigen presenting cells such as macrophages and dendritic cells are F_1 in origin. In contrast, non-bone marrow derived tissues including the myelin sheath and the associated vascular endothelium remain BN in origin. Once adequate time elapsed for the turnover of accessory cells, these (BN X LEW)F_1->BN chimeras were used as recipients of culture activated Lewis spleen cells. Lymphocytes derived from Lewis donors immunized with MBP have learned to recognized MBP in association with Lewis MHC complex, and should not recognize MBP presented by BN cells, including

the endothelium. However, the transferred Lewis cells should be able to recognize MBP on bone marrow derived antigen presenting cells in the chimeras, since they express MHC molecules from both parental strains. The transfer of culture activated Lewis cells into chimeric recipients resulted in expression of clinical EAE, with comparable severity as Lewis into Lewis cell transfers. The development of EAE in the (BN X LEW)F_1->BN chimeras following transfer of Lewis cells implies lymphocyte trafficking in an allogeneic environment. Antigen recognition on the vascular endothelium could not facilitate lymphocyte egress into the CNS since the transferred lymphocytes were allogenic to the endothelium in the (BN X LEW)F_1->BN chimeras. Activation of MBP reactive lymphocytes in the (BN X LEW)F_1->BN chimeras must have taken place on semi-syngeneic bone marrow derived antigen presenting cells, perhaps following their arrival in the CNS.

Results from experiments with monoclonal antibodies directed against the TCR of MBP reactive T cells also question the hypothesis that antigen presentation by the vascular endothelium facilitates cellular infiltration of the CNS. The limited idiotypic repertoire responsive to MBP has allowed investigators to prepare monoclonal antibodies that react with most MBP reactive T cells, but not lymphocytes with other specificities (154). When rats were treated with anti-idiotypic monoclonals, clinical EAE was prevented, although histologic examination of the CNS revealed extensive mononuclear cell infiltrates. Inhibition of clinical disease by anti-idiotypic antibodies is consistent with the need for encephalitogenic cells to recognize MBP in order to gain full effector function. However, the presence of mononuclear infiltrates in the CNS demonstrates that this antigen recognition by MBP reactive

cells follows lymphocyte extravasation into the CNS. If antigen presentation by capillary endothelial cells occurs and this facilitates lymphocyte trafficking into the CNS, then anti-idiotypic antibodies should block histologic EAE. Therefore, antigen presentation to MBP reactive T cells by capillary endothelial cells is not consistent with the findings of these studies. There must be a alternative mechanism which allows activated lymphocytes to cross the BBB and is not linked to antigen recognition.

Evidence for Vascular Permeability Changes in EAE.

At the onset of clinical EAE, there is a generalized increase in vascular permeability which allows soluble serum proteins to move across the endothelial layer and into the CNS. When [^{125}I] labeled BSA is injected i.v. into normal rats, very little of the label finds its way into the CNS. However, in rats with clinical EAE, relatively high concentrations of [^{125}I] labeled BSA can be found in the CNS, demonstrating that the integrity of the microvasculature is severely compromised during the disease (155). The increase in vascular permeability seems to occur regionally and coincide with the ascending paralysis. Early in EAE, the highest concentrations of label were found in the lumbar section of the cord, while label became more prevalent in the anterior regions of the cord as the disease progressed.

This generalized increase in vascular permeability may then facilitate the cellular infiltration into the CNS. Studies have demonstrated that activated lymphocytes, infiltrate the CNS at the onset of EAE regardless of their antigen specificity. This was demonstrated by co-immunizing Lewis rats with tetanus toxoid (TT) at the same time they

were injected with MBP (156). At the onset of clinical disease, 12 days following sensitization, spinal cords were removed from the immunized rats and the infiltrating lymphocytes were isolated. The frequency of lymphocytes responsive to the sensitizing antigens was determined by limiting dilution analysis and found to be 3.36 cells per 10^4 for MBP, compared to 7.60 per 10^4 for TT. These findings demonstrate that during clinical EAE, activated lymphocytes can leave the vasculature and infiltrate the CNS regardless of their antigen specificity.

Vascular Permeability and Vasoactive Amines.

While VAA, including serotonin and histamine have different effects in different animal species, it is generally agreed that these preformed mediators can produce dramatic changes in vascular permeability. Histamine is a small, dibasic molecule, usually produced in MC by the decarboxylation of histidine by histidine decarboxylase, while serotonin is formed by hydroxylation and decarboxylation of tryptophan (157).

In man and most other animals, the predominant effects of histamine release include the dilation of small blood vessels, and the disruption of endothelial cell junctions (158). Vasodilatation increases blood flow by reducing peripheral resistance and serves to flush the capillary beds, while constriction and separation of endothelial cells increase vascular permeability (159). Gaps formed between adjacent endothelial cells allow an outward flow of plasma proteins and fluid into the extracellular spaces, producing edema. Histamine exerts its effect through two distinct receptors, referred to as H_1 and H_2 receptors, which are expressed at varying levels in tissues sensitive to this VAA (160). The

H_1 receptor, which exhibits the higher affinity of the two receptors, mediates a rapid but short lived vasodilatation when it binds histamine. In contrast, activation of the H_2 receptor results in a slow, but sustained vasodilatation (159). Increases in capillary permeability are thought to be mediated primarily though activation of the H_1 receptor, although the effects of the H_2 receptor activation have not been fully elucidated.

Histamine is found primarily in MC and basophils, where it is stored in secretory vesicles. Activation of these cells results in the release of histamine which produces dramatic changes in vascular permeability. Histamine concentrations as high as 10^{-3} M are achieved during degranulation (161), although rapid degradation of extracellular histamine keeps the inflammation localized (162). Within seconds of its release, histamine is ether inactivated or degraded by enzymes in the serum and various tissues. Histamine can be inactivated by N-3 methylation, which is followed by oxidative deamination producing 3-methylimidazole-5-acetic acid (159). Alternatively, histamine is degraded by diamine oxidase or histaminase, to form imidizole-5-acetic acid (159).

Serotonin may also be released by activated MC. However, unlike histamine, quantities of serotonin found in MC granules vary considerably in different species. Both rats and mice contain large amounts of serotonin while this mediator is absent in MC isolated from human lung (163,164,165) In species where serotonin is present, its release causes dramatic changes in vascular permeability, perhaps exceeding those mediated by histamine. Majno and Palade, found that mouse endothelial cells were 100 times more sensitive to serotonin than they were to histamine (166). Other investigators have implicated serotonin as the principle VAA which mediates the changes in vascular

permeability associated with cutaneous delayed-type hypersensitivity (167).

The Release of Vasoactive Amines by Mast cells and Basophils.

Circulating basophils along with their tissue counterpart the MC, act as important storage reservoirs for potent mediators of inflammation. The stores, which represent the major source of vasoactive amines in most tissue (159), are released by activated MC and basophils to increase vascular permeability. While these cell types differ in distribution, morphology and origin they do possess a number of similarities. Both cell types contain large numbers of electron dense, metachromatic granules, which stain with basic dyes such as toluidine blue and alcian blue (168). The basis for the unique staining properties of the granules result from the large amounts of acidic proteoglycans found within the storage granule (169). These proteoglycans are thought to act as an ion exchange resin which concentrates basic molecules such as histamine and cationic proteases in the storage granules (170).

Both MC and basophils originate from multipotential hematopoietic stem cells (171). Basophils, like neutrophils and eosinophils, complete their differentiation in the bone marrow and enter circulation where they normally reside (172). Like other granulocytic cells, mature basophils range in size from 10-15 microns, have a polylobed nucleus with condensed chromatin, and contain many small cytoplasmic vesicles and granules (173,174). These cells also have large granules (1.0-1.2 μm) that stain with metachromatic dyes and are rich in vasoactive amines (174). During many cell mediated immune responses

basophils are recruited from circulation and localize in tissues where they function and eventually die (171).

In contrast to basophils, MC leave the marrow as undifferentiated precursors that migrate through the blood and invade different tissues where they differentiate in response to microenvironmental stimuli (171,175). Once morphologically differentiated, MC retain the capacity to divide and can proliferate extensively during some immune responses (176). Mature MC are long lived, sessile cells, which are distributed primarily near blood vessels, lymphatics and connective tissue (177). These cells seem particularly abundant in tissues which are exposed to the external environment, including the skin, lungs, and gastrointestinal track, where they can rapidly respond to environmental antigens (178). The release of MC mediators is thought to be responsible for wheal and erythema responses of the skin, and allergic asthma in the lung (179).

Mature MC have a number of common features. The cells are 14-20 μm in size, with an eccentric nucleus (173). The surface has narrow, regularly placed processes or philopodia, while the cytoplasm contains numerous cytoplasmic granules which are smaller yet more abundant than those found in basophils (173). Mast cells do not, however, constitute a single homogeneous population. Cells isolated from different tissues can differ morphologically, biochemically, and functionally (180). One of the most striking differences is seen when rat connective tissue MC (CTMC) are compared with MC from the gastrointestinal mucosa, which have been termed mucosal MC (MMC). Compared to CTMC, MMC tend to be smaller, more variable in shape, and usually contain fewer granules which are more variable in size and shape (180). Significant differences are also seen in the staining

properties of the two MC types. In fixed tissues stained sequentially with alcian blue and safranin, MMC granules stain blue, while CTMC granules stain red (180). Furthermore, CTMC granules become fluorescent when stained with berberine, while MMC do not. These staining differences appear to be the result of different granule glycosaminoglycans, where CTMC granules contain primarily heparin sulfate, while MMC granules have oversulfated chondroitin sulfate (180). Other biochemical differences between MC subpopulations include distinct types of granule proteases, and histamine/serotonin levels which are considerably higher in CTMC than their MMC counterparts (180).

Numerous functional differences between MC subpopulations have also been reported. CTMC are thought to have a long life span, perhaps exceeding 6 months, while MMC generally live less than 40 days (180). MMC proliferation during the course of immune responses is thymus dependent, requiring IL-3 and IL-4, while it is unclear what cytokines may stimulate CTMC division (181,182). Finally, while both MMC and CTMC are stimulated by IgE receptor cross-linking, MMC do not degranulate in response to stimulation with polyamines such as compound 48/80 and polymyxin B, which are potent activators of CTMC (183,184).

Mast cells and basophils both posses large numbers of receptors which allow the cells to bind soluble IgE. With a disassociation constant approaching 1×10^{10}, these receptors bind IgE with such avidity that nearly all of the IgE found in an animal is bound to the surface of MC and basophils. And once exposed to IgE, MC may remain sensitized for a period of several months (185). Sensitization with IgE alone does not, however, stimulate MC degranulation. Mediator release requires the

cross-linking of surface bound IgE, which is accomplished by a multivalent antigen, anti-IgE immunoglobulin, or various lectins including Con A, PHA, ricin and wheat germ agglutinin (157,186-188).

Within seconds of receptor cross linking, a series of biochemical events takes place within the stimulated MC and basophils. 1) Proteolytic enzymes become activated (189), 2) phospholipid methylation occurs (190), 3) intracellular cyclic AMP levels increase (191), 4) phosphorylation of membrane lipids increases (192), 5) a Ca_2^+ influx occurs (192), 6) phospholipase is activated, resulting in the release of arachidonic acid (170), and 7) protein phosphorylation occurs (193). While the significance of may of these intracytoplasmic events remains ambiguous, they appear to work in concert, facilitating MC degranulation (194,195) and stimulating the release of inflammatory mediators (189-191).

On activation, MC granules located immediately under the cell surface begin to fuse with the cell's plasma membrane. This is followed by the formation of numerous channels or lacunae and canaliculae within the granule system that increase the exposed surface of the cell by as much as 3.5 times (196,197). Solubilization of the granule matrix (173), results in the release of granule contents into surrounding medium, although intact granules may also be extruded from activated cells. Following activation MC are able to recover, reforming storage granules and repackaging much of the granule's original contents (197). In activated basophils degranulation is usually slower and less dramatic. Cytoplasmic granules fuse directly to the plasma membrane without the formation of lacunae and canaliculae (173).

It is unclear how MC storage granules fuse with the cytoplasmic membrane. It has been proposed that hydration of granule proteoglycans is the force which drives granule fusion and exocytosis of stored mediators (198). Membrane fusion may be aided by fusogenic lipids produced by phospholipid metabolism which occurs in activated MC. During MC activation phosphatidyl serine is methylated and eventually converted to phosphatidyl choline, which is known to promote membrane fusion. Other fusogenic phospholipids, 1,2 diacylglycerol and its breakdown product monoacylglycerol are also produced when Ca_2+ dependent, phospholipase C becomes activated (196,199).

Cross-linking IgE-R is not the only mechanism which can trigger MC degranulation. A number of immunologic and non-immunologic agents also stimulate MC. Receptors for some IgG subtypes can trigger MC degranulation, however, unlike IgE the half-life of IgG on the mast cell surface is rather short. MC also posses receptors for C3a, C3b, C4a, and C5a, all of which have been reported to produce mast cell degranulation (157,200). Several neuropeptides, including substance P (201), calcitonin gene-related peptide (202), somatostatin (203), and vasointestinal peptide (203), have been reported to cause mast cell degranulation. The activities of neuropeptides on MC indicates that the central nervous system may modulate inflammatory responses. Interactions between MC and sensory nerves is supported by histologic sections which show nerve fibers connected directly to tissue MC. Non-immunologic agents capable of mediating mast cell degranulation include agents such as compound 48/80 (204), calcium ionophore A23187 (204), and basic peptides including polylysine (205) and even MBP (127).

While mast cell degranulation is usually associated with an explosive release of mediators, other forms of degranulation also exist. Under some conditions that remain ill defined, a slow, progressive loss of granule materials occurs, resulting in partially filled or completely empty granule chambers (173). This degranulation is thought to occur through shuttling of granule contents to the cell surface by small cytoplasmic vesicles (206). Other studies have found that rat peritoneal MC exposed to various psychotropic agents and stimulated with suboptimal concentrations of compound 48/80 also undergo slow degranulation that does not involve granule exocytosis (207). Interestingly, this slow degranulation results in the release of serotonin without significant histamine release.

Other Mediators of Inflammation.

While serotonin and histamine are the most well characterized of the mediators released by activated MC, other products may be equally important in inflammation. Mast cell activation results in the metabolism of arachidonic acid or eicosatetraenoic acid, into a number of potent mediators of inflammation. Arachidonic acid is a C_{20} fatty acid with double bonds at the 5-6, 8-9, 11-12 and 14-15 positions which is present in large amounts within phospholipids of cell membranes. Arachidonic acid can be liberated directly from the cell membrane by phospholipase A_2 (208) or through the sequential action of phospholipase C and diacylglycerol lipase (209). Following its release arachidonic acid can be metabolized through either the lipoxygenase or cyclooxygenase pathway. Activation of the lipoxygenase pathway results in the production of leukotrienes and other lipid mediators, while

activation of the cyclooxygenase pathway leads to the production of prostaglandins and thromboxanes (196).

Activation of the lipoxygenase pathway results in the conversion of arachidonic acid to a variety of potent lipid mediators. Lipoxygenases initially convert arachidonic acid to the corresponding hydroperoxyeicosatetraenoic acids (HPETEs) (196). Within different cell populations, distinct lipoxygenases preferentially attack certain double bonds in arachidonic acid. 12-lipoxygenase (12-LO) is the best characterized of these enzymes, found primarily in platelets, where it metabolizes arachidonic acid to 12-HPETE (209). The calcium dependent 5-lipoxygenase (5-LO) (210), which generates 5-HPETE is prominent in MC, basophils, monocytes and neutrophils (211), while 15-lipoxygenase is found in lung tissues (212). While the biological significance of many arachidonic acid metabolites has yet to be elucidated, studies have demonstrated that products of the 5-LO pathway play important roles in allergic and inflammatory responses.

Following its generation by 5-LO, 5-HPETE can by further metabolized though one of at least two diverging pathways. 5-HPETE can be metabolized through the glutathione peroxidase system to produce 5-mono-HETE (213), or used as a precursor for the generation of leukotrienes, a term applied to lipoxygenase derivatives with at least three conjugated double bonds. To generate leukotrienes, 5-HPETE is converted both enzymatically and nonenzymatically to form an unstable epoxide intermediate, leukotriene A_4 (LTA_4) (211). The addition of water to LTA_4 by epoxide hydrolase leads to the formation of 5-12-dihydroxyeicosatetraenoic acid or LTB_4 (214), which is subsequently converted to the less active 20-OH and 20-COOH derivatives (215).

Alteratively, glutathione can be attached to LTA_4 at position C-6 by glutathione-S-transferase, producing a peptido-lipid conjugate referred to as leukotriene C_4 (LTC_4) (216). Once formed, LTC_4 is rapidly released by the cell into the surrounding medium where it is normally converted to leukotriene D_4 (LTD_4) and then to leukotriene E_4 (LTE_4), by enzymes present in activated leukocytes and the plasma (217,218). Conversion of LTC_4 to LTD_4 and finally to LTE_4, is the result of modifications to glutathione involving the sequential loss of glutamic acid and glycine resides.

A wide variety of immunologic stimuli can stimulate leukocytes to release 5-LO products. Following cross-linking of surface bound IgE, some types of MC metabolize arachidonic acid through the lipoxygenase pathway (219). Monocytes, eosinophils and neutrophil also activate the 5-LO pathway in response to aggregated immunoglobulins, products of the complement cascade, and many microorganisms. These products mediate a diverse variety of biologic activities that are important in many immune functions. LTB_4 is prominent mediator of leukocyte activation, enhancing chemotaxis in many cell types including neutrophils, eosinophils, lymphocytes, and monocytes (213). Exposure to LTB_4 also triggers neutrophils to aggregate, release lysosomal enzymes, and generate superoxides (216). LTB_4 has been considered as a possible modulator of T cell function. LTB_4 promotes the proliferation of $CD8^+$ lymphocytes while blocking the expansion of $CD4^+$ cells (220,221), and may even convert CD4+ cells into the $CD8^+$ phenotype (222). By regulating the balance between helper and suppressor lymphocytes, LTB_4 may play a significant role in controlling how an immune response develops.

The peptido-lipid leukotrienes, LTC_4-LTE_4 are collectively known as slow reacting substance of anaphylaxis, (SRS-A). Members of the SRS-A family can alter vascular permeability and may potentiate the action of other vasoactive agents (223). When applied to hamster cheek pouchs, LTC_4, LTD_4 and LTE_4 all induce a long lived vasoconstriction that allows extravasation of macromolecules into surrounding tissues (216). In dose response curves, LTC_4 the most potent mediator, was found to be approximately 5000 times more effective than histamine (216). In the guinea pig both LTC_4 and LTD_4 administered exogenously produce vasoconstriction and increase vascular permeability (224,225). Although LTD_4 was found to be less potent in the guinea pig, its activity was dramatically increased when administered with prostaglandin E (PGE). The combination of LTD_4 and PGE_1 or PGE_2 was found to be approximately 400 times more potent than histamine at promoting plasma leakage. LTC_4 and LTD_4 administered subcutaneously into rats also cause a marked increase in vascular permeability (226), although PGE does not seem to potentiate this activity.

Metabolism of AA through the cyclooxygenase pathway also results in the production of potent inflammatory mediators. Activation of the cyclooxygenase pathway initially results in a bis-dioxygenation reaction of arachidonic acid which forms of an unstable endoperoxide intermediated, prostaglandin G_2 (PGG_2) (213). Subsequent reduction of PGG_2 by hydroperoxidase forms a second unstable intermediate, prostaglandin H_2 (PGH_2). PGH_2 nonenzymatically converts to either prostaglandin D_2 (PGD_2) or prostaglandin E_2 (PGE_2)(210). In rat CTMC, a glutathione-dependent PGH-D isomerase (227), results in preferential production of PGD_2 by MC stimulated with either calcium ionophore or

anti-IgE. (228,229). In addition to these reactions PGH_2 and PGG_2 can also be converted to prostaglandin I_2 (PGI_2) by PGI_2 synthetase or thromboxane A_2 (TXA_2) by TXA_2 synthetase (230). Both PGI_2 and TXA_2 are unstable and spontaneously break down to 6-keto prostaglandin (231) and thromboxane B_2 (232), respectively, products devoid of biologic activity.

Products of the cyclooxygenase pathway have a variety of biologic activities. Fever triggered in response to inflammatory stimuli is mediated primarily by PGE_1 and PGE_2, although PGF_{2a}, PGF_{1a} and PGA_1 are slightly active (233). PGI_2 functions as a vasodilator and inhibits platelet aggregation, while TXA_2 has opposing activities causing vasoconstriction and promoting platelet aggregation (234). Although exogenously administered prostaglandins are poor inducers of edema in many species, PGE_2 does produce edema in rats and humans (235,236), while PGE_2 and PGI_2 increase blood flow to the skin of rabbits (237,238). PGE has also been found to act synergistically with other mediators such as histamine, bradykinin and products of the complement system resulting in increased vascular permeability and edema regardless of the species (236,237,239). PGD_2 increases vascular permeability in a response that persists for up to 2 hours and is accompanied by marked capillary and venule dilation, and endothelial cell activation (179).

There is some indication that prostaglandins play an important role in EAE. Quantitation of prostaglandin and thromboxane levels in the spinal cords of rats and guinea pigs with EAE has shown that PGE and 6-oxo-PGF levels increase at the onset of neurologic impairment (240). In rats both PGF_{1a} and PGE levels return to normal as the disease progresses, although PGE levels continue to increase in guinea pigs.

These differences may be attributable to the course of clinical EAE in these two models, where rats recover from EAE while guinea pigs become progressively sicker. It is interesting that inhibitors of the cyclooxygenase pathway do not prevent paralysis but actually exacerbate the disease (241). This may be the result of arachidonic acid being shunted through the lipoxygenase pathway were it is used to generate leukotrienes which have the potential to produce more severe inflammation in the CNS than products of the cyclooxygenase pathway.

Mast Cell Proteases.

While MC have long been regarded as the major source of histamine in different tissues, they also appear to be the major source of neutral serine proteases. Estimates indicate that enzyme levels are between 10 and 25 $\mu g/10^6$ MC or roughly 20 mg/ml of cells (242-244). In rat CTMC, chymase, a 29 Kd, neutral serine endoprotease with a specificity for aromatic amino acids (245-248), constitutes approximately 50% of the protein found within the mast cell granules (240). A second neutral protease, carboxypeptidase A, accounts for an additional 30% of the protein found in rat CTMC secretory granules (249). This 35 Kd exoprotease, removes C-terminal amino acids with aromatic side chains (250). Other enzymes localized in the secretory granules of rat CTMC include tryptase, a trypsin "like" protease (251), and the acid hydrolases including, B-hexosaminidase, B-galactosidase, B-glucuronidase and arylsulfatase A (198,252,253).

Many of the mast cell granule constituents including trypase, acid hydrolases and vasoactive amines are fully soluble upon release (251,254-256). Other granule components including carboxypeptidase,

chymase and a few uncharacterized proteins remain associated with heparin (257). The close association of these different proteases in a macromolecular complex may facilitate sequential modification of enzyme substrates (250). In addition, the low solubility of these protein/proteoglycan complexes limits enzyme diffusion and allows high protease concentrations to be maintained near the surface of the activated MC (250).

The function of mast cell proteases has not been fully elucidated. Trypase has activity similar to Factor Xa, suggesting that it may have a role in prothrombin activation during the blood clotting process (251). Trypase has also been shown to activate the complement cascade by cleaving C3 to generate C3a and C3b (258). CTMC chymase has been implicated in a variety of activities including the production of chemotactic factors (254,255), selective degradation of basement membrane collagen (type IV) (256), degradation of connective tissue proteoglycans (257), and increased vascular permeability (259). Intriguingly, neutral serine proteases found in mast cell storage granules may play an important role in the activation and degranulation of MC. Ishizaka has reported that serine protease inhibitors and substrates for trypsin and α-chymotrypsin block phospholipid methylation induced by bridging of IgE receptors (260,261). This action inhibits the subsequent Ca_2^+ influx and the release of histamine (260,261). Other investigators have demonstrated that rat CTMC release their secretory granules and metabolize endogenous arachidonic acid to PGD_2 when exposed to purified chymase (262). In light of these findings, it has been proposed that proteolytic enzymes transduce the signal for mediator release by

associating with the MC cell membrane and cleaving unknown membrane substrates (262).

Mast Cell Cytokines.

In the past, MC and basophils have been implicated in tumor cell killing (263-265) although the basis for this tumoricidal activity was unclear. Recently investigators have demonstrated that cytotoxic activity found in rat CTMC can be attributed to the release of tumor necrosis factor (TNF) (266). Murine MC have also been found to produce a cytotoxic factor that is immunologically related to tumor necrosis factor (TNF) (267). Supernatants from stimulated murine MC were found to lyse TNF sensitive target cells and this activity could be partially blocked by antibodies against human and mouse TNF or human lymphotoxin. Subcellular fractionation of MC components showed that the TNF "like" activity was located in both the cytosol and granule fractions. Localization of mast cell TNF in the granules may facilitate the rapid release of this mediator during the degranulation of activated MC.

The release of mast cell TNF has been found to play an important role in the elicitation of cutaneous inflammation (268). The release of TNF by dermal MC causes up regulation of the endothelial cell adhesion molecule, ELAM-1 (268). Expression of ELAM-1 by activated endothelial cells allows circulating leukocytes to adhere to the lining of the blood vessels and facilitates leukocyte egress into the perivascular space. TNF also elicits the expression of other adhesion molecules important to lymphocyte trafficking. Lymphocytes have been found to adhere to cultured human umbilical vein endothelial cells stimulated with either TNF and lymphotoxin. Furthermore, TNF/lymphotoxin induced

adhesion between lymphocytes and endothelial cells was found to increase in both a time and dose dependent manner (269).

In an attempt to understand how lymphocytes might traffic into the CNS during infection and autoimmune reactions, Hughes et al. (270) have studied the adhesion of lymphocytes to cerebral endothelium in vitro. The level of adhesion between lymphocytes and endothelial cells was found to increase in a dose-dependent manner when endothelial cells were treated with either interferon-gamma or TNF. This increased adhesion was inhibited by an antibody to the common β-chain of the lymphocyte functional antigen-1 (LFA-1) family of molecules.

Selmaj and Raine tested recombinant human TNF (rhTNF), for its effect on myelinated mouse spinal cord tissue cultures (271). Following an 18-24 hour exposure to rhTNF, many myelinated nerve fibers exhibited alterations which included ballooning of the myelin sheath. Damage to the myelin sheath continued to progress, resulting in demyelination of some nerve fibers by 72 hours. Nearby astrocytes were often seen phagocytosing debris resulting from the degeneration of the myelin sheath. Oligodendrocyte necrosis also accompanied TNF mediated damage to the myelin sheath. In explants exposed to TNF, oligodendrocytes frequently became rounder with fewer processes while the cytoplasm and nucleus became necrotic and granular.

The mechanism by which TNF mediates damage to the myelin remains unclear. In other systems TNF has been found to alter the activity of sodium channels (271), which causes water and electrolytes to move out of the cell and into the extracellular space (272). Exposure of myelinated fibers to TNF could have similar effects on the activity of oligodendrocyte sodium ion channels. The resulting electrolyte

imbalance could shrink axons and disrupt the intimate association between the axon and oligodendrocyte, causing the myelin sheath to bubble (271). In support of this, peripheral nervous system tissue exposed to sodium channel blocking agents such as batrachotoxin results in significant myelin damage (273), similar to what is seen in the early stages of demyelination induced by an electrolyte imbalance (274).

Inhibition of Mast Cell Function.

Normal mast cell function can be altered by a number of pharmacological agents. Compounds which raise intercellular cAMP levels, including theophylline and dibutyryl cAMP, inhibit phospholipid methylation, Ca_2+ influx and subsequent histamine release (275,276). Theophylline, a methylxanthine, elevates cAMP levels by interfering with cAMP phosphodiesterase activity. Theophylline's effect on cAMP levels is augmented by isoproterenol, a catecholamine which triggers an increase in cAMP synthesis (277). Antigen-induced mast cell degranulation and the production of leukotrienes can also be blocked by cromolyn and its derivative proxicromil (159). It is generally accepted that primary mode of action for cromoglycate is to stabilize the mast cell membrane and prevent the release of inflammatory mediators (278).

Depletion of vasoactive amines by reserpine also effectively blocks normal mast cell function. This basic compound is taken up by MC where it competes with VAA for binding spaces, forcing serotonin and histamine from the granules into the cytoplasm. Once in the cytoplasm, serotonin is quickly catabolized by monoamine oxidase (MAO), effectively depleting MC of this important, preformed mediator. Reserpine's effect on MC can be partially blocked by pretreating cells with pargyline, an

inhibitor of MAO (279). Following pretreatment with pargyline, reserpine still forces serotonin and histamine into the cytoplasm, however, they are no longer degraded by MAO. These pharmacologic manipulations leave mast cell storage granules devoid of VAA, thereby preventing IgE mediated release which occurs through the fusion of storage granule and cytoplasmic membranes (279). However, other forms of mast cell degranulation may still proceed. Mast cells can undergo more subtle forms of degranulation, where preformed mediators are shuttled between the storage granules and the mast cell surface. This form of degranulation can still proceed when serotonin and histamine are in the cytoplasm following reserpine/pargyline treatments (280).

The effects of mast cell degranulation can also be blocked by serotonin and histamine antagonists. H_1-blocking drugs effectively antagonize the action of histamine, preventing changes in vascular permeability and the formation of edema and wheal (159). Frequently used H_1 antagonists include diphenhydramine, pyrilamine, promethazine, chlorpheniramine cyproheptadine and chlorcyclizine (159). The effects of serotonin release can be blocked with serotonin antagonists, including ketanserin, methysergide and cyproheptadine (159). It is of interest that cyproheptadine can antagonize both H_1 and serotonin receptors. This agent has been found to block the induction of passive EAE (281), and the reinduction of EAE with pertussis toxin (282), suggesting that both histamine and serotonin play an important role in the induction of EAE.

The Role of Basophils in Cell Mediated Immunity.

Basophils are generally associated with immediate type

hypersensitive reactions mediated by IgE. However, these cells can participate in other types of immune responses. In appropriately sensitized guinea pigs, basophils accumulate at the site where antigen has been introduced and release their stored mediators. This type of immune response is known as cutaneous basophil hypersensitivity (CBH), or the Jones Mote reaction in man (283,284). Basophil infiltrations have also been demonstrated in a number of delayed-type reactions including: contact dermatitis (285), immunity to viruses (286) and ticks (287), allograft rejection (288) and tumor immunity (263-265). While being delayed in onset, CBH reactions differ considerably from tuberculin or classic DTH sensitivity. The CBH reaction elicits only slight induration, lacks the production of macrophage migration inhibition factor (289) and can be elicited without the use of mycobacterium adjuvants (290).

Although CBH would appear to be a unique form of cell mediated immunity it could be an important component of classic DTH reactions. In animals skin tested one week after immunization with CFA the test site was found to contain substantial basophil infiltration although reduced infiltration was seen in skin biopsies done at later time points (285). Dvorak et al. concluded that classical "delayed hypersensitivity included a basophilic component early in its evolution that diminished with time".

The Role of Mast Cells in Cell Mediated Immunity.

Tissue MC may play a similar and perhaps more extensive role than basophils in the development of DTH reactions. MC are particularly prevalent at sites likely to contact the external environment, including

the lungs, skin, airways and the gastrointestinal track. Because of their wide distribution, these cells are often first cells from the immune system to contact antigen. The importance of the MC in DTH reactions can be demonstrated with skin test reactions. When Ag is injected into the flank of an appropriately sensitized, guinea pig, monkey or man, an erythematous, indurated lesion, typical of a delayed-type reaction forms at the site 24-48 hours later (291). Mice, while exhibiting other normal manifestations of cell mediated immunity fail to produce a DTH response in the flank (292-294). This non-responsiveness can be reversed if peritoneal cells, rich in serosal MC, are injected into the site along with the challenge antigen. With the transfer of peritoneal cells, skin test reactions in the flank become morphology indistinguishable from DTH reactions elicited at other sites in the animal (293).

In rodents it is believed that MC contribute to the induction of DTH responses through the release of serotonin. Serotonin release is thought to open gaps between endothelial cells, allowing cellular diapedesis and the subsequent accumulation of mononuclear cells at the skin test site (167,291). To further elucidate the role of MC in cell mediated responses, the mast cell deficient mouse strains W/W^v and Sl/Sl^d, were evaluated for their ability to express DTH. W/W^v mice have a defect in the bone marrow precursor population which blocks the development of MC, while a defect in the peripheral tissue microenvironment of Sl/Sl^d mice prevents MC precursors from differentiating into mature cells (295,296). In both the W/W^v and Sl/Sl^d mouse strains, skin test responses could not be elicited to antigens such as sheep erythrocytes and picryl chloride following appropriate sensitization with the challenge antigen (297). To demonstrate that the lack of DTH responsiveness was

not from a defect in effector T cells, adoptive transfer experiments were done. Lymphocytes from normal sensitized littermates were transferred into mast cell-deficient mice. However, normal skin test reactions could not be elicited in the recipient mice, demonstrating that the development of cutaneous DTH is dependent on the release of vasoactive mediators from MC.

Numerous studies with pharmacologic agents that alter MC function, have demonstrated the paramount role of MC in DTH, confirming the work done with mast-cell-deficient mice (297): 1) Treatment of mice with proxicromil, a cromolyn-like drug that prevents the release of mast cell mediators blocks both the early and late skin thickening associated with DTH reactivity in sensitized mice (298); 2) Depleting MC of serotonin with reserpine treatments abolishes DTH responses, although this effect can be reversed by inhibiting MAO (116); 3) DTH responses are inhibited with methysergide, ketanserin and cyproheptadine, serotonin receptor antagonists which prevent activation of the endothelium and subsequent increase in vascular permeability (167,299); 4) Pretreatment of cutaneous sites with serotonin results in desensitization of vascular serotonin receptors and inhibits DTH on subsequent challenge with specific antigen (300).

Mast cell degranulation has also been implicated in the progression of cell mediated responses other than cutaneous DTH reactions. For example, when mice are sublethally irradiated and given allogenic spleen cells i.v. they develop chronic graft-vs-host disease (GVHD). Manifestations of this disease include the development of dermal fibrosis and the presence of mononuclear cell infiltrates in the skin (301). In several studies mast cell degranulation has been

implicated in the inflammation and fibrotic changes associated with GVHD (302,303). Examination of skin sections using light microscopy has revealed the disappearance of connective tissue MC (303). In these studies, tissue was stained with toluidine blue, which is specific for heparin contained in intact mast cell granules and not for MC themselves. Further examination of similar sections by electron microscopy found that MC were still present in the tissue but they had undergone granule depletion and cellular activation (303). Double immunofluorescent staining for IgE receptors and cytoplasmic granules (with avidin) has also demonstrated that MC are present but severely degranulated during the course of GVHD. The mast cell degranulation seen in GVHD is qualitatively different from mast cell degranulation seen after anaphylactic stimuli. In contrast to the explosive degranulation mediated by IgE, MC are believed to slowly release their granule contents during GVHD (301).

Mast Cell Involvement in Autoimmune Diseases.

A disappearance of toluidine blue positive MC, similar to what was observed in GVHD, has also been reported in both adjuvant arthritis (304,305) and experimental allergic neuritis (EAN) (306). Toluidine blue is a basic dye which selectively stains MC by binding to heparin within the mast cell granules. During mast cell degranulation the granule contents, including heparin are extruded from the MC resulting in the loss of staining by toluidine blue. Therefore, in these quantitative studies, the apparent reduction in mast cell numbers is attributable to degranulation and not the emigration of these cells.

In other studies, sections of sciatic nerve were examined by electron microscopy to visualize changes in MC that are present in the endoneurial interstitium. Ten days following the induction of EAN, when edema and endoneurial fluid pressure are present, MC in the nerve were found to contain fewer than normal numbers of granules. Electron lucent vacuoles in these cells provides additional evidence that mast cell degranulation was taking place (307). By day 14, MC appeared more prevalent, suggesting cell proliferation. Individual granules within these MC showed significant enlargement and were often seen fusing with adjacent granules, while exocytosis of some granules was seen.

Mast cell degranulation has also been reported in experimental autoimmune uveoretinitis (EAU) (308), an intraocular inflammatory disease which can be induced in certain rat strains by immunization with a retinal-specific protein. Researchers working with the disease in rats have reported a correlation between a particular strains susceptibility to the disease and the number of MC in the choroid plexus (309). Interestingly the same rat strains which are susceptible to EAU are also susceptible to EAE.

During the induction of adjuvant arthritis, changes in MC have been reported (310). MC are the first cells to increase in the inflamed tissues, infiltrating the synovial layers of the joint 5 days following the induction of adjuvant arthritis. Later in the disease, MC devoid of cytoplasmic granules were identified, demonstrating that mast cell degranulation was taking place during the disease. The appearance of lymphocytes and neutrophils also coincides closely with mast cell degranulation, indicating that MC contribute to the induction of adjuvant arthritis.

Considering the similarities between the autoimmune diseases, EAN, EAU, collagen arthritis, and EAE, it seems reasonable to suggest that degranulation of MC takes place in rats with EAE. In contact dermatitis, products released during the degranulation of MC are thought to contribute to increased vascular permeability and accompanying edema (311). Similar mast cell activity in EAE would account for the change in blood-brain-barrier permeability and the accompanying edema.

Mast Cells in the Central Nervous System.

In comparison to MC in other tissues, relatively little is known about the occurrence and activity of MC in the CNS. MC are generally abundant in the dura and leptomeninges of the CNS, where they are located primarily along blood vessels as they are in other tissues (312). MC can also be identified within the peripheral nervous system where they often are situated between nerve fibers. Detailed studies have demonstrated that two types of MC, type I and type II exist within the CNS (128). Type I MC are morphologically and functionally similar to CTMC. In adults, type I MC are oval with a central or rounded nuclei and contain many round uniform granules. Type I MC occur primarily in the leptomeninges, choroid plexuses and occasionally in the CNS parenchyma (128).

Type II MC are the more prominent MC type, with their frequency being numerically proportional to the blood supply of the region. These cells are particularly prominent along vessels ranging from 50-100 μm in diameter. Consequently, grey matter which has a greater abundance of vessels of this size, tends to contain more type II MC then does white

matter (128). Morphologically, type II MC have granules which are larger, more variable in size, and stain with greater homogeneity than the granules seen in type I MC. The different staining properties probably reflect a higher lipid content in type II MC, which has lead some investigators to refer to them as neurolipomastocytoid cells.

Studies indicate that the numbers of MC in the CNS are sufficient to contribute to the pathology of EAE. Brain slices incubated with the mast cell activator compound 48/80, develop areas of demyelination (313). Furthermore, when guinea pigs and rats were injected with compound 48/80, many of the animals developed varying degrees of limb paralysis within 2 weeks of the injection (314). Histologic examination revealed that neurolipomastocytoid cells, like MC outside the CNS, had responded to compound 48/80 resulting in vacuolation, changes in granule size, and degranulation. Swelling, splitting and folding of the basal lamina system were also seen, indicating that increased vascular permeability and edema had accompanied MC degranulation.

Studies with encephalitogenic viruses provide additional proof that CNS MC contribute to the inflammation associated with EAE. Sindbis virus infection of the CNS triggers a mononuclear inflammatory response similar to what is seen in EAE and cutaneous DTH reactions (315). The mononuclear cell infiltration which begins 3 days after virus injection (316,317), is both immunologically specific and T cell mediated (318). To establish that MC were important pathologic component of the disease, mast cell deficient mice (WBB6 F_1 W/W^v) were infected with Sindbis virus and compared with normal littermates. Histologic examination found a significant reduction in inflammation of the brain parenchyma and meninges in mast cell deficient mice. Depletion of mast cell

vasoactive amines in normal mice with reserpine, also significantly reduced the amount of inflammation present in the CNS. Collectively these findings demonstrate that the presence of functional MC in the CNS is required for the full development of a mononuclear inflammatory response to Sindbis virus infection.

Mast Cell Involvement is Facilitated by an Antigen Specific T cell Factor (TCF).

Askenase et al (319) have proposed that an antigen specific factor is responsible for the MC participation in delayed type hypersensitivity. DTH is generally characterized by cellular infiltration causing induration at the site 24-72 hours following antigen deposition (298). Closer observations have determined that DTH responses are actually biphasic and that a temporary thickening of the site precedes the cellular infiltration. The early portion of the response has been attributed to the release of vasoactive mediators by the MC sensitized with an antigen-specific T cell derived factor or TCF (167).

Most of this work has employed a contact dermatitis system in which small reactive molecules such as picryl chloride or oxazolone are painted on the animal's skin. Covalent binding between the hapten and skin proteins leads to a strong cellular response against the altered host protein. Following sensitization, animals respond with a strong DTH reaction on subsequent contact with the same hapten as demonstrated by a skin test. To derive TCF, spleen and lymph node cells from sensitized mice are placed into culture where they continue to secrete TCF (298,319). After a 48 hour culture period, TCF can be extracted from the cell supernatant using an affinity column to which the specific

antigen has been attached. This partially purified T cell factor has been used to transfer an immediate hypersensitivity-like reaction into naive recipient mice (298,319). To test the TCF activity, recipients are skin tested by placing a small quantity of the appropriate hapten on the ear. In response to the challenge, a strong local swelling at the skin test site develops in recipient animals but not normal controls. The swelling mediated by the TCF generally peaks two hours following the application of picryl chloride, and has normally subsided by four hours (298). The local swelling is attributed to changes in the endothelial cell permeability as demonstrated by accumulation of label in the ears of animals pretreated with [^{125}I]-BSA (319). As would be expected, the response generated by the factor is antigen specific. Picryl chloride factor sensitizes mice to only picryl chloride and not other contact inducing agents such as oxazolone (319).

To demonstrate that TCF works in conjunction with the mast cell to mediate vasoactive amine release, experiments were done with mast-cell-deficient mouse strains. The W/W^{v} and Sl/Sl^{d} mouse strains have substantial reductions in the quantity of tissue MC when compared to their normal littermates (+/+) (295,296,320). Both sheep erythrocyte-induced footpad DTH and picryl chloride-induced contact sensitivity responses were significantly reduced in mast-cell-deficient mice (297). Adoptive transfer experiments confirmed that the diminished responses were attributable to the mast cell deficiency and not due to a defect in T cell induction. Sensitized cells from +/+ mice failed to transfer DTH reactivity to the mast-cell-deficient mice, yet cells from W/W^{v} and Sl/Sl^{d} mice were found to sensitize normal +/+ littermates (297). When the mast-cell-deficient stains were sensitized with TCF, the animals failed to

respond with the antigen specific hypersensitivity seen in normal animals. These experiments serve to illustrate the importance of an early mast cell component, facilitated by TCF, during DTH responses.

Characterization of TCF.

TCF has been further characterized, and can be distinguished from IgE by its biological activities and immunochemical properties. Using an IgE specific, radioimmune assay, TCF did not compete with labeled IgE for binding sites (319). Affinity columns specific for IgE, were likewise unable to retain TCF, while a TCF specific column could not bind IgE. The duration that passive recipients remain sensitized also differs with TCF and IgE (319). When an animal is given IgE then challenged 48 hours later, the measured response is nearly as strong as what had been seen two hours after IgE is administered. This prolonged sensitization is not seen with TCF. While a strong response is seen at two hours following sensitization, by 48 hours the animal is only able to give a response slightly above background. Additional biochemical characterization of TCF has determined that it has a molecular weight of approximately 70 kd, and is stable at 56°, further demonstrating that it is not an immunoglobulin of the IgE isotype (319).

Characteristics of TCF Mediated Mast Cell Degranulation.

Vasoactive amine release from MC armed with TCF is thought to proceed by a degranulative process similar to that observed in GVHD. The MC at the skin test site of TCF sensitized animals form numerous filipodial extensions and elongate, features which result in an increase of the surface area to volume ratio (167). These morphologic alterations are

accompanied by cytoplasmic changes leading to increased prominence of mitochondria, Golgi apparatus, and the formation of numerous cytoplasmic vesicles (167). These MC appear to no longer function as storage cells, but instead are actively secreting products as suggested by large numbers of Golgi apparatus and abundant rough endoplasmic reticulum (302,166). The newly formed vesicles, which contain primarily serotonin (207), may selectively transport storage granule contents to the cytoplasmic membrane in a process that may take up to 18 hours before extensive MC degranulation can be seen (321).

Evidence for Antigen Specific T cell Factors (TCF) in Other Models.

Demonstration of antigen specific T cell factors is not limited to the contact dermatitis system. Askenase et al. have successfully demonstrated that similar T cell factors are produced during immune responses in tumor model systems (322). Other investigators have demonstrated that an antigen specific factor which sensitizes tissue MC is produced in animals immunized with SRBC (323). Biphasic skin test responses, similar to those seen in by Askenase et al. have been reported in skin test responses to soluble protein antigens (324). Presumably the early swelling is mediated by mast cell degranulation in response to the sensitizing antigen.

There is also increasing evidence that arthritis is mediated through a soluble, antigen specific, T cell derived factor, designated arthritogenic factor (AF) (325). AF was first detected in culture supernatants of LNC from rats immunized with type II collagen. Crude AF containing supernatants injected intraarticularly into the knees of syngeneic rats produced profound changes in the synovium and subjacent fat tissue

(326). LNC from rats immunized with antigens other that type II collagen failed to produce arthritogenic activity, indicating that AF was antigen specific. Accordingly, AF activity was purified from the supernatants of $W3/25^{+}$, type II collagen specific T cell lines, by affinity chromatography (326).

The synovitis produced by purified AF has been characterized by examining the knee joints by light and electron microscopy at different time points following the injection (326). At six hours following injection of AF, there was considerable disruption of the synovium. Adhesion of normal synoviocytes to adjacent cells was reduced, and a generalized edema was apparent. In many regions, synoviocytes had become completely detached from the underlying extracellular matrix, exposing collagen fibers to the joint space. In the connective tissue, PMNs accompanied by some mononuclear cells, were seen infiltrating from the post capillary venules into the surrounding tissues. Furthermore, storage granules in synovium MC were metachromatic and swollen, evidence that degranulation was taking place. At 24 hours, the cellular infiltrate was predominantly mononuclear cells. Edema was still present and large masses of fibrin were seen deposited in the synovium.

Comparisons of MC in treated and control rats showed a clear increase in the number of discharging MC following AF injection. These observations lead the investigators to propose that AF mediates mast cell exocytosis either directly or indirectly (326). AF mediated degranulation is consistent with earlier reports where MC were reported to increase in number and to degranulate approximately two days prior to the onset of clinical arthritis.

Recently AF has been isolated from rats with adjuvant induced arthritis (327). AF isolated from both arthritis models has been found to be both functionally and biochemically identical. SDS-PAGE analysis of AF from both models has determined a common MW of 65 Kd from the protein (327). Injected into a knee joint, AF in either model, produces a long lasting inflammation which is not dependent on the complement system (325), resulting in extensive mast cell degranulation and cellular infiltration.

Antigen Specific T cell Factors (TCF) in EAE.

In EAE, there is evidence that an antigen specific T cell factor can mediate changes in CNS vascular permeability. P.Y. Paterson et al described the transfer of histologic EAE with supernatants derived from LNC of Lewis rats immunized with MBP/CFA (328). LNC from rats actively immunized seven days earlier were suspended at a high cell concentration (2.5×10^8 cells/ml) for one hour. Cell free supernatants from the culture were collected and injected into normal syngeneic recipients. Although clinical disease was not observed in any of these recipients, histologic examination of the brains and spinal cords of the recipients revealed lesions typical of EAE. Some additional work attempted to characterize the factor responsible for altering vascular permeability in the CNS (329). Molecular weight fractionation determined that the factor responsible for biological activity was in excess of 100 Kd, while its activity was found to be stable for at least 4 days at -20°C, 18 hours at 4°C and 1 hour at 56°C.

The activity described in these experiments may have been an antigen specific TCF, similar to those described in the contact dermatitis

and collagen type II arthritis systems. TCF present in the LNC supernatants could have sensitized MC in the dura mater of the CNS, facilitating degranulation in response to MBP shed from the myelin sheath. The release of VAA in response to MBP by sensitized MC would increase vascular permeability in the CNS and allow activated lymphocytes to infiltrate the CNS. Rats used as recipients of the LNC supernatants had not been previously exposed to exogenous MBP and would be expected have a low frequency of MBP reactive lymphocytes. Therefore, cells infiltrating the CNS are not MBP reactive but represent a pool of activated lymphocytes reactive to any number of antigens that the rat has recently encountered. Once in the CNS these infiltrating cells fail to encounter their specific antigen and fail to invoke the inflammatory response needed for clinical paralysis.

Summary.

The goal of this thesis is explore the issue of how lymphocytes move out of circulation and into the CNS during the course of EAE. In numerous studies described above, investigators have made observations which are consistent with the model proposed by Askenase et al. based on work done in the contact dermatitis system. In this model, interactions between antigen reactive T cells, soluble T cell factors and MC are required for the elicitation of classic cutaneous delayed-type immune responses. We have proposed that similar events are required for the induction of EAE and attempt to demonstrate a significant role for MC the induction of this autoimmune disease.

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MANUSCRIPT #1

Title: The Role of Mast Cells in the Development of Allergic Encephalomyelitis.

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ABSTRACT

In the rat model of Experimental Allergic Encephalomyelitis (EAE) we have been able to adoptively transfer clinical disease into bone marrow chimeras constructed by infusing bone marrow cells derived from F1 (Lewis X Brown-Norway) animals into lethally irradiated Brown-Norway (BN) recipients. These chimeras developed paralytic disease 5 days following transfer of basic protein sensitized spleen cells. The spleen cells were obtained from Lewis animals previously injected with myelin-derived basic protein emulsified in Complete Freund's Adjuvant. The development of disease in these chimeras questions the mechanisms of lymphocyte trafficking since the transferred cells are interacting in vivo with allogenic endothelium. A possible explanation of disease transfer in this donor-recipient combination evokes the involvement of the mast cells as a source of mediators that alter the endothelial barrier and allow semi-allogenic cell interactions at the developing CNS lesion. We have completed a series of experiments that indicate a key role for the mast cell in the initial cell interactions leading to clinical disease. Moreover, all of our observations are consistent with the existence of mast cells with receptors for T cell derived antigen specific factors, When mast cells come into contact with antigen a triggering event ensues which leads to capillary permeability and subsequent lymphocyte interaction with antigen in the extravascular space.

INTRODUCTION

EAE is a neurologic disorder resulting from an immune response directed toward neural antigens. In this autoimmune disease the immune system is presumably activated against autologous myelin basic protein (BP), a constituent of the myelin sheath. The ensuing immune response leads to perivascular infiltration of the CNS by inflammatory mononuclear cells and varying amounts of demyelination (1). Demyelination is accompanied by edema and fibrin deposition in the CNS, both of which are suspected of contributing to disease progression (2,3). The damage and associated inflammation of the CNS during the disease episode results in the clinical manifestations of the disease, which can be characterized by an acute ascending paralysis.

EAE can be induced in guinea pigs and some rat strains, with a single injection of guinea pig spinal cord or purified BP emulsified in complete Freund's Adjuvant (CFA). Murine models of the disease also exist but require the use of Bordetella pertussis as an additional adjuvant (4). Following immunization disease progresses to involve the hind quarters and culminates in complete hindquarter immobilization with incontinence fourteen days after the injection of BP. A rapid recovery follows this nearly fatal paralysis, despite the persistence of inflammatory lesions within the CNS.

EAE can be adoptively transferred by antigen specific lymphocytes. Relatively few cells are required to cause clinically apparent disease especially if the culture enhancement step as originally reported by McFarlin is used (5). Although T lymphocytes are required for the successful passive transfer of EAE, the nature of the in vivo cell

interactions required for disease development are unknown. In order to gain some insight into these in vivo events we have recently used bone marrow reconstituted radiation chimeras as recipient for the adoptive transfer of EAE (6). The results of these studies are consisting with a crucial role for the mast cell and its products for the development of EAE. In this report we show additional data in support of mast cell involvement in the pathogenesis of EAE.

METHODS

Inbred Lewis (LEW) female rats used for all of the experiments reported with the exception of the chimera transfer recipients. The chimeras were obtained by lethally irradiating (1200 rads) Brown-Norway (BN) and then reconstituting these animals with 5×10^7 bone marrow cells obtained from $(LEW \times BN)_{F1}$ donors. The chimeras were used six months following reconstitution.

BP was prepared and used to induce clinical disease as previously reported (5).

Rats used in the adoptive transfer experiments were evaluated daily for clinical signs of neurologic impairment. The grading system used was as follows; 1 -- flaccid tail, grade 2 -- hindquarter weakness, grade 3 -- hindquarter paralysis, grade 4 -- complete hindquarter paralysis with incontinence.

For the adoptive transfer of EAE we used spleen cell suspensions obtained from LEW animals injected 12 days earlier with BP-CFA and expanded in culture as previously described.

Delayed type hypersensitivity (DTH) reactions to BP were quantitated using a 10 μl injection of BP injected intradermally into the ear. In all cases the left ear was injected with an equal concentration of irrelevant antigen. The ear thickness was measured prior to the skin test an at regular intervals using a spring loaded caliper.

RESULTS

The successful cell transfer of clinical EAE into bone marrow chimeras by LEW derived lymphocytes (Table 1) is a result mediated in part by antigen sensitive lymphocytes which must "escape" from an allogeneic vascular compartment in order to initiate disease.

Within the chimera environment the transferred LEW lymphocytes first encounter the endothelium of the BN strain. This interaction does not alter disease development and these chimeras develop clinical EAE to the same end point as seen in the syngeneic and semisyngeneic recipients. These observations are at odds with commonly accepted endothelial-lymphocyte interactions thought to take place in vivo. Furthermore, it would appear that any cell trafficking necessary for the development of adoptively transferred EAE is similarly not influenced by the allogenic vasculature experienced by the transferred LEW cells within the BN <-(LEW X BN) bone marrow chimera

One explanation for these observations involves a central role for the mast cell which has been postulated by P. Askenase (7). This model assumes that the mast cell may specifically interact with antigen. This specific interaction is mediated by a T cell derived factor which binds to mast cells in a manner conceptually similar to the binding of IgE. Following interaction with antigen the sensitized mast cell releases some of its granule contained mediators with a resultant alteration of vascular permeability. This model, if applicable to the rat response to BP, would predict an influence of inhibitors of mast cell activity on EAE and would also predict that skin test reactions would have an early component due to the immediate changes in vascular permeability as caused by

mediators released from antigen reactive mast cell.

Figure 1 is a summary of skin test reactions that developed in recipients of 2×10^7 spleen cells. The skin test was administered 3 days following adoptive transfer and measurements of the skin test response were made one hour after the skin test and at the indicated intervals.

The early skin test response develops quickly and is initially edematous in nature however by 15-24 hours the skin test response is significantly more indurated in character. The nature of the skin test response is in keeping with a role for the mast cell in the response to BP. In order to assess the possible role of the mast cell on the development of clinical EAE we treated cell recipients with the various reagents listed in Table 2 and subsequently assessed these animals for the development of clinical disease.

DISCUSSION

It is evident from this study that the mast cell does play a significant role in the response to clinical disease. The specific role for the mast cell in this disease is unclear however mast cell degranulation may lead to localized alteration of vascular permeability, edema accumulation and resultant temporary nerve dysfunction. This possibility would also explain the many reports of a lack of correlation of clinical disease with severity of inflammatory lesions within the CNS.

The reagents that we used in Table 2 are known to alter mast cell release of vasoactive amines. Reserpine effectively depletes mast cells of vasoactive amines (8). No disease was evident in cell recipients treated with reserpine. Inhibition of monoamine oxidase activity by pargyline decreased the effectiveness of reserpine as would be expected if mast cell derived mediators are of importance. Inhibition of mast cell degranulation by theophylline as well as by proxicromil also inhibits the development of clinical disease in cell recipients treated with these reagents.

The results of these studies all point to a role for the mast cell in the development of clinical EAE especially as it develops following adoptive transfer of BP specific lymphocytes. These studies also imply that the vascular endothelium may play a less significant role in antigen presentation and cell trafficking at least as it relates to effector function of antigen activated T cells.

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TABLE 1

TRANSFER OF EAE INTO BONE MARROW CHIMERAS

DONOR[a]	RECIPIENT	CLINICAL DISEASE INDEX
Lewis	Lewis	4.0
Lewis	BN	0.0
Lewis	BN (irradiated)	0.0
Lewis	$(LEW \times BN)_{F1}$	3.8
Lewis	$BN<-(BN \times LEW)_{F1}$ chimera[b]	3.7

[a] 2×10^7 spleen cells from BP-CFA immunized LEW rats were transferred iv to all recipients.

[b] The bone marrow reconstituted chimeras were used 6 months after reconstitution.

TABLE 2

ALTERATIONS IN THE DEVELOPMENT OF ADOPTIVELY TRANSFERRED EAE CAUSED BY REAGENTS THAT INFLUENCE MAST CELL ACTIVITY[a]

TREATMENT[a]	SICK/TOTAL	DISEASE SEVERITY[b]
Control	6/6	3.8
Reserpine[c]	0/6	0.0
Pargyline-reserpine[d]	4/5	1.5
Theophylline[e]	2/7	1.0
Proxicromil[f]	0/5	0.0

[a] recipients received 2 x 10^7 spleen cells cultured for 72 hrs with 1 μg/ml BP.

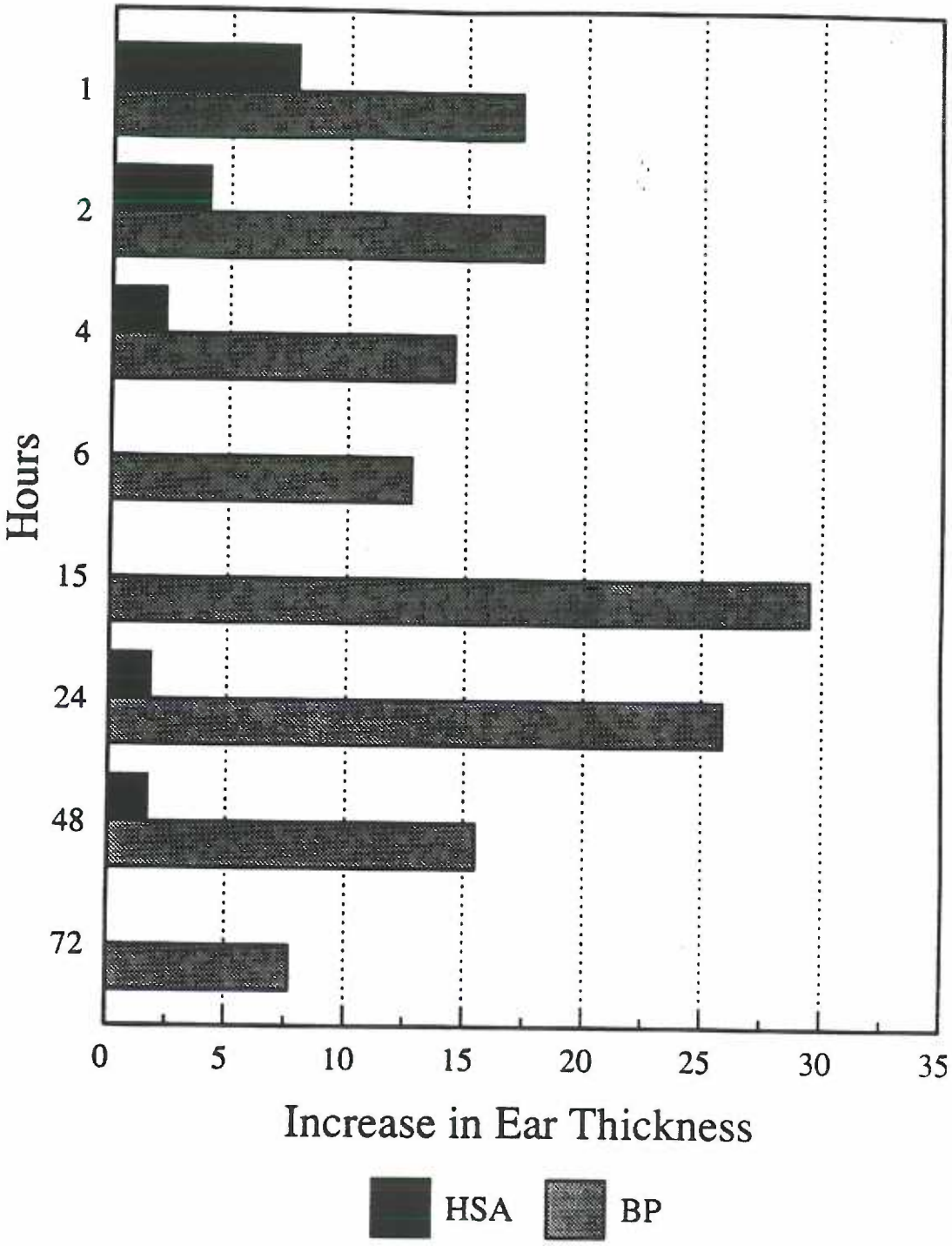
[b] Maximum clinical disease is graded 4.0

[c] 5 mg/kg of reserpine was given on day 3 following cell transfer.

[d] 100 mg/kg of pargyline was given prior to treatment with reserpine.

[e,f] Administered at 50 mg/kg twice daily.

Figure 1. Skin test response in cell recipients



MANUSCRIPT #2

TITLE:

Transfer of Experimental Allergic Encephalomyelitis to Bone Marrow Chimeras: Endothelial Cells are not a Restricting Element

Running Title:

Endothelial cells do not restrict passive EAE

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INTRODUCTION

Ia^+ cells of non-bone marrow origin (e.g., endothelial cells, astrocytes) have been proposed to play a significant and perhaps determining role in the expression of a cell-mediated response to neuroantigens (1-4). To test this premise in vivo we prepared F_1-to-parent bone marrow chimeras and used these chimeras as recipients in the adoptive transfer of experimental allergic encephalomyelitis (EAE). Our results indicate that the development of EAE within the recipient is dependent upon MHC compatibility between adoptively transferred lymphocytes and bone marrow-derived cells. MHC compatibility between transferred lymphocytes and non-bone marrow-derived cells was not a requirement for adoptive transfer of clinical disease.

MATERIALS AND METHODS

Animals. Inbred Lewis [LEW (RT-1^{l})], Brown Norway [BN (RT-1^{n})], Fischer-344 [F-344 (RT-1^{lvl})], Buffalo [BUF (RT-1^{b})], and ACI (RT-1^{a}) rat strains were obtained from Microbiological Associates, Walkersville, MD, and from Simonson Laboratories Inc., Gilroy, CA. The following (LEW x Parent-2)F_1 rat strains were bred and maintained locally : (LEW X BN)F_1 (RT-$1^{l/n}$), (LEW x BUF)F_1 (RT-$1^{l/b}$), and (LEW x ACI)F_1 (RT-$1^{l/a}$). Animals were provided access to food and water without restriction and were watered by hand during periods of paralysis.

Purification of Myelin Basic protein. Guinea pig brains were purchased from Pel Freez Biologicals (Rogers, AR) and stored at -70°C until extracted for basic protein (BP). BP was prepared according to a procedure modified from Diebler et al. (5).

Immunization with BP-CFA. Myelin basic protein was rehydrated in saline at a concentration of 1 mg/ml and emulsified in an equal volume of CFA containing 10 mg of nonviable, desiccated *Mycobacterium tuberculosis* H37 RA per ml. Active immunization with BP was accomplished by injecting 0.1 ml of the BP-CFA divided equally between the two front foot pads.

The adoptive transfer of EAE. The procedure was followed as described (7).

Production of bone marrow chimeric animals. Animals received 900-1000 rad of irradiation utilizing a ^{60}Cobalt source. After irradiation, hematopoietic function was restored by bone marrow reconstitution.

Chimeric rats were used as recipients not sooner than 6 mo after bone marrow engraftment.

Irradiation of Cell recipients. Where indicated, cell recipients received 900-1000 rad of irradiation using ^{60}Cobalt source 24 h before cell transfer.

Clinical evaluation. Rats used in adoptive transfer experiments were evaluated daily for clinical signs of neurologic impairment and graded for clinical signs of neurologic impairment and graded on a scale of 1-3: animals with flaccid tails were given a grade 1; animals with hindquarter weakness or paralysis were given a grade 2; and hindquarter paralysis with incontinence was given a grade 3.

Histology. Tissue was removed from selected recipients at the time of maximum clinical disease. After fixation in 10% buffered formaldehyde solution, spinal cords were carefully isolated from the surrounding tissue. The cords were subdivided into 3 segments and embedded in paraffin. At least 8 sections were cut from each block and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

The adoptive transfer of clinical EAE is readily achieved with spleen cells obtained from BP-sensitized donors if these cells are stimulated in vitro with specific antigen before transfer. Table I shows the development of adoptively transferred clinical EAE in syngeneic and semi syngeneic, but not allogeneic recipients of BP-stimulated spleen cells obtained from BP-CFA-immunized Lewis (LEW) and (ACI x LEW)F_1 rats. These results are consistent with other reports of MHC restriction in the transfer of EAE in the rat (6,7). In addition to exhibiting a requirement for MHC compatibility, presumably reflecting the need for *in vivo* antigen presentation requirements, transferred cells must escape host rejection mechanisms during the inductive phase of the disease. For example, clinical EAE develops in experiments where LEW cells are transferred to F344 rats or (LEW X BN)F_1 cells are transferred to LEW rats only if the recipient is irradiated before transfer. While these donor-recipient combinations have shared MHC antigens, recipient recognition of foreign non-MHC antigens on the transferred cells apparently inhibits donor cell function in the nonirradiated recipient. Irradiation of recipients before transfer, however, does not allow MHC-incompatible cells to transfer EAE, indicating a requirement for proper antigen presentation within the recipients.

The restriction patterns seen in Table I do not separate the APC role of Ia^+ nonhematogenous cells from that of the bone marrow-derived mononuclear cell populations in the development of adoptively transferred EAE. The data presented in Table II demonstrate that (LEW X BN)F_1 -> BN bone marrow chimeras developed clinical signs of disease

after transfer of LEW cells similar to those seen with syngeneic transfer of BP-activated LEW spleen cells. In contrast, transfer of allogenic (ACI) BP-activated spleen cells to these bone marrow chimeras did not result in clinical or histopathologic signs of EAE. However, (ACI X LEW)F_1 ->LEW bone marrow chimeras developed clinical disease when infused with BP-reactive ACI-derived spleen cells. In all cases, histologic disease routinely associated with clinical EAE (8) was evident in animals that had exhibited signs of clinical disease (results not shown).

The chimeras used in this study were designed to test the potential influence of non-bone marrow-derived cells on the development of adoptively transferred EAE. Endothelial cells isolated from the cerebral vasculature express Ia when isolated from SJL mice exhibiting clinical EAE, but Ia^+ endothelial cells are not found in similar preparations from normal syngeneic mice (1). In guinea pigs immunized with BP, endothelial cells become Ia^+ just before disease onset (9). However, in (Strain 2 X Strain 13)F_1 hybrids, only the high-responder strain 13 haplotype is expressed on endothelial cells of BP-sensitized guinea pigs (10). Large numbers of Ia^+ cells are also found in multiple sclerosis brain lesions, especially in the peripheral areas of the expanding plaque (11). These observations have led some investigators to suggest that endothelial cells may have a relevant role in antigen presentation in vivo (9,12), making them an active and central participant in the expression of antigen-specific delayed-type hypersensitivity responses (13) in general and in the development of autoimmune response to antigens of the central and peripheral nervous tissue.

Our results question the need for MHC-restricted antigen presentation by endothelial cells *in vivo*. Table I shows that LEW donor

cells transfer clinical EAE only to recipients that share the LEW MHC and that the (LEW X BN)F_1 recipient develops a severity of clinical disease similar to that seen with syngeneic recipients. In recipients that develop disease, antigen presentation could be the responsibility of endothelial cells or astrocytes as well as bone marrow-derived cells. However, chimeric recipients of the (LEW X BN)F_1->BN construct would possess non-bone marrow-derived cells allogenic to the transferred LEW spleen cells. Since Table II shows that these chimeric recipients develop and recover from clinical disease with the same kinetics as syngeneic recipients, any MHC-restricted antigen-presenting function by these non-bone marrow-derived cells is not required for the development of adoptively transferred EAE.

We have previously shown (7) that rats which have recovered from adoptively transferred EAE can serve as a source of transfer-active cells and that this transfer-active population is derived from the original donor cell inoculum. It is evident from the data presented in Table II that recovery from adoptively transferred clinical disease in the BN chimeras is temporally consistent with recovery after syngeneic transfer of clinical disease. Furthermore, spleen cells obtained from chimeric recipients of transfer-active LEW cells can serve as a population of transfer-active cells (Table III). Notably, serial transfer of EAE by this cell population is still dependent on *in vitro* activation by spleen cells. The recipient combinations exhibiting successful serial transfer of clinical disease also suggest that BP-specific LEW cells survive in the chimeric environment and can subsequently transfer clinical disease to LEW recipients. In contrast serial transfer to BN or ACI recipients was not successful. These latter results also support our previous observations (7) that the

development of adoptively transferred EAE does not result in the recruitment of BP-reactive cells derived from the recipient's lymphoid compartment.

If the endothelium does not play an active, antigen-presenting role in the development of EAE, how then does the inflammatory lesion develop? The ability to transfer EAE to a recipient in which the endothelium is allogenic to the transferred cells may be explained by an interaction of T cells, T cell products, and mast cells (14-17). In support of this model we (18) and others (19-22) have reported that the clinical signs of EAE can be prevented in animals that have received compounds that alter mast cell release of histamine and serotonin.

In addition to lending indirect support of the Askenase-Loveren model of DTH (17), our results would also suggest that the clinical development of EAE is not caused by a direct interaction of MHC-restricted cytotoxic cells and the central nervous system (CNS) tissue. The response of chimeric recipients to the transfer of BP-specific lymphocytes (Table II) indicates that the presence of semisyngeneic F_1 bone marrow-derived accessory cells are sufficient for disease induction, even though the transferred lymphocytes are allogenic to the CNS. In these donor-recipient combinations the only possible MHC compatibility is with the transferred lymphocytes and the established bone marrow. Non-bone marrow-derived CNS tissue would not display MHC compatibility with the transferred lymphocytes. Consequently, MHC-restricted cytotoxic cells would not be expected to function in the development of clinical disease.

Our observation that the endothelium need not be MHC compatible with BP-specific lymphocytes to have these cells function *in*

vivo may be relevant to general considerations of the role of Ia^+, non-bone marrow-derived cells and the antigen-presenting requirement for delayed-type hypersensitivity responses. It is probable within the syngeneic system that all Ia^+ cells, independent of origin, are involved in antigen presentation at some point in the immune response. However, the results of our studies would argue that any antigen-presenting function of endothelial cells *in vivo* is secondary to that of bone marrow-derived cells and that the development of EAE is not influenced by the lack of antigen presentation, or of compatible Ia^+ expression, by cells of the endothelial barrier.

SUMMARY

The adoptive transfer of clinical and histopathologic signs of experimental allergic encephalomyelitis (EAE) requires MHC compatibility between cell donor and cell recipient. The results of adoptive transfer studies using F_1 to parent bone marrow chimeras as recipients of parental-derived BP-sensitive spleen cells indicate that this restriction is not expressed at the level of the endothelial cell but is confined to the cells of bone marrow derivation. Furthermore, these results indicate that the development of EAE is not dependent on the activity of MHC-restricted cytotoxic cells.

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Table I

MHC Restriction of Adoptively Transferred EAE

Donor[a]	Recipient[b,c]	Nonirradiated		Irradiated[d]	
		Disease/Total[e]	Symptoms[f]	Disease/Total[e]	Symptoms[f]
LEW	LEW	8/8	2.7	6/6	2.8
	BN	0/4	0.0	0/4	0.0
	BUF	0/4	0.0	0/4	0.0
	F-344	0/6	0.0	6/6	2.7
	(BN X LEW)F_1	6/6	2.6	5/5	2.4
	(ACI X LEW)F_1	7/7	2.8	6/6	2.6
(ACI X LEW)F_1	(ACI X LEW)F_1	6/6	2.4	5/5	2.6
	LEW	0/7	0.0	6/6	2.7
	ACI	0/5	0.0	6/6	2.2
	BN	0/4	0.0	0/4	0.0

a) Spleen cells were obtained from donors 12-14 days following BP-CFA immunization. Cells were cultured in the presence of BP for 72 hours and were subsequently transferred to recipients.

b) Recipients received 2×10^7 cells.

c) MHC haplotypes of recipient animals are as follows: LEW, RT-1^l ; F-344, RT-1^{lvl} ; BN, RT-1^n ; BUF, RT-1^b.

d) Irradiated recipients received 900-1000 rads of irradiation 24 hrs prior to cell transfer.

e) Number of animals with clinical signs of disease/total animals per group; clinical disease indices are described in materials and methods.

f) Average maximum clinical signs that develop following disease onset.

Table II

Adoptive Transfer EAE in Bone Marrow Chimeras

Donor[a]	Recipient[b]	Disease/Total[c]	Symptoms[d]	Disease[e] Onset	Disease[f] Recovery
LEW	LEW	6/6	2.6	5.2	8.1
LEW	(LEW X BN)F_1->BN bone marrow chimera	11/11	2.7	5.4	8.5
LEW	BN	0/4	0.0	---	---
ACI	ACI	4/4	2.2	5.5	8.2
ACI	(ACI X LEW)F_1->LEW bone marrow chimera	5/6	2.4	5.3	8.3
ACI	(LEW X BN)F_1->BN bone marrow chimera	0/3	0.0	---	---

a) Spleen cells were obtained from donors 12-14 days following BP-CFA immunization. Cells were cultured in the presence of BP for 72 hours and were subsequently transferred to recipients.

b) Recipients received 2×10^7 cells.

c) Number of animals with clinical signs of disease/total animals per group; clinical disease indices are described in materials and methods.

d) Average maximum clinical signs that develop following disease onset.

e) Average time of onset (days) of grade 2 clinical signs.

f) Average time (days) at which recovery from clinical disease was complete.

Table III

Persistence of Donor Derived BP Sensitive Cells in Bone Marrow Chimeras

Donor[a]	Primary Recipient[b]	Secondary Culture[c]	Secondary Recipient	Disease[d]/Total	Symptoms[e]
LEW	(LEW X BN)F_1->BN bone marrow chimera	BP	LEW	4/4	2.2
	(LEW X BN)F_1->BN bone marrow chimera	BP	BN	0/4	0.0
	(LEW X BN)F_1->BN bone marrow chimera	---	LEW	0/4	0.0
LEW	(ACI X LEW)F_1->ACI bone marrow chimera	BP	LEW	4/4	2.4
	(ACI X LEW)F_1->ACI bone marrow chimera	BP	ACI	0/3	0.0
	(ACI X LEW)F_1->ACI bone marrow chimera	BP	ACI_{irr}	0/4	0.0

a) Spleen cells were obtained from donors 12-14 days following BP-CFA immunization. Cells were cultured in the presence of BP for 72 hours and were subsequently transferred to recipients.

b) Recipients received 2×10^7 cells.

c) Spleen cells were obtained from 1^o recipients 10-21 days after recovery from the initial episode of clinical disease. The cells were cultured with BP for 72 hrs prior to transfer to 2^o recipients.

d) Number of animals with clinical signs of disease/total animals per group; clinical disease indices are described in materials and methods.

e) Average maximum clinical signs that develop following disease onset.

MANUSCRIPT #3

Title: The Role of Mast Cells in the Elicitation of Experimental Allergic Encephalomyelitis

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Abbreviations used in this paper:

EAE, experimental allergic encephalomyelitis; BP myelin basic protein, EP, encephalitogenic peptide; pen penicillin; strep, streptomycin; MAO, monoamine oxidase.

ABSTRACT

Experimental allergic encephalomyelitis (EAE), a T-cell mediated autoimmune disease can be transferred with lymphoid cells from actively immunized rats into naive recipients. In the mouse, previous studies have suggested a role for histamine/serotonin in the development of active EAE. We have found that myelin basic protein-reactive cells transfer a biphasic skin test response to naive rats analogous to what has been described in the mouse contact dermatitis system, where mast cell sensitization by Ag-specific T cell factors is required for the induction of skin test responses. Treatment of passive transfer recipients with the serotonin receptor antagonists, cyproheptadine or methysergide, blocked or significantly reduced the development of EAE. Furthermore, it was found that treatment with cyproheptadine was effective in blocking clinical disease when administered day 3 to day 6 after cell transfer. In contrast, cyproheptadine treatments before induction of paralysis day 0 to day 3, failed to alter the course of clinical disease. The inhibitor of mast cell degranulation, proxicromil, was also found to effectively block the elicitation of adoptively transferred EAE and was found to be effective when administered just before the onset of clinical disease. Reserpine, a compound known to deplete mast cells of their stored vasoactive amines by forcing granule contents into the cytoplasm where they are degraded by cell enzymes, was also effective in blocking both active and adoptively transferred EAE. Disease inhibition was found to be partially reversed with pargyline, and inhibitor of monoamine oxidase. In addition lymphocytes from treated animals were capable of transferring disease to

naive recipients and appeared to have normal activity as assessed by Ag or mitogen-driven proliferation in addition to IL-2 production.

INTRODUCTION

EAE is an autoimmune inflammatory disease which clinically progresses in rats to severe hindquarter paralysis. The disease develops following immunization with CNS tissue or purified CNS Ag, primarily BP (1).

Development of EAE is dependent on activation of BP reactive T-cells (2), which presumably recognize autologous BP within the CNS of the immunized rat (3). The ensuing immune response directed against BP leads to mononuclear cell infiltration of the CNS, particularly in the lumbar and thoracic regions (4). The inflammatory response triggered by BP reactive lymphocytes leads to increased vascular permeability and fibrin deposition which may correlate more closely with clinical EAE than the cellular infiltration (5). Disease-associated breakdown of the vasculature surrounding the CNS has led some investigators to propose that endothelial cells may play a significant role in Ag presentation and this interaction with BP reactive T cells could facilitate the apparent changes in vascular permeability (6,7). However, in previous studies we have presented findings which argue against a MHC-restricted, Ag-presenting role for the vascular endothelium during the development of EAE (8).

In the mouse model of contact dermatitis the cell-mediated response to the sensitizing Ag has been reported to be dependent on interactions between Ag-specific T cells, soluble T cell factors and mast cells (9). Mast cell involvement and subsequent release of vasoactive amines have been proposed to play a role in the development of actively induced EAE in the mouse (10). Localized release of vasoactive amines

would be consistent with the reported changes in vascular permeability (11) and with our previous report demonstrating the lack of MHC restriction at the endothelial level in adoptively transferred disease (8). In this study, we present evidence supporting the premise that mast cells have as active role in the elicitation of EAE.

MATERIALS AND METHODS

Animals. Inbred Lewis female rats were obtained from both Harlan Sprague Dawley (Indianapolis, Indiana) and the Laboratory Animal Resources Center (Washington State University, Pullman, WA). Animals were housed under standard conditions and offered feed and water ad libitum.

Purification of basic protein. Guinea pig brains were purchased from Pel Freez Biologicals (Rogers, AK) and stored at -70° until extracted for BP. Extraction of the BP from the neural tissue was carried out using a modification of a previously reported method (12). The neural tissue was homogenized in a Waring blender (Waring Products Div., New Hartforf, CT.) and suspended in a chloroform-methanol solution at 4°C to extract lipid material from the tissue. Further removal of undesirable material was accomplished by suspending the tissue in acetone at 4°C. The BP was then extracted at pH 3.0 from the tissue homogenate lyophilized, and stored at -20°C. Further purification of myelin BP was achieved by FPLC (Pharmacia Fine Chemicals, Piscataway, NJ) ion exchange chromatography . Approximately 300 mg of lyophilized extract was suspended in 50 ml of buffer A (2M urea, 0.08M glycine pH 10.4) and applied to a Mono-Q 10/10 column (Pharamacia) previously equilibrated with the buffer. The column was then eluted at 4 ml/min for 30 min with buffer A, followed by a gradient of 0% to 100% buffer B (2M urea, 0.08M glycine, 0.5 M NaCl, pH 10.4) over 60 min. BP was found to elute at approximately 30% buffer B, with a purity of greater than 95% as determined by SDS polyacryamide gel electrophoresis.

Production of encephalitogenic peptide. A peptide of BP sequence 68-86 (YGSLPQKSQRSQDENPV), previously reported to be encephalitogenic (13), was synthesized by the Merrifield solid phase method on a manual synthesizer. Purification of the peptide was accomplished by gel filtration with Sephadex G-50 (Pharamacia), followed by C_{18} reverse phase chromatography. The amino acid sequence of the synthesized peptide was confirmed by Edman degradation with an Applied Biosystems (Foster City, CA) 470A protein seqencer.

Induction of EAE. EAE was induced by a single injection of 50 μg of guinea pig BP emulsified in CFA, containing 10 mg/ml of heat-killed Mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, MI). A total of 0.1 ml of the BP/CFA emulsion was divided equally between the two front footpads.

Clinical evaluation. Rats used in adoptive transfer experiments were evaluated daily for clinical signs of neurologic impairment. Animals were graded as follows; a flaccid tail was given a grade 1; hindquarter weakness, characterized by an abnormal gait was given a grade 2; hindquarter paralysis was given a grade 3; while complete hindquarter paralysis with incontinence was graded as a 4.

Adoptive transfer of EAE. Spleens cell suspensions were obtained from Lewis animals injected 12 days earlier with BP-CFA. The cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 U/ml pen, 100 μg/ml streptomycin (strep), and 5×10^{-5} molar 2-ME, at a concentration of 2×10^6 cells/ml in $75cm^2$ tissue culture flasks (Falcon 3023, Becton Dickinson, Oxnard, CA). The cells were stimulated during the in vitro culture period with BP at 1 μg/ml and maintained in a humidified 5% CO_2 - 95% air atmosphere at 37°C.

After a 3 day culture, the cells were collected, washed twice and viability determined by dye exclusion. Cell recipients received 2 X 10^7 viable cells injected via the tail vein.

For transfer into secondary recipients, day 7 spleen cells from primary recipients were placed into the culture conditions previously described. Cell recipients received 2 X 10^7 viable cells injected via the tail vein.

Lymphocyte proliferation assay. Spleen cells were assessed for their ability to respond to specific Ag and T cell mitogens by a standard lymphocyte proliferation assay. Log 10 dilutions of the Ag PPD and BP in addition to the mitogen Con A ranging from 20 to .02 µg/ml were made in RPMI supplemented with pen/strep and plated in triplicate into microtiter plates (Vangard Int'l, Portland, OR), at 100 µl/well. Spleen cells were suspended in RPMI supplemented with 10% FCS, pen, strep, and 1.0 X 10^{-7} M 2-ME. A 100 µl volume of the spleen cell suspension was then added to the microtiter wells with the dilutions of Ag or mitogen. The plates were allowed to incubate for 72 h in a humidified 5% CO_2 - 95% air atmosphere incubator at 37°C. For the final 4 h of the culture the spleen cells were pulsed with [^{3}H]-TdR (New England Nuclear, Boston, MA), 1 µCi/well. Cells were harvested using a Titertek 550 Cell Harvester (Flow Laboratories, McLean, VA). Samples were counted in scintillation cocktail, 6 g/M PPO, 0.1 g/M POPO dissolved in toluene and counted on a Packard model 3255 liquid scintillation counter.

IL-2 assay. IL-2 production was assessed by placing spleen cells in culture with RPMI supplemented with 5% FCS, pen/strep and stimulated with Con A at 1 µg/ml for 24 hours at 37°. The cells were

removed from the supernatant by centrifugation and assayed for IL-2 with HT-2 cells, a IL-2 dependent cell line. Doubling dilutions of the cell supernatant were made in microtiter plates using RPMI with each well containing 100 µl. To each well 100 µl of HT-2 cells suspended at 6 X 10^6 cells/ml in RPMI supplemented with 10% FCS, pen/strep and 2-ME were added to each well. The assay was allowed to go for 24 hours with 1 µCi of [^{3}H] TdR being added to each well for the final four hours. After sample preparation and counting, the units of IL-2 were determined where 1 U was equal to the dilution of supernatant which produced half the maximum proliferative response.

Skin test responsiveness. DTH reactivity to BP or to EP was quantitated using a skin test reaction, where 10 µl of a BP solution was injected under the skin of the ear. In all cases the left ear was injected with BP or EP, whereas the right ear was used as a control and was injected with an equal concentration of the irrelevant Ag, human serum albumin. The ear thickness was measured prior to the skin test and at regular intervals using a spring loaded caliper (Ralmikes, Plainfield, NJ.) whereas the rats were anesthetized with ether. The skin test response was reported as the net increase in ear thickness.

Treatment with pharmacologic agents. Cyproheptadine, and pargyline, were obtained from Sigma Chemical Company (St. Louis, MO). Reserpine (Serpasil) was obtained from CIBA (Summit, NJ). Methysergide was a gift from Sandoz Inc. (Hanover, NJ) and proxicromil was a gift from Fisons Corporation (Bedford, MA). Stock solutions of cyproheptadine, methysergide and proxicromil were prepared daily in saline at the concentration of 50 mg/ml. Treated rats were given indicated dosages of pharmacologic agents by i.p. injection, and control

rats were given a equal volume of saline. Then 5 mg/kg of the stock reserpine solution was injected i.p.

Histology. Sections of spinal cord were removed from recipients at the height of disease. After fixation sections were stained with hemotoxylin-eosin and assessed for cellular infiltration as previously described (8).

RESULTS

Previous reports in the mouse model of contact dermatitis (14), have shown that delayed-type hypersensitivity reactions are biphasic skin test responses with an early component dependent on the release of mediators from tissue mast cells. As Table 1 shows, recipients of BP-cultured spleen cells show a positive skin test response to BP at 24 and 48 hrs. Skin test sites in these recipients were also monitored immediately after challenge to determine if an "early" response could be detected in the EAE system. In all recipients there was an early swelling event which peaked at 1 to 2 hours and was not attributable to the volume of fluid that was injected. We also found that the response of naive animals to skin tests with BP elicited an early swelling response. The early response to BP in cell recipients was always larger than the response to BP seen in naive animals. However, variability of response and high background values in the naive group caused probability values to exceed $P > 0.05$ for all measurements made earlier than 20 h.

It has been reported that BP, perhaps due to its charge characteristics, can directly cause mast cell degranulation (15). To potentially reduce this general effect and the background skin test response in naive animals we used an EP in order to evaluate the early skin test response. In these recipients we also observed early skin test responses at the 1- and 2-h measurement times. These responses were significant at the $P < 0.05$ and $P < 0.001$ values respectively.

Effects of vasoactive amine antagonists on passive EAE. The biphasic skin test response seen in recipients of BP-activated cells is

consistent with findings in other model systems where mast cell activation is required for induction of delayed type hypersensitivity (17,18). To determine if mast cell release of vasoactive amines may play a role in the elicitation of clinical EAE, recipients were treated with the serotonin antagonist methysergide at a dosage of 50 mg/kg administered twice daily. Treatments beginning either at the time of cell transfer or 3 days after cell transfer were found to significantly reduce disease severity, although complete disease inhibition was infrequent (Table II).

A second vasoactive amine antagonist, cyproheptadine, was also assessed for its capacity to block EAE in the passive transfer model. Cyproheptadine treatment was found to completely arrest the development of disease when administered daily following cell transfer (Table II). Administration of cyproheptadine could be delayed until three days post cell transfer and still remain completely effective (Table III). However, treating recipients for a period of 3 days commencing at the time of cell transfer did not alter the course of clinical disease.

Effects of inhibitors of mast cell degranulation on passive EAE. The cromolyn derivative, proxicromil, which blocks mast cell degranulation was also tested for its influence on adoptively transferred EAE. Concentrations of proxicromil which had effectively blocked skin test reactions in the murine contact dermatitis system (18), also prevented clinical EAE (Table III). Histologic sections taken from treated recipients lacked the perivascular infiltration associated with the disease (data not shown). As with cyproheptadine, initiating proxicromil treatments just prior to the onset of disease effectively blocked disease

induction. Treatments stopped prior to disease onset failed to alter the course of clinical disease (Table III).

Serial transfer using proxicromil treated recipients as donors. Clinical EAE can be serially transferred from primary recipients into secondary recipients provided that BP is used to drive the required intervening in vitro activation step (20). Serial transfer of EAE was used to further evaluate the possibility that proxicromil treatments had exerted a direct influence on effector cell function in the treated recipients. Passively transferred disease was blocked in primary recipients with proxicromil treatments on days 3-7, at which time these animals were used as donors for adoptive transfer into naive recipients. As shown in Table IV, secondary recipients were found to undergo an episode of clinical disease similar in magnitude to what was seen in recipients of control donors, an observation consistent with proxicromil inhibition of mast cell function in the expression of EAE rather than an alteration of lymphocyte function.

Inhibition of EAE using reserpine. Reserpine, a plant alkaloid, has been found to effectively inhibit cell mediated responses in a number of experimental systems, including experimental allergic neuritis (20). It has been proposed that this drug also mediates its effect on delayed-type hypersensitivity by impairing mast cell function (21). Exposure of mast cells to reserpine causes vasoactive amines such as serotonin and histamine to be forced from storage granules into the cell cytoplasm where they are rapidly degraded by the enzyme MAO. To establish the activity of reserpine in the EAE model, we treated cell recipients as well as actively immunized rats at various time points with reserpine (Table

V). The results demonstrate that reserpine effectively inhibits both active and passive EAE. In the active system EAE can be blocked with reserpine treatments beginning as late as day 6, while a single treatment on day 3 is sufficient to block adoptively transferred EAE, indicating that the drug exerts its activity at the effector stage of the response.

Reserpine does not inhibit effector cell function. Within hours after injection profound systemic side effects of reserpine can be seen in the treated rats. Treated animals fail to eat or drink but instead huddle in the corner of the cage unresponsive to touch. The severity of these side effects caused by the reserpine treatment created concern as to whether observed inhibition of EAE could be attributed to activities other than depletion of vasoactive amines from mast cells (22). To insure that lymphocyte function had not been impaired in the treated animals, adoptive transfer experiments were conducted with reserpine-treated donors. BP/CFA immunized donors receiving 5 mg/kg reserpine on days 6, 8 and 10, failed to develop any of the symptoms associated with EAE. A group of donors were similarly treated with reserpine and used as spleen cell donors on day 12. After the standard 72 hour culture with BP the spleen cells from these animals were found to effectively transfer EAE into naive recipient animals (Table VI).

Further support for full effector cell capacity in reserpine-treated rats was derived from lymphocyte proliferation assays. Treating immunized rats with reserpine at levels which blocked active disease did not significantly influence the capacity of spleen cells to respond to specific Ag or the T cell mitogen, Con A (results not shown).

Reversal of reserpine's effects with pargyline. The action of reserpine on mast cells can be partially reversed with the use of pargyline, an inhibitor of MAO (23). To further support the observation that reserpine blocks EAE by interfering with the effector function of mast cells, pargyline was administered to cell recipients in an attempt to counteract the effects of reserpine and allow for the expression of EAE in the recipient animals (Table VII). Pre-treatment with pargyline was found to partially reverse the inhibitory effect of reserpine and allow the development of clinical disease in the passive transfer system. This is consistent with the known action of pargyline, because MAO would have to be inactivated before the administration of reserpine for the drug to prevent the degradation of vasoactive amines in the mast cells.

DISCUSSION

In the Lewis rat model of EAE a single injection of BP emulsified in CFA provides the signals necessary to evoke a T cell response to BP which leads to the subsequent development of EAE. Although the cascade of events leading to apparent disease remains unclear it is presumed that once activated, this effector cell population infiltrates the CNS causing inflammation and accompanying fibrin deposition, which results in an acute, ascending paralysis, characteristic of the disease (24). Current evidence involving mAB depletion of lymphoid cell subpopulations and successful transfer with cloned T cell lines expressing helper cell markers, indicates that the clinical paralysis and histologic lesions seen in EAE are apparently the result of a delayed type hypersensitive immune response. However, it is not clear how BP-reactive lymphocytes are allowed to traffic from circulation into the CNS through the highly restrictive blood brain barrier.

The expression of Ia on the surface of endothelial cells (6) and electron micrographs showing intimate contact between Ag-primed endothelial cells and lymphocytes (25) argues in favor of an MHC-restricted endothelial cell regulation of cell trafficking. However, these findings are not consistent with our recent observations of adoptive transfer of clinical EAE into chimeric animals (8). To explain these later results an alternative mechanism must be proposed to allow lymphocytes to cross endothelial barriers in a non-MHC-restricted manner. A model by Askenase and Van Loveren (9), involving a T cell factor that works in conjunction with tissue mast cells to facilitate effector cell trafficking into Ag sites provides an explanation for cellular infiltration into the CNS.

This model proposes that a T cell subset produces an Ag-specific factor which can bind to tissue mast cells (26). Sensitized mast cells stimulated by free Ag then undergo a slow release of vasoactive amines, leading to increased endothelial cell permeability (27). The circulating T cell population is then able to move from circulation into the tissues where subsequent contact with Ag presumably upregulates lymphokine production to attract circulating monocytes to the site.

A similar mechanism involving mast cell degranulation offers an explanation for cell infiltration of the CNS during the onset of EAE. Activation and subsequent release of vasoactive amines from mast cells within the CNS would be expected to alter the integrity of blood brain barrier, facilitating lymphocyte permeation of the CNS. In support of this model, I^{125} labeled proteins which would normally be excluded from the CNS, permeate the endothelial barrier surrounding the CNS during the onset of disease (28). Furthermore, it has recently been reported that a very small percentage of the lymphocytes found in the perivascular infiltrate are actually reactive towards BP (29). These finding suggest that a generalized increase in vascular permeability takes place before to the onset of clinical EAE.

The existence of a biphasic skin test response (Table I) in cell recipients of culture-activated spleen cells is consistent with mast cell contribution to the developing skin test. However, the issue of the mast cell as a principle component in the expression of clinical disease can only be indirectly ascertained by the in vivo use of pharmacologic agents which alter mast cell activity. Thus reserpine, a molecule that effectively depletes mast cells of vasoactive amines was found to effectively inhibit both active and adoptively transferred EAE in the Lewis rat (Table V)

Although reports of reserpine inhibition of lymphocytes function exists (22), reserpine does not appear to block EAE by altering lymphocyte activity. Comparison of the lymphocyte populations obtained from control and reserpine treated donors in Table V has demonstrated no significant differences in IL-2 production, lymphocyte proliferation, and adoptive transfer potential, which could account for the inhibition of disease in reserpine-treated rats. The capacity of pargyline to reverse the action of reserpine thus allowing for the onset of EAE as seen in Table VII, lends further support to the hypothesis that mast cell products play a role in disease elicitation.

Other pharmacologic agents which impair mast cell release or vasoactive amine activity were also found to be effective in inhibiting the expression of clinical disease in cell recipients. Blocking histamine and serotonin receptors by the vasoactive amine antagonist cyproheptadine (Table II and III), was found to completely block clinical symptoms in recipient animals. Another serotonin antagonist, methysergide, was found to be reduce but not inhibit clinical symptoms. The inability of methysergide to completely block adoptively transferred disease may in part be attributed to structural similarities it shares with serotonin. In blocking the serotonin receptor methysergide may actually activate the serotonin receptor and produce many of the same effects as serotonin which could reduce the effectiveness of the treatment, explaining why cell transfer is rarely blocked by this agent even at high dosages (30). Alternatively, heterogeneity in serotonin receptors may account for disparate effects of serotonin inhibitors on the induction of EAE (31). This difference in disease inhibition could also be attributed to the fact

that methysergide is only an anti-serotonin whereas cyproheptadine is both anti-serotonin and anti-histamine.

Although the pharmacologic agents used in this study alter mast cell activity, there is the concern that lymphocyte function in vivo may also be modified. Support for alteration of mast cell activity and not lymphocyte function comes from the results of both time course experiments and serial transfer experiments (Tables III to V). With both proxicromil and cyproheptadine, complete disease inhibition was observed when treatments began as late as day 3 after cell transfer, 24 h before the onset of clinical disease. When similar treatments were initiated at the time of cell transfer and terminated before the onset of paralysis, recipients develop severe clinical disease, indicating that the in vivo activity of transferred lymphocytes was not influenced by the various pharmacologic treatments. One may speculate that the in vitro environment that we use for the development of transfer-active cell allows recovery from the in vivo drug-induced inhibition. This is unlikely because the kinetics of IL-2 production, proliferation and adoptive transfer activity are virtually identical in drug-treated and control groups.

It is likely that development of clinical EAE requires an alteration in the endothelial barrier surrounding the CNS. The mast cell release of vasoactive amines could contribute to increased vascular permeability. The demonstration of Ag-specific T-cell factor sensitization of mast cells in other models of cell-mediated responses may explain the proposed involvement of mast cells in the rat model of EAE. Increased sensitivity of mast cells armed with a T cell-derived factor specific for BP could trigger increased vascular permeability after contact with BP present

from normal turnover of membrane proteins. This mast cell release of vasoactive amines then provides a mechanism for lymphocyte egress into the CNS in a non-MHC-restricted manner. Collectively these results would argue against a direct lymphocyte effect and would support our premise that mast cells play a key role in the elicitation EAE.

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TABLE I. Skin test response to Myelin Basic Protein and to an Encephalitogenic Fragment[a]

Whole BP		
	Cell Recipient	Control
1 hr	18.3 ± 3.6	12.3 ± 2.1
2 hr	14.2 ± 4.3	9.0 ± 1.4
4 hr	6.1 ± 0.5	5.8 ± 2.2
24 hr	24.2 ± 5.9*	2.1 ± 1.4
36 hr	10.5 ± 3.8*	2.8 ± 1.8
Encephalogenic Fragment		
	Cell Recipient	Control
1 hr	14.3 ± 2.8*	8.5 ± 3.5
2 hr	7.8 ± 1.8*	3.5 ± 1.7
4 hr	4.3 ± 2.3	3.0 ± 1.5
8 hr	6.5 ± 3.3	4.7 ± 4.5
12 hr	10.3 ± 2.7	4.0 ± 3.2
24 hr	14.0 ± 2.5*	5.5 ± 4.0
36 hr	12.7 ± 2.8*	4.5 ± 2.0

[a]Recipients of 2×10^7 culture activated spleen cells were skin tested three days following cell transfer. Basic protein or encephalogenic peptide was injected intradermally into the ear of cell transfer recipients or naive controls. The ears of both test groups were measured at regular intervals with the measured thickness being subtracted from

the ear thickness prior to the skin test to give: Increase in Ear Thickness (Units X 10^{-3} cm) presented as mean $\pm$ one std deviation.

*Skin test response different from control $p \leq 0.05$

TABLE II. Effects of serotonin antagonists on the adoptive transfer of EAE[a]

Treatment	Sick/Group	Disease Severity[b]
Control	8/8	2.6 ± 1.2
Methysergide (50 mg/kg - twice daily days 0-7)	2/3	1.0 ± 0.0
Methysergide (50 mg/kg - twice daily days 3-7)	6/8	1.1 ± 0.5
Cyproheptadine (15 mg/kg - daily, days 0-7)	0/6	0.0 ± 0.0

[a]Recipients received 2×10^7 spleen cells cultured for 72 hours with 1 μg/ml BP.

[b]Disease severity of rats showing symptoms of EAE, graded on a 1-4 scale and presented as mean ± one std deviation.

TABLE III. Time dependent influence of cyproheptadine and proxicromil in the inhibition of EAE.

Treatment	Sick/Group[a]	Disease Severity[b]
Cyproheptadine (15 mg/kg twice daily)		
Control	6/6	3.6 ± 0.5
Days 0 to 7 post cell transfer	0/7	0.0 ± 0.0
Days 0 to 3 post cell transfer	4/4	3.0 ± 0.0
Days 3 to 7 post cell transfer	0/6	0.0 ± 0.0
Proxicromil (50 mg/kg - twice daily)		
Control	9/9	3.1 ± 0.7
Days 0 to 7 post cell transfer	0/8	0.0 ± 0.0
Days -1 to 2 post cell transfer	4/4	2.5 ± 1.1
Days 0 to 3 post cell transfer	2/3	2.5 ± 0.5
Days 3 to 6 post cell transfer	0/7	0.0 ± 0.0

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 µg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

TABLE IV. Serial transfer using proxicromil treated donors

Donor Treatment	Sick/Group[a]	Disease Severity[b]
Proxicromil - Days 0 to 7 post cell transfer (50 mg/kg - twice daily)		
Recipient of 2 x 10^7 cells	4/4	1.7 ± 0.5
Control		
Recipient of 2 x 10^7 cells	3/3	2.3 ± 0.9

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 μg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

TABLE V. Use of reserpine to block EAE

Treatment[a]	Sick/Total[b]	Disease Severity[c]
Actively Induced EAE		
Controls	7/7	2.6 ± 0.53
Day 6	2/3	1.5 ± 0.71
Day 8	6/7	1.7 ± 0.81
Days 6 and 8	1/3	1.0 ± 0.0
Days 6,8 and 10	2/7	1.0 ± 0.0
Days 6,8,10 and 12	0/3	0.0 ± 0.0
Passively Induced EAE		
Controls	4/4	4.0 ± 0.0
Day 3	0/6	0.0 ± 0.0

[a]All animals received 5 mg/kg Reserpine on the indicated days following injection with 50 mg BP emulsified in CFA.

[b]Number of animals which showed symptoms of active EAE as a function of the total number of animals in the group.

[c]Disease severity of the rats which show symptoms of EAE ± one standard deviation. Rats were graded on a scale of 1-4

TABLE VI. Assessment of reserpine on the development of transfer active cells

Donor	Recipients[a] Sick/Total	Disease Severity[b]
Control	4/4	3.25 ± 0.5
Reserpine[c] Treated Donors	9/9	2.90 ± 0.8

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 µg/ml BP.

[b]Data is presented as mean ± one std deviation.

[c]Reserpine treated donor animals received injections of reserpine 5 mg/kg on days 6, 8, and 10 following immunization. With BP/CFA none of the reserpine treated rats developed signs of clinical EAE. Control animals received injections of carrier on days 6, 8, and 10.

TABLE VII. Influence of pargyline on the reserpine-mediated inhibition of adoptively transferred EAE[a]

Treatment	Sick/Total	Disease Severity[b]
Control	3/3	4.0 ± 0.0
Reserpine[c] Treated	0/6	0.0 ± 0.0
Pargyline[d] - Reserpine (pargyline given 2 hr prior to reserpine)	0/3	0.0 ± 0.0
Pargyline - Reserpine (pargyline given 6 hr prior to reserpine)	4/5	1.5 ± 1.0

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 μg/ml BP.

[b]Disease severity of rats showing symptoms of EAE, graded on a 1-4 scale and presented as mean ± one std deviation.

[c]5 mg/kg of reserpine was given on day 3 following cell transfer.

[d]100 mg/kg of pargyline given prior to treatment with reserpine.

MANUSCRIPT #4

Title:

Mast Cell Proteases Liberate Stable Encephalitogenic Fragments from Intact Myelin

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Abbreviations:

EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; GP-MBP, guinea pig myelin basic protein; Rt- MBP rat myelin basic protein; CFA, complete Freund's adjuvant; BBB, blood brain barrier; RP-HPLC, reverse phase high pressure chromatography; OPA, ortho-phthalaldehyde; FMOC, 9-fluorenylmethylchloroformate.

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a paralytic autoimmune disease, induced by a single injection of Myelin Basic Protein (MBP), and mediated by lymphocytes responding to MBP found within the Central Nervous System (CNS). In the rat model of this disease guinea pig MBP (GP-MBP) is used for primary sensitization. Rat T cell lines, derived in response to GP-MBP, adoptively transfer clinical disease seemingly by recognition and cross reaction with rat MBP (Rt-MBP) from the myelin sheath of the cell recipient (1). This recognition results in the accumulation of mononuclear cells in the post capillary venules of the CNS. Cellular infiltration in conjunction with the accompanying edema and fibrin deposition leads to an acute ascending hindquarter paralysis in the animal (2).

The mechanism by which BP reactive T cells permeate the blood brain barrier (BBB) of the CNS is unclear. Studies exist which suggest that other events, preceding the onset of cellular infiltration into the CNS must take place for the induction of EAE (3,4). For example, mast cell degranulation is apparently required for the elicitation of EAE. Both inhibitors of mast cell degranulation and antagonists of vasoactive amines, have been found to effectively inhibit adoptively transferred EAE (5). Mast cell degranulation early in the development of EAE could result in the release of potent vasoactive amines which subsequently alter the BBB, allowing unrestricted entry of proteins and cells from the surrounding capillary beds into the CNS.

Although rodent mast cell granules contain large amounts of vasoactive amines , serine proteases are also major constituents of the

storage granules (6,7). In a previous report, the enzymes contained within mast cell secretory granules were found to exhibit significant myelinolytic activity (8) although the fate and activity of the resulting cleavage products was not determined. In this study we describe the capacity of rat mast cell enzymes to degrade proteins within the rat myelin sheath, and show that the sequence of MBP known to be encephalitogenic to the Lewis rat is cleaved from myelin but not subject to additional degradation. The encephalitogenicity of the peptides released following the proteolytic degradation of myelin was determined by active immunization and by stimulation of an encephalitogenic T cell line.

MATERIALS AND METHODS

Mast Cell Preparation. Mast cells were collected from the peritoneal cavities of Lewis Rat retired breeders by lavage with Tyrodes buffer containing 0.1% BSA. Mast cells were isolated from the resident peritoneal cell population by centrifugation over a 22.5% metrizamide gradient as previously reported (9). The purifed mast cells (90-95% mast cells) were resuspended in Tyrode's buffer at 1 x 10^4 cells/ml and stimulated to degranulate by incubating the cells for 30 min at 37 C in the presence of 10 μg/ml of compound 48-80. Supernatants from the stimulated mast cell preparations were then used to digest rat spinal cord myelin.

Myelin Treatment and Analysis. One ml of rat mast cell supernatant or Tyrodes buffer was added to 50 mg of purified rat myelin (10), which had been washed 3X in 0.15 M NaCl, 2mM $CaCl_2$ and 10 mM HEPES, pH 7.4. Following incubation for 3 hours at 37 C the undigested myelin was removed by centrifugation and the remaining supernatant was lyophilized and stored at -20 C until analysis. Samples were analyzed on a Vydac C4 wide pore column, using a Hewlet Packard 1090 HPLC. The separation was achieved using dH_2O/0.1% TFA (buffer A) and 70% ACN/0.1 % TFA (buffer B), in a gradient which consisted of 5% B for the first 5 min, followed by a linear gradient going to 35% B over the next 50 min. One minute fractions were collected during the course of the separation, lyophilized and stored at -20 C. Additional purification of the peptides was done with a Vydac wide pore C18 column using the following mobile phases A: 0.05 M Ammonium Acetate, pH 6.0, B: 40% 0.125 M Ammnium Acetate pH 6.0, 60% Acetonitrile. The separation

was done at 50 C with a flow rate of 1 ml/min using a linear gradient going from 10% B to 35% B over 40 minutes.

BP-3 Cell Line Stimulation. BP-3, is a highly encephalitogenic T cell line derived from lymph node cells of Lewis rats immunized with a synthetic peptide consisting of GP-MBP sequence 68-86, (11) (YGSLPQKSQRSQDENPV). At the time of the assays the BP-3 cell line had been maintained in continuous culture for 3 months by repeated stimulations with the 68-86 peptide, using a previously reported method (1). To determine if encephalitogenic peptides had been released during the digestion of myelin by mast cell enzymes, HPLC fractions of the digest were tested for the capacity to stimulate the proliferation of cell line BP-3. Individual fractions were plated in triplicate into 96 well microtiter plates. Fractions were sterilized by exposing the plate to UV light for 45 min. One hundred microliters of a suspension consisting of 5×10^4 BP-3 cells and 1×10^6 irradiated syngeneic thymocytes was then added to each well in stimulation media. The proliferation assay was incubated for 72 hours with 1 μCi of [^{3}H]-TdR being added to each well for the final 24 hours. Counts for the triplicate wells were averaged and expressed as a stimulation index.

Clinical Disease Assessment. Assessment of clinical signs of EAE was graded as follows: a flaccid tail was given a grade 1; hindquarter weakness, characterized by a abnormal gait and difficulty supporting the weight of hind quarters was given a grade 2; Complete hindquarter paralysis was given a grade of 3; Completely immobile rats with front quarter involvement was given a grade of 4.

Determination of Peptide Stability. Purified rat MBP fragment 69-88 was suspended at 100 μg/ml in 0.15 M NaCl, 2mM $CaCl_2$ and

10mM Hepes, pH 7.4 and mixed with a equal volume of supernatant from 1 x 10^5 mast cells/ml degranulated with 10 μg/ml compound 48/80. At thirty minutes, 25 ml of peptide suspension was analyzed by RP-HPLC using a Vydac wide pore C4 column. The analysis was done with mobile phases consisting of A: H_2O with 0.12% TFA, B: 70% acetonitrile with 0.1% TFA. With a flow rate of 1 ml/min, a linear gradient from 5% to 35% B, was run over 30 minutes. Under these conditions, purified rat MBP peptide 69-88 elutes at 12.282 minutes, figure 2a.

RESULTS

Purified rat myelin, following treatment with supernatants from activated rat mast cells yielded a number of HPLC-determined products absent in the control digestion, (Figures 1a and 1b). To determine if any released peptides contained the encephalitogenic region of Rt-MBP, fractions from the RP-HPLC separation were initially tested in proliferation assays using a MBP reactive T cell line, referred to as BP-3. A major peak of activity was found within HPLC fractions eluting at 22-24 minutes, figure 1c. The stimulatory activity appeared to coincide with a predominant peptide peak eluting at 23 minutes on the chromatograph, (see arrow figure 1b). This peak at 23 minutes was not detected in the control chromatograph, (figure 1a) and fractions from the control digest were unable to stimulate BP-3 cells to proliferate.

In larger scale preparations two peaks eluting at 21 and 23 minutes were routinely found to induce the proliferation of the BP-3 cell line. The two peptides were initially isolated by C4 RP-HPLC and further purified by C18 RP-HPLC. Following acid hydrolysis, the amino acid composition of the two peptides was determined by C18 RP-HPLC, using OPA/FMOC pre-column derivitization (12). The amino acid composition of the peptide at 21 minutes was consistent with the 69-87 sequence of Rt-MBP, (GSLPQKSQRTQDENPVV), while the peptide eluting at 23 minutes was consistent with the 69-88 sequence, (GSLPQKSQRTQDENPVVH).

The existence of these two similar peptides in the crude myelin digest suggested that the 69-88 peptide could be an intermediate which undergoes additional degradation to yield a peptide consisting of residues

69-87. To determine the stability of Rt 69-88, the purified peptide was subjected to high levels of mast cell derived enzymes. When purified Rt 69-88, shown in figure 2a, was exposed to the enzyme containing mast cell supernatants for 30 minutes a considerable amount of degradation had taken place resulting in a new peak with a retention time consistent with that for Rt 69-87, shown in figure 2b. After a two hour exposure to the mast cell supernatants, the Rt 69-88 peptide had been completely converted to Rt 69-87 (figure 2c), however, there was no evidence indicating that additional proteolytic activity was taking place. To confirm the stability of Rt 69-87, the purified peptide was incubated for 24 hrs in supernatants obtained from mast cells degranulated at a cell concentration of 10^6 mast cells/ml. No degradation of the Rt 69-87 sequence was detected. The amino acid composition of the peptide eluting at 10.9 minutes was consistent with Rt 69-87 (GSLPQKSQRTQDENPVV). These results indicate that histamine on the COOH terminus of the peptide is susceptible to enzymatic cleavage, but the remainder of the peptide, residues 69-87, is stable in the presence of the enzymes released by activated mast cells.

Within the encephalitogenic region of the MBP molecule, Rt-MBP differs from GP-MBP by a single amino acid substitution Table 1, which may account for the reduced encephalitogenicity of Rt-MBP (13, 14). Purified rat peptide 69-87 when emulsified in CFA and injected into naive Lewis rats was also found to be considerably less encephalitogenic than GP-MBP, GP 69-86 or even Rt-MBP. Nevertheless, immunization with Rt 69-87 did produce clinical disease in 40% of the immunized rats.

Encephalitogenic T cell lines maintained in IL-2 containing growth media, lose their ability to adoptively transfer clinical disease (15).

Stimulation of these cells with MBP or the encephalitogenic region of the molecule is required for the cells to regain the capacity to adoptively transfer clinical disease. After culture in IL-2 rich growth media, BP-3 cells were stimulated with either the original sensitizing peptide GPMBP (68-86), whole GP-MBP, whole Rt-BP or purified Rat 69-87. Following antigen induced activation, 3×10^6 BP-3 cells were transferred to naive recipient rats, as shown in Table 2. Cells cultured in the absence of MBP failed to adoptively transfer clinical disease. BP-3 cells stimulated with Rt-MBP and the Rt 69-87 fragment adoptively transferred maximum clinical disease in recipient rats. In addition, GP-MBP or GP 69-87 at concentrations as low as 0.1 μg/ml produced severe clinical disease in all transfer recipients.

DISCUSSION

The issue of in situ activation of MBP reactive cells within the CNS remains unclear. MBP is an integral membrane protein found in high concentrations in oligodendrocytes within the CNS. In some models of EAE, investigators found Ia positive cells, including endothelial cells near areas of cellular infiltration (16,17). It has been proposed that these cells could be presenting MBP to responsive T cells. However, it is not clear why MBP would be present in a form that allowed it to be pinocytosed, processed and expressed on the surface of Ia expressing cells. Our findings, which demonstrate that proteases stored within the mast cell secretory granules are capable of releasing an encephalitogenic peptide from MBP within the myelin sheath, may suggest a mechanism which results in the release of the encephalitogen. This proteolytic activity may explain the loss of MBP from myelin in the early stages of EAE (18).

Previous studies using MBP fragments prepared by enzymatic cleavage of purified GP-MBP found that amino acid residues 69-89 (GSLPQKSQRSQDENPVVHF) contained the entire encephalitogenic activity for the Lewis rat (19). Enzymatic removal of amino acids from the COOH terminus, to give peptide 69-85 (GSLPQKSQRSQDENP), reduced the encephalitogenic activity by an order-of-magnitude, as did removal of the NH2 terminus to give peptide 73-89 (QKSQRSQDENPVVHF). These observations lead Chou et al to conclude that the shortest sequence possessing the full encephalitogenic activity of GP-MBP was peptide 72-86 (PQKSQRSQDENPV) (19). Our results demonstrate that mast cell enzymatic activity releases a peptide from myelin corresponding to the Rt-MBP sequence 69-87. The release of this

encephalitogenic peptide from myelin may serve as an antigen source and thus influence the influx of MBP reactive lymphocytes into the CNS.

Rat peptide 69-87 produces modest clinical symptoms in actively immunized rats, and this peptide effectively activates MBP reactive T-cells. Although rat sequence 69-88 is susceptible to loss of the COOH terminal histamine, the resulting peptide 69-87, was resistant to further enzymatic activities contained within the mast cell granules. The stability of the rat 69-87 peptide lends support to the argument that the release of these peptides may by crucial in the activation of MBP reactive lymphocytes infiltrating the CNS during the course of EAE. Released peptide could be presented to MBP reactive T cells through direct or indirect association with antigen presenting cells and thus serve as a stimulus to MBP reactive T cells.

Although speculative, the release of MBP peptide fragments by mast cell proteases may suggest an additional role for the mast cell in the pathogenesis of EAE. The activity of these proteases may serve to explain why only certain regions of the MBP molecule are encephalitogenic. For instance, in the Lewis rat the encephalitogenic region of the molecule is contained within amino acid sequence 72-86. While the T cell repertoire of the Lewis rat may include T cells with specificities to other regions of the MBP molecule, these regions may not be liberated by mast cell enzymes at the onset of the disease. Alternatively other regions of MBP which could serve as T cell epitopes may be rapidly degraded in vivo, thereby preventing the activation of T cells responsive to these regions of the MBP molecule.

SUMMARY

Protease containing supernatants from activated rat mast cells were found to degrade purified rat myelin with a subsequent release of a stable encephalitogenic peptide. The two most abundant peptides were identified as resides 69-87 (GSLPQKSQRTQDENPVV) and resides 69-88 (GSLPQKSQRTQDENPVVH). While additional exposure to the mast cell supernatants removes the COOH terminal histamine from peptide 69-88 to yield peptide 69-87, additional proteolytic degradation of the 69-87 peptide was not detected. Immunization with this peptide emulsified in CFA caused the development of clinical EAE in Lewis rats. In addition this 69-87 sequence was found to activate resting encephalitogenic MBP reactive T cell lines to adoptively transfer clinical EAE. The release of stable encephalitogenic peptides from the myelin sheath by mast cell proteases may play a role in activation of encephalitogen-specific T-cells during the progression of EAE.

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Figure 1a

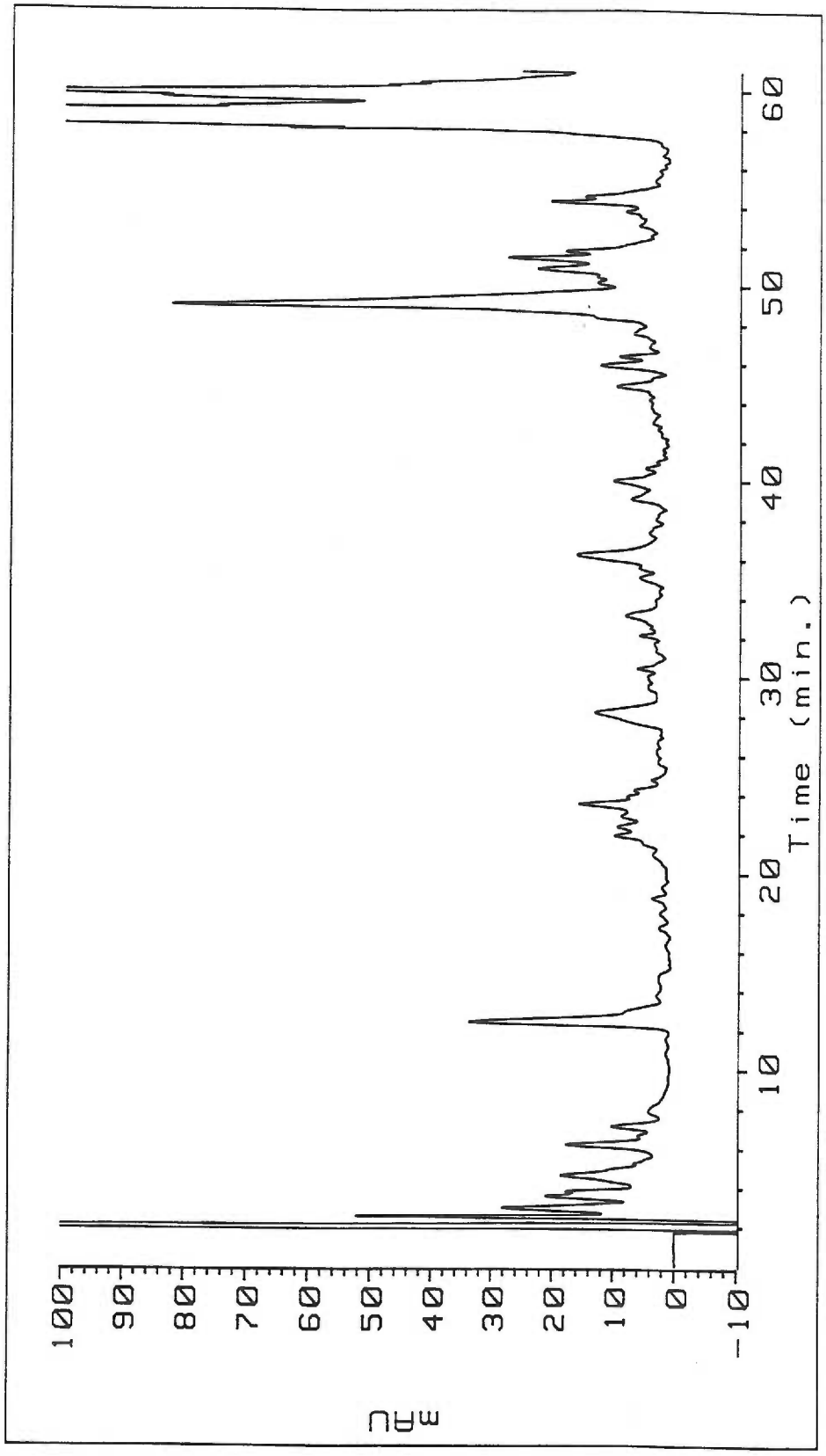


Figure 1b

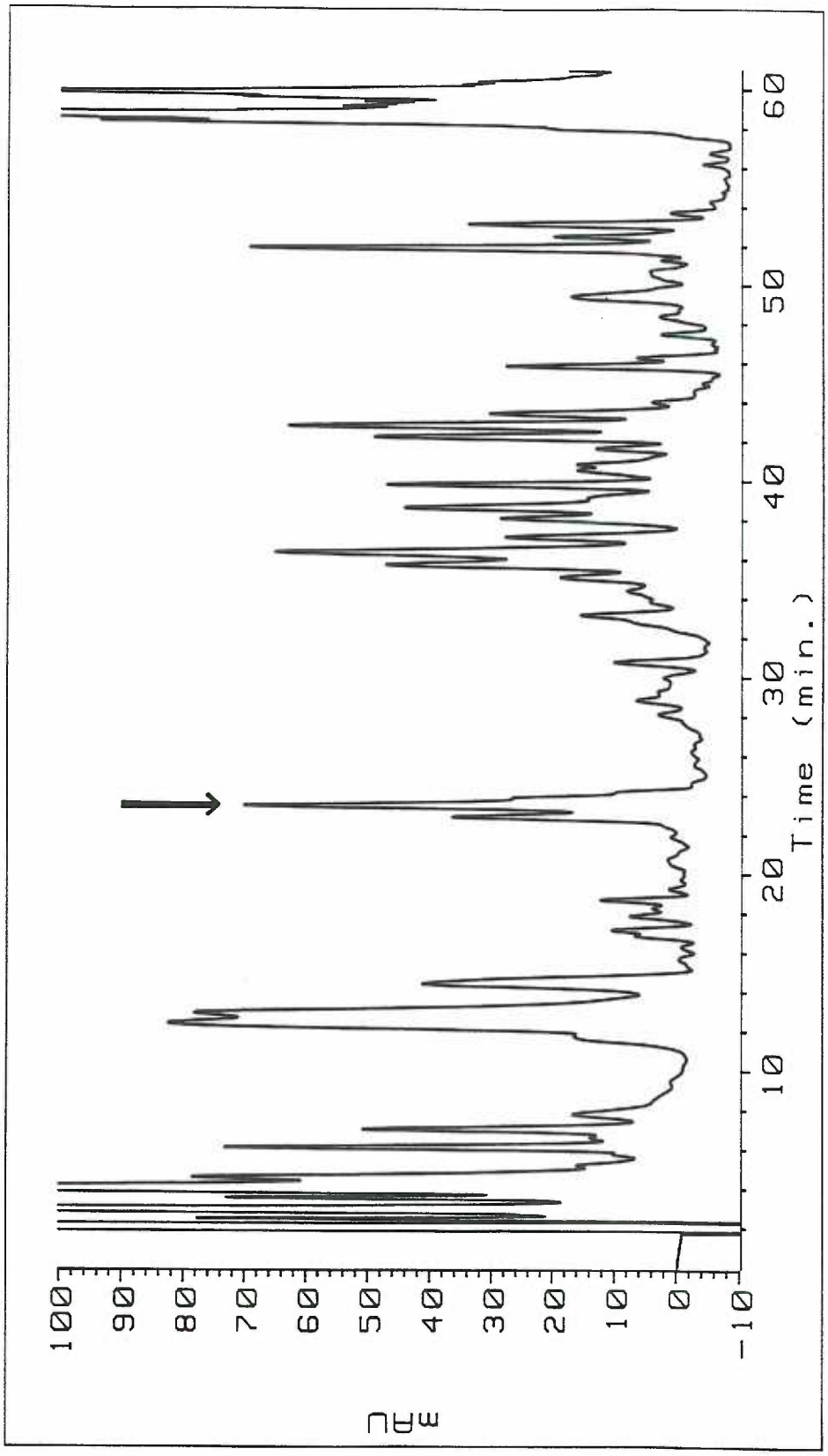
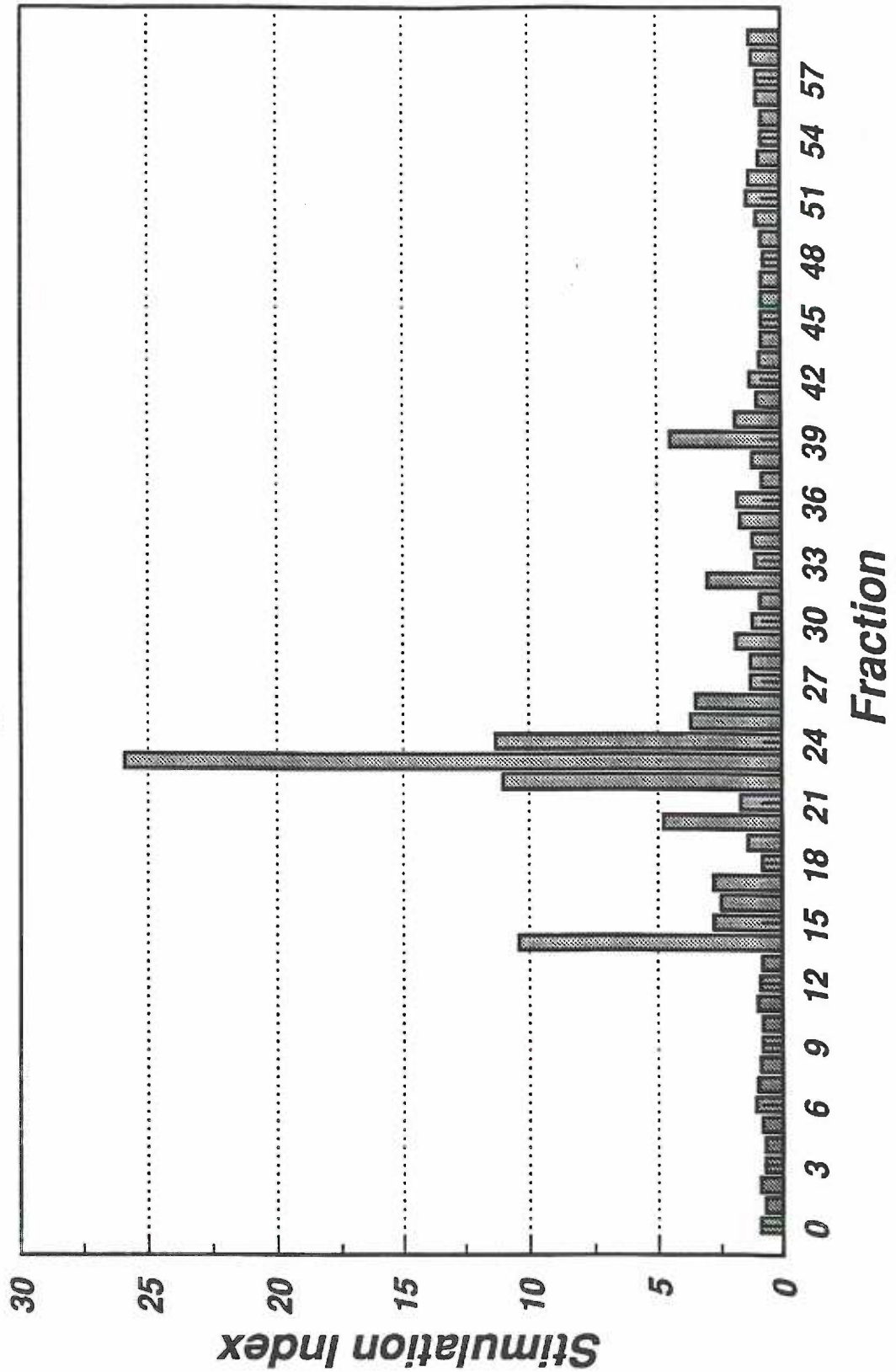


Figure 1c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30
25
20
15
10
5
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57
Fraction

Figure 1

HPLC and lymphocyte proliferation analysis of rat myelin following digestion with mast cell enzymes. (A) HPLC profile of supernatant from rat myelin incubated in the absence of mast cell enzymes. (B) HPLC profile following 3 hours incubation with mast cell enzymes. (C) Proliferation of BP-3 cell line in response to HPLC fractions obtained from digest in B. Maximum stimulation seen with HPLC fraction eluting at approximately 23 minutes, arrow-B.

Figure 2a

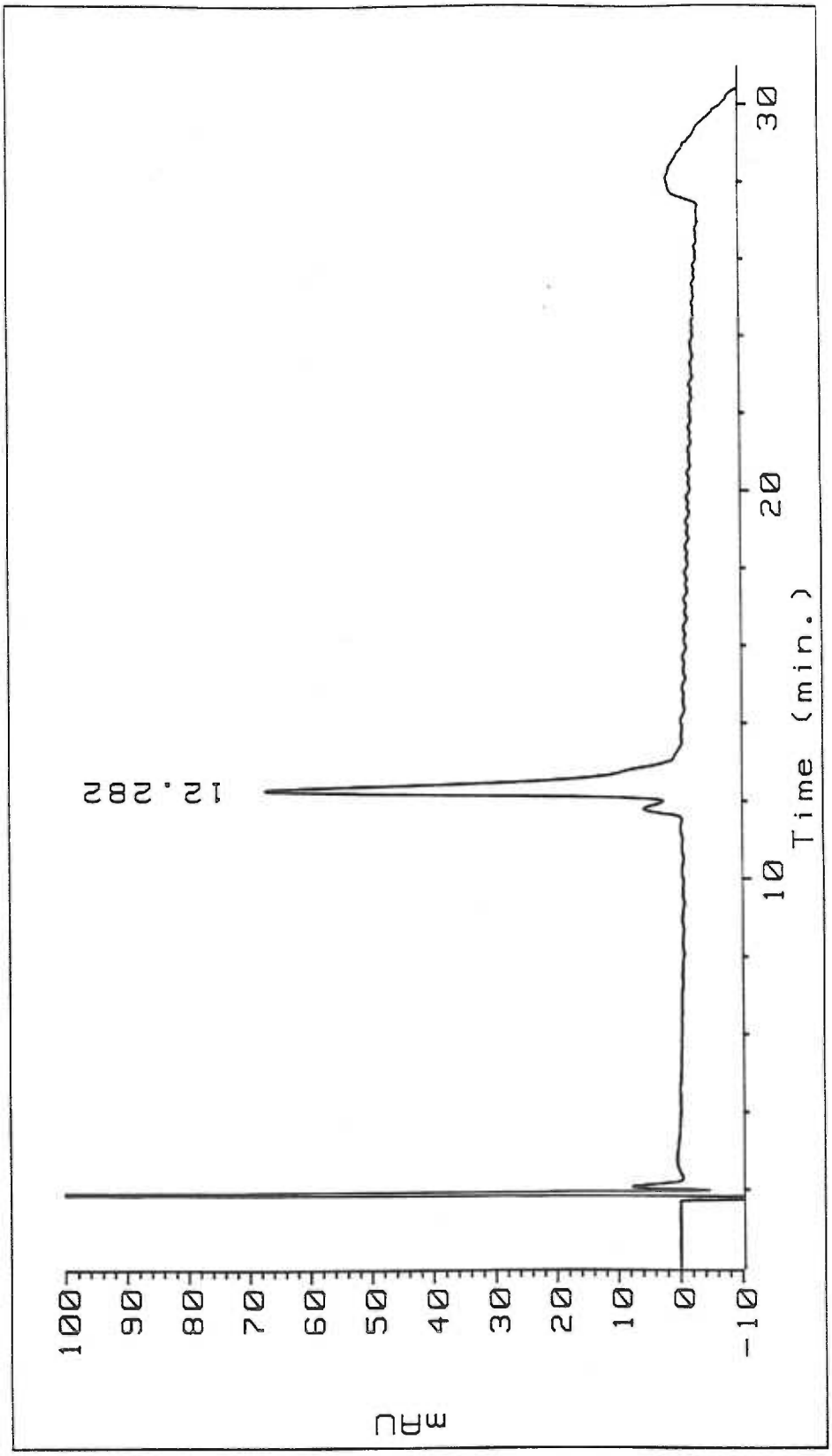

Figure 2b

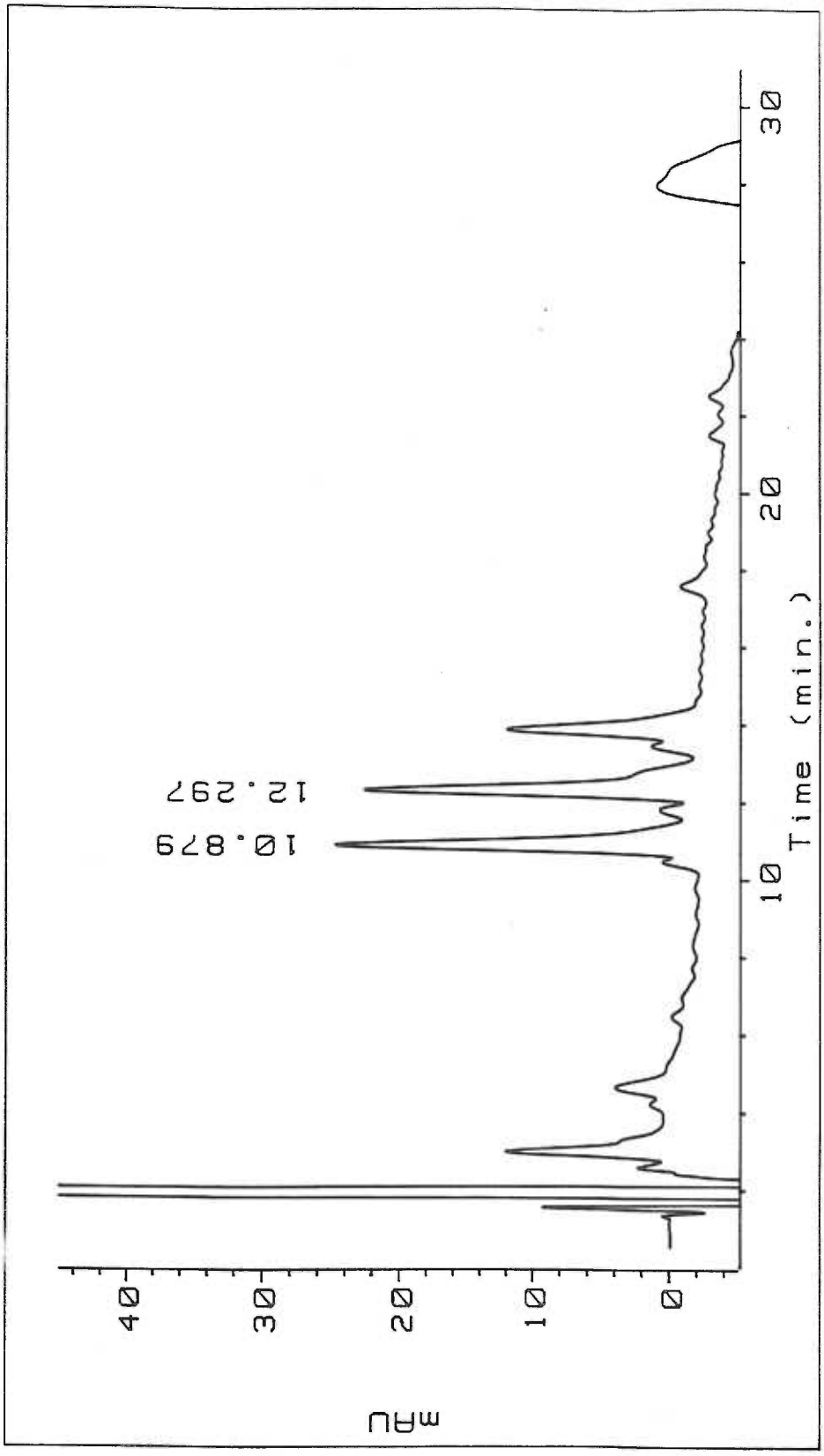

Figure 2c

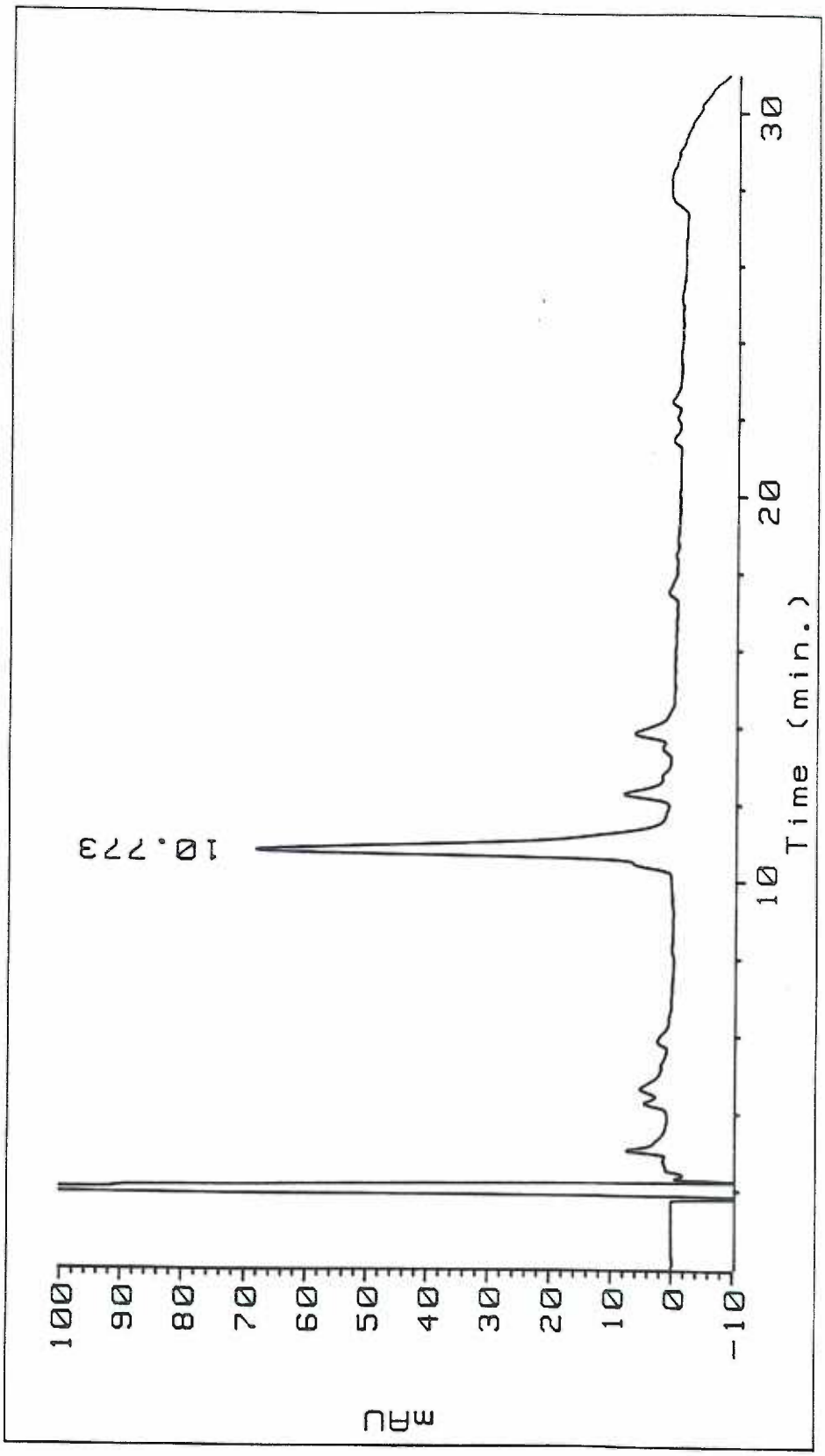

Figure 2

Stability of RT-MBP fragment 69-87. (A) Elution profile of rat MBP fragment 69-88. (B) Exposure of fragment 69-88 for 30 minutes to mast cell enzyme containing supernatant. Note appearance of new peak with elution at 10.879. (C) Elution profile following incubation of peptide 69-88 with mast cell enzymes for 2 hrs. Complete conversion to elution peak at 10.854. This shift in retention time is consistent with rat MBP peptide 69-87.

Table 1

Species	Residues[a]	Sequence
		70 80 90
Bovine	67–91	HYGSLPQKAQGHRPQDENPVVHFFK
Guinea Pig	67–91	HYGSLPQKSQ--RSQDENPVVHFFK
Rat	67–91	HYGSLPQKSQ--RTQDENPVVHFFK

[a]The amino acid reside numbering system is that originally published by Eylar et al. in J. Biol Chem 246:5770. Rat and guinea pig resides are numbered with respect to bovine MBP that contains a glycine-histidine insertion at positions 77 and 78.

Table 2

Induction of Adoptively Transferred EAE with BP-3 Cells+

Activating Antigen	Concentration µg/ml	Incidence	Disease Severity of Sick rats
GP BP	10	7/7	3.3 ± 1.2
	1	7/7	3.3 ± 0.9
	0.1	7/7	4.0 ± 0.0
GP 68-84	10	7/7	4.0 ± 0.0
	1	6/6	2.7 ± 1.1
	0.1	5/5	4.0 ± 0.0
Rat BP	10	6/6	4.0 ± 0.0
	1	7/7	4.0 ± 0.0
Rat 69-87	1	14/14	3.3 ± 1.1
	0.1	8/8	3.8 ± 0.4
No antigen	---	0/5	0.0 ± 0.0

+ BP-3 cells were stimulated for 3 days in the presence of various preparations of BP. Following stimulation, 3×10^6 activated BP-3 cells were transferred into naive recipients by intravenous injection. Beginning on day three, recipients were examined twice daily for neurologic impairment. Clinical disease score as defined in Methods.

MANUSCRIPT #5

TITLE: Inhibition of Experimental Autoimmune Encephalomyelitis with Isoproterenol and Theophylline.

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RUNNING TITLE: Inhibition of EAE with Isoproterenol and Theophylline.

FOOTNOTES:

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[3]Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; CNS, central nervous system; ISO, Isoproterenol; THEO, Theophylline; SI, Stimulation index.

ABSTRACT

During the induction of experimental allergic encephalitis (EAE), autoaggressive T lymphocytes infiltrate the CNS and cause inflammation which results in clinical paralysis. In many cellular immune responses the release of vasoactive amines by tissue mast cells is believed to play an integral role in the induction of the response. To demonstrate that normal mast cell function is needed during the elicitation EAE, Lewis rats developing EAE were treated with isoproterenol (ISO) and theophylline (THEO). ISO and THEO are pharmacologic agents which elevate cAMP levels and prevent stimulated mast cells from responding. ISO a B adrenergic receptor agonist stimulates cAMP production, while THEO interferes with the action of cAMP phosphodiesterase and prevents cAMP breakdown. The independent activities of these two agents on cellular cAMP levels predicts a synergistic effect on mast cell function when the agents are administered concurrently. In both actively induced and adoptively transferred EAE, ISO/THEO treatments were found to either prevent disease expression or significantly reduce clinical severity. Additional studies have established that ISO/THEO treatments do not prevent EAE by altering normal lymphocyte function. Lymphocytes from actively immunized rats treated with ISO/THEO were found to adoptively transfer EAE to naive controls. Furthermore, MBP reactive T cell lines activated *in vitro* in the presence of ISO/THEO, proliferate normally and adoptively transferring severe EAE. We also report that ISO/THEO treatments at concentrations which block the development of clinical EAE do not inhibit the development of active immunity to the intracellular parasite *Listeria monocytogenes*.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE), is a paralytic autoimmune disease that develops in susceptible strains of laboratory rodents following sensitization with myelin basic protein (MBP). The clinical signs of EAE are associated with inflammatory responses in the central nervous system (CNS) that include; perivascular infiltration by mononuclear cells (1), edema (2,3), fibrin deposition (2,3) and often damage to the myelin sheath (4). The induction of these immune responses within CNS the implies that the integrity blood-brain-barrier (BBB) is altered to the extent that circulating cells and serum proteins move from circulation into the CNS (5).

Since endothelial cells can present antigen in the context of class II MHC to responsive lymphocytes *in vitro*, it has been proposed that the vascular endothelium is an active component in the progression of EAE (6). In some models vascular endothelial cells within the CNS have been found to express Ia just prior to the onset of clinical EAE (7). Endothelial cells expressing Ia on their lumen side could present MBP to circulating cells responsive to MBP and this interaction might facilitate lymphocyte egress into the CNS (8). However, studies have demonstrated that cognate interaction between the vascular endothelium and MBP reactive lymphocytes is not a requirement for the induction of EAE (9).

A generalized breakdown of the BBB occurring during EAE (10), provides an alternative mechanism to explain how lymphocyte infiltration into the CNS is facilitated during the disease. In this hypothesis, alterations in the BBB could be accomplished by mast cell degranulation, which has previously been reported to play an integral role in the induction of EAE (11). Activation of "serosal" like mast cells

found in the CNS (12,13), would lead to the release of stored vasoactive amines and de novo synthesis of arachidonic acid metabolites (14,15). Collectively, these mast cell products would act on capillary endothelial cells to increase vascular permeability, and in effect compromise the integrity of the BBB.

Previously we have reported that inhibitors of mast cell degranulation block an important step in the elicitation of clinically apparent EAE (11). In this study we extend these observations by demonstrating that THEO, an inhibitor of mast cell degranulation, can reduce the clinical severity of EAE. This effect is markedly potentiated by the synergistic action of ISO, a catecholamine also know to impair mast cell function. Administered together these agents completely block or significantly clinical expression of both actively induced and adoptively transferred EAE. We have been unable to demonstrate a direct effect of these agents on lymphocyte activity. Lymphocyte activation in the presence of THEO alone or combination with ISO had little effect on *in vitro* proliferation or subsequent effector cell function *in vivo*. These findings underscore the important role of the mast cell in the elicitation of actively induced and adoptively transferred EAE.

MATERIALS AND METHODS

Animals. Inbred Lewis female rats were obtained from Charles river and housed under standard conditions in the Animal research facility at the Veterans administration Hospital, Portland OR.

Materials. THEO and ISO were obtained from Sigma Chemical Co. (St. Louis. MO), fetal calf serum from Tissue Culture Biologicals (Tulare, CA), NU serum was obtained from Collaborative research incorporated (Bedford, MA), RPMI, L-glutamine, Sodium pyruvate, penicillin, streptomycin, and horse serum from Gibco Laboratories (Grand Island, NY).

Purification of BP. Guinea pig brains were purchased from Pel-Freez Biologicals (Rogers, AK) and stored at -70 prior to extracting MBP. Extraction of MBP was carried out by previously reported methods (16). MBP was further purified from co-extracted contaminants by ion exchange column chromatography, as previously reported (11). Purity of the MBP preparation was assessed by SDS-PAGE, and determined to be greater that 95%.

Induction of active EAE. Purified MBP dissolved at 1 mg/ml in saline was emulsified in an equal volume of complete Freund's adjuvant containing 10 mg/ml of heat killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit MI). Eight to twelve week old Lewis rats were immunized with 100 µl of the BP/CFA preparation, distributed equally between the two front footpads.

Adoptive transfer of EAE. Spleens were removed from rats immunized with BP/CFA twelve days earlier and placed into a single cell suspension. The spleen cells were suspended at 2×10^6 cells/ml in RPMI supplemented with 5% Fetal calf serum, 1 mM glutamine, 1 mM

pyruvate, 5 x 10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell cultures were stimulated with 1 μg/ml purified MBP in 75 cm^2 tissue culture flasks (Falcon 3023, Becton Dickinson, Oxnard CA.), and allowed to incubate at 37° for 72 h in a humidified incubator with 7% CO_2-93% air. Following stimulation, cells were washed, enumerated, and 2 x 10^7 viable cells were injected i.v. into the tail vein of naive Lewis recipients.

Clinical evaluation. Rats used in active immunization and adoptive transfer experiments were evaluated daily for clinical signs of neurologic impairment. Animals were graded as follows; a flaccid tail was given a grade 1; hindquarter weakness, characterized by a abnormal gait was given a grade 2; hindquarter paralysis was given a grade 3; and complete hindquarter paralysis with incontinence was graded as a 4.

Generation and maintenance of BP-3 cell line. The BP-3 cell line was generated by a modification of previously reported methods (17). In brief, naive Lewis rats were immunized with a synthetic peptide consisting of the region of MBP known to be encephalitogenic in the Lewis rats, resides 68-84 (YGSLPQKSQRSQDENPV) (18). Nine days following sensitization, the rats were sacrificed, draining lymph nodes were removed, and the cells were placed into a single cell suspension. The cells were washed and suspended at 2 x 10^6 lymph node cells/ml in RPMI supplemented with 2% autologous rat serum, 1 mM glutamine, 1 mM pyruvate, 5 x 10^{-5} M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and placed into 75 cm^2 tissue culture flasks, and stimulated with 1 μg/ml of the GP-MBP fragment 68-84. Following a three day culture period the cells were washed and resuspended in T cell growth media consisting of 5% Horse serum, 5% Nu Serum, 1 mM

glutamine, 1 mM pyruvate, 5 x 10^{-5} M 2-ME, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone and 10% conditioned media. Conditioned media was previously prepared by stimulating normal spleen cells suspended at 5 x 10^6 cells/ml in RPMI supplemented with 5% FCS, 5 x 10^{-5} M 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin with 5 µg/ml Con A for 24 hours. Following four days on T cell growth media, viable lymphocytes were restimulated by suspending 5 x 10^6 lymphocytes with 1 x 10^8 irradiated thymocytes in 10 ml of stimulation media with 10 µg/ml of the 68-84 peptide. The cell line, BP-3, generated by this weekly stimulation/growth cycle has been in continuous culture for 10 months at the time it was used for these studies.

Lymphocyte proliferation assay. BP-3 cells were assessed for their ability to respond to specific antigen in the presence of ISO and or THEO in a standard lymphocyte proliferation assay. ISO and THEO were dissolved in RPMI at 4X the indicated concentrations and 50 µl volumes of the solutions were plated into flat welled microtiter plates (Falcon) in triplicate. 100 µl of a suspension consisting of 5 x 10^4 BP-3 cells and 1 x 10^6 irradiated syngeneic thymocytes in 2X stimulation media was then added to each well. The plates were allowed to incubate for 72 h in a humidified 5% CO_2 - 95% air atmosphere incubator at 37°C. For the final 4 h of the culture, cells were pulsed with [^{3}H]-TdR (New England Nuclear, Boston, MA), 1 µCi/well. Counts for the triplicate wells were averaged and expressed as a stimulation index, based on the proliferation of BP-3 cells cultured in the absence of MBP.

Spleen clearance assay for evaluating protection to Listeria monocytogenes. Lewis rats were immunized with 0.1 LD_{50} of *Listeria monocytogenes*, strain 10403, (approximately 2 x 10^5 CFU). Twelve days

post immunization, rats were by challenged with 10 LD_{50}s of strain 10403 (2 x 10^6 CFU) given by i.v. injection. Immunity to the challenge of strain 10403 was evaluated two days later by an immune clearance assay, as previously reported (19). In brief, spleens of challenged rats were homogenized and log dilutions were made from the homogenate. 100 µl of the suspension from the appropriate dilutions was plated onto BHI agar plates. Plates were incubated for 24 hours, at which time the number of colonies was enumerated and the values were used to calculate the log10 protection.

RESULTS

Isoproterenol in combination with Theophylline blocks passive EAE. Initially, ISO and THEO independently and in combination were tested for their effects on adoptively transferred EAE. Spleen cells from actively immunized donors were activated *in vitro* with MBP and transferred to naive recipients. Recipients of culture activated spleen cells were treated twice daily with the indicated concentrations of ISO, THEO or a combination of the two reagents, beginning at the time of cell transfer. Shown in Table 1, ISO administered alone at either 0.75 mg/kg and 2.5 mg/kg had little if any effect on the development of adoptively transferred EAE. Treatments of THEO at 10 mg/kg also failed to alter disease progression, although clinical EAE was blocked in 5 of the 7 transfer recipients when 50 mg/kg of THEO was used. Although dosages of 0.75 mg/kg of ISO and 10 mg/kg THEO given independently do not alter the course of clinical EAE, in combination they were found to block clinical disease in 5 of 7 rats. By increasing the dosage of THEO to 50 mg/kg, the ISO/THEO treatments prevented adoptively transferred disease in 100% of the recipients. In each case treatments were continued for seven days following cell transfer, at which time untreated controls had recovered from disease. Following termination of ISO/THEO treatments, rats were evaluated for 10 days for the appearance clinical EAE, although disease was never observed.

The effects of ISO/THEO treatments were then tested on adoptively transferred EAE mediated by MBP reactive T cell lines. Recipients were given the indicated inoculum of BP-3 cells immediately following cell activation, shown in Table 2. With 2×10^6 BP-3 cells, all untreated

normal recipients developed severe clinical disease, while 4 of 5 recipients treated daily with ISO/THEO failed to develop clinical EAE. Although ISO/THEO treatments failed to block clinical EAE when higher dosages of BP-3 cells were given to recipient rats, disease severity was reduced considerably. Transfer of 3×10^6 BP-3 produced severe clinical EAE that resulted in the death of untreated controls, while ISO/THEO treated recipients developed only modest clinical disease.

Timing of isoproterenol/theophylline treatments. To further explore the effects of clinical disease inhibition by ISO/THEO, different treatment regimens were followed. Adoptive transfer recipients were divided into four groups which were treated as follows. Group 1 received ISO/THEO injections twice daily from the time of cell transfer until day 8; group 2 received treatments beginning on day 3 and extending through day 7; group 3 received treatments for only the first three days, beginning on the time of cell transfer and extending through day 2; and group 4 received treatments for four days beginning the time of cell transfer and extending through day 3. Rats in group 1 which were treated from the time of cell transfer through day 7, failed to express clinical disease, shown in Table 3. Delaying administration of ISO/THEO until three days following cell transfer was also effective, preventing clinical disease in 4 of the 4 rats in group 2. In contrast, ISO/THEO treatments given only prior to the normal onset of clinical disease, groups 3 and 4, did not prevent clinical EAE.

Isoproterenol in combination with Theophylline blocks induction of active EAE. To test the effects of ISO/THEO treatments on actively induced EAE, naive rats were immunized with GP-MBP/CFA.

Beginning 7 days following the sensitization, the rats were given two daily injections of ISO/THEO. On day 10, ISO/THEO treatments were reduced to a single daily injection given for the remainder of the test period, which extended through day 15 for group A, and through day 20 for group B. Untreated controls developed normal clinical disease, where hindquarter weakness was noted in most of the animals by days 9-10. Clinical disease reached maximum severity between days 11-14, shown in figure 1, and was followed by a rapid and complete recovery by day 15 or 16. ISO/THEO treatments effectively blocked clinical disease in 16 of 16 rats in group A, while 13 of 17 rats in group B were protected and only modest clinical disease was apparent in the rats which did develop clinical EAE, shown in figure 1. Following the withdraw of the treatments the rats were observed for a period of two weeks, with only 3 of the total 33 rats experiencing clinical disease beyond this point. In an identical experiment, spinal cords were removed from treated rats 14 days following immunization and evaluated for histologic EAE. Mononuclear infiltrates around a post capillary venules, characteristic of EAE, were not found in the cords from rats treated with ISO/THEO, data not shown.

Adoptive transfer with spleen cells from isoproterenol/theophylline treated donors. To demonstrate that the ISO/THEO treatments do not alter the effector function of MBP reactive lymphocytes, treated rats were used in adoptive transfer experiments. MBP immunized rats were treated with the same ISO/THEO treatment regiment which was previously used to block active EAE. On day 12 post sensitization, all treated rats had failed to show signs of clinical EAE,

while untreated controls had developed clinical paralysis. At this time point, spleen cells from both treated and untreated groups were placed into culture with MBP. Following the 3 day *in vitro* culture step, 2×10^7 activated spleen cells were transferred into naive Lewis rats. Spleen cells derived from both ISO/THEO treated and untreated controls adoptively transferred clinical disease to all recipients, shown in Table 4.

Isoproterenol/Theophylline do not alter lymphocyte proliferation or capacity to adoptively transfer disease. To determine if THEO and or ISO could prevent T cell activation, these agents were tested in a standard lymphocyte proliferation assay. As shown in Table 5, THEO at concentrations up to 100 μg/ml had only a small effect on the response of the BP-3 cell line to the antigen MBP. The addition of ISO to the cultures at concentrations up to 20 μg/ml did not effect the cell proliferation. Similar effects were seen when ISO and THEO were tested on an ovalbumin specific T cells line (data not shown).

To evaluate the development of lymphocyte effector function in the presence of ISO/THEO, BP-3 cells were stimulated for three days in the presence of 100 μg/ml THEO and 2 μg/ml ISO. Following culture, 3×10^6 activated cells from either the control or ISO/THEO treated cultures were adoptively transferred to naive recipients. Severe clinical EAE was seen in both recipients groups demonstrating that lymphocyte activation in the presence of ISO/THEO had not alter the cells normal immune function, shown in Table 6.

The Effects of Isoproterenol/theophylline treatments on the development of anti-Listeria immunity. To demonstrate that ISO/THEO blocks clinical EAE without interfering with lymphocyte function, we tested the effects the these agents on the development and expression of immunity to *Listeria monocytogenes*. In these experiments, two groups of rats were immunized with 0.1 LD_{50}. One group of immunized rats was also treated with 0.75 mg/kg ISO and 50 mg/kg THEO, twice daily beginning 7 days following immunization. A non-immune control group was also treated with ISO/THEO in an manner identical to that used for the Listeria immunized rats. Twelve days following immunization, all groups which included rats which had been immunized, immunized and treated, non-immunized but treated and normal rats were challenged with 10 LD_{50} of the immunizing strain of *L. monocytogenes*. Two days post challenge, the immune status of the animals was evaluated by a standard spleen clearance assay, shown in Table 7. In two independent experiments non-immunized rats that had been treated with ISO/THEO expressed little if any protection. In contrast both the immunized rats and immunized rats treated with ISO/THEO, expressed more than four log10 units of protection. The similar levels of protection seen in the immunized and treated rats compared to the non-treated, immunized rats suggests that ISO/THEO treatments had no direct effect on the development of the primary immune response to Listeria.

DISCUSSION

The sequence of events that allows BP reactive lymphocytes to infiltrate the CNS and cause EAE, remains unclear. It has been proposed that capillary endothelial cells may play an active role in T cell activation and could facilitate lymphocyte egress into the CNS during the onset of EAE. While isolated endothelial cells can be induced to express Ia (20) and stimulate lymphocytes *in vitro* (7), this interaction is not required for the induction of EAE. Adoptive transfer studies with chimera rats have demonstrated that MHC compatibility between transferred lymphocytes and capillary endothelial cells is not a requirement for the development of EAE (9).

Studies monitoring vascular permeability by i.v. injections of ^{125}I-labeled BSA, indicate that integrity of the BBB is compromised at the onset of clinical EAE (5). This generalized breakdown of the BBB could be the mechanism which allows MBP reactive cells to egress through the endothelial layer and enter the CNS. A generalized increase in vascular permeability would predict that activated lymphocytes could gain access to the CNS in the initial stages of EAE, regardless of their antigen specificity. Non-specific trafficking was demonstrated by Lisak et al (21), who concurrently immunizing rats with tetanus toxoid and MBP, and subsequently isolated lymphocytes from the spinal cord at the onset of clinical EAE. Limiting dilution analysis of the isolated lymphocytes revealed that BP and TT reactive cells infiltrate the cord with a similar frequency. Thus, the implication is that prior to clinical EAE the vascular endothelium becomes leaky, allowing both serum proteins and lymphocytes to non-specifically infiltrate the CNS.

What then is the mechanism that is responsible for altering vascular permeability? In some model systems, mast cell degranulation plays an integral role in cell extravasation an event required for clinical manifestation of many cellular immune responses (14,22,23). The release of vasoactive amines by activated mast cells increases vascular permeability and facilitates lymphocyte infiltration into the tissue (24). In previous studies we have demonstrated that agents which block normal mast cell function prevent the induction of passive EAE. We have hypothesized that mast cells, perhaps sensitized by antigen specific T cell factors (9), release vasoactive amines which cause a generalized breakdown of the BBB during the course of EAE. This event allows lymphocytes to infiltrate the CNS and mediate the subsequent immune events that result in clinical paralysis.

In this report we demonstrate that ISO given with THEO effectively inhibits both adoptively transferred and actively induced EAE. THEO has been reported to elevate intracellular cAMP in mast cells by inhibiting cAMP phosphodiesterase (25), making the cells less responsive to stimuli (26,27,28). Acting as a beta adrenergic agonist, ISO, also increases cAMP levels by activating adenylate cyclase (29), making mast cells less responsive to stimuli (26,27,28). By using ISO to increase cAMP levels, and THEO to block normal degradation of this intracellular messenger, these compounds should act synergistically to markedly inhibit mast cell function. When tested alone for there effects on adoptively transferred EAE, ISO failed to alter the progression of clinical EAE, while THEO produced only modest reduction in clinical disease, Table 1. However, when administered together these agents were found to work synergistically. Independently ISO treatments of 0.75 and THEO

treatments of 10 mg/ml had no effect on passive EAE, yet when these agents administered in combination clinical disease was blocked in 5 of 7 recipients. Increasing THEO treatments to 50 mg/kg resulted in complete disease inhibition in all cell transfer recipients. The course of adoptive EAE mediated by MBP reactive T cell lines was also altered by ISO/THEO treatments. ISO/THEO treatments were able to block the expression of clinical EAE when 2×10^6 BP-3 cells were transferred into the recipients. However, the treatments did not prevent clinical EAE when a larger inoculum of BP-3 cells (3×10^6 cells) were used, although the clinical symptoms were significantly reduced when compared to untreated recipients.

Adoptive EAE represents a situation where MBP reactive cells are fully developed and are capable of mediating EAE in the recipients. Therefore, the effects of ISO/THEO do cannot be attributed to a blockade in normal lymphocyte development. Furthermore, it is generally accepted that the activity of culture activated cells is not easily down regulated prior to the course of clinical EAE. Rats which have recovered from active EAE and are resistant to a second disease episode induced by MBP/CFA, have been found to be fully susceptible to episodes of adoptively transferred EAE using culture activated spleen cells (30). Therefore, it is unlikely that ISO/THEO treatments abrogate clinical EAE by down regulating activated MBP reactive T cells. To further support our view that ISO/THEO treatments prevent clinical EAE indirectly by preventing lymphocyte trafficking and not by altering lymphocyte activity, MBP specific T cell lines were activated in the presence of these agents. Although, *in vivo* concentrations of ISO and THEO attained in treated rats was not known, concentrations of 100 μg/ml THEO and 2

μg/ml ISO, which are several fold higher than the daily administered dosage, did not significantly alter lymphocyte proliferation (Table 5). While *in vitro* proliferation generally corresponds to lymphocyte activation, it could be argued that exposure of lymphocytes to ISO/THEO could compromise function without reducing proliferation. To demonstrate that ISO/THEO treatments do not block lymphocyte activation at concentrations used to prevent clinical EAE, BP-3 cells were stimulated with MBP in the presence of these agents and used in adoptive transfer studies. Recipients of cells activated in the presence of ISO/THEO developed clinical disease with severity comparable to recipients of untreated BP-3 cells, seen in Table 6. These experiments lend additional evidence that ISO/THEO treatments do not prevent or down regulate normal lymphocyte function, but block expression of EAE by altering mast cell function and preventing lymphocyte traficking into the CNS.

If ISO/THEO alter lymphocyte function, then treatments given following cell transfer but terminated prior disease expression should prevent EAE. However, if ISO/THEO block EAE by preventing lymphocyte trafficking, administering these agents during the period preceding cellular infiltration of the CNS, should not interfere with the normal course of disease. Likewise, if only lymphocyte trafficking is affected by ISO/THEO then it should be possible to delay the treatments with these agents until just prior to the disease onset and still successfully block clinical disease. When ISO/THEO was administered to adoptive transfer recipients for days 0-2 or days 0-3 following cell transfer, the expression of clinical disease was not altered (Table 3). And as expected delaying treatments until day 3 still provided adequate

protection against adoptively transferred EAE. These findings support the argument that ISO/THEO do not mediate there effect directly on the MBP reactive cell population, but block EAE by preventing autoaggressive cells from reaching the CNS. This is consistent with our proposal that mast cell activation normally occurs during the induction of EAE, and the resulting release of vasoactive amines alters vascular permeability and facilitates lymphocyte egress into the CNS.

The effects of ISO and THEO treatments were evaluated in the development of active EAE. Previously it has been demonstrated that lymphocytes taken from the draining lymph nodes seven days post immunization can be used to transfer EAE into naive recipients (31). This indicates that MBP reactive cells have developed and are capable of mediating EAE by day seven, the time point where ISO/THEO treatments were initiated. Following immunization with BP/CFA, ISO/THEO treatments were started on day 7, two or three days prior to the usual onset of active EAE. Administering ISO/THEO to actively immunized rats prevented both the mononuclear cell infiltration of the CNS and the expression of clinical disease, shown in Figure 1. Following the termination of ISO/THEO treatments a small percentage of the rats did developed clinical disease although the clinical scores were very modest. This indicates that normal immunoregulatory events which down regulate the effector function of autoaggressive lymphocytes are still functional in the ISO/THEO treated rats.

In other experiments, rats treated with ISO/THEO were used in adoptive transfer experiments to demonstrate the presence of MBP reactive lymphocytes in the treated donors. Spleen cells from both treated and non-treated rats immunized with MBP/CFA were collected

on day 12 and cultured in the presence of MBP. When adoptively transferred into naive recipients, spleen cells from both treated and non-treated donors transferred severe clinical disease (Table 4), demonstrating the presence of functional MBP reactive cells in immunized rats treated with ISO/THEO.

We have also measured the influence of ISO/THEO treatments on the development of immunity to Listeria. This intracellular parasite is eliminated *in vivo* only following the development of T lymphocytes capable of producing lymphokines which activate macrophages. ISO/THEO was administered to rats immunized with sub-lethal numbers of *L. monocytogenes* (strain 10403) using a treatment regiment similar to what was used to block active EAE. Rats were given ISO/THEO treatments twice daily beginning on day 7 and extending through day 13. On day 12 the rats were challenged with 10 LD_{50} of the immunizing strain of Listeria, and two days later protection to the pathogen was evaluated in a standard spleen clearance assay. Immunization with a the sublethal inoculum of Listeria, resulted in more than 4 logs of protection, when immunized controls were challenged with *L. monocytogenes*. Treatments with ISO/THEO were found to have little if any effect on the development and expression of immunity to *L. monocytogenes*, immunized rats treated with ISO/THEO also expressed over 4 logs of protection.

Generation of an immune response L. monocytogenes does not require the same cell traficking events that must occur in the induction of EAE. During the course of EAE, MBP reactive lymphocytes must leave the circulation, cross the highly restrictive blood-brain-barrier (BBB) and initiate inflammatory events. The release of vasoactive amines and other

potent mediators from tissue mast cells appears to be central in the disease process (11). Inhibitors of mast cell degranulation including ISO and THEO would then be expected to block clinical EAE by preventing lymphocyte trafficking into the CNS. Without increasing vascular permeability of capillary beds in the CNS events that result in clinical paralysis including; mononuclear cellular infiltration, fibrin deposition and edema, can not occur. In contrast, *L. monocytogenes* is an facultative intracellular parasite which resides in phagocytic cells in the infected host. Challenges of *L. monocytogenes* give by i.v. challenge, places the organism directly in circulation where it is quickly removed by phagocytes in the spleen, liver and other organs of the reticuloendotheial system. While antigen specific T cells are still required for the successful eradicate of the organism, these lymphocytes need not leave circulation to provide help to infected phagocytes. Therefore, altering mast cell function with ISO/THEO treatments would not be expected to affect the hosts capacity to deal with *L. monocytogenes*.

These findings lend additional support to our earlier observations that mast cell degranulation is required for the induction of EAE. Treatments with ISO/THEO significantly alter the course of both actively induced and adoptively transferred EAE. Furthermore there is no evidence indicating that this inhibitory effect is the result of ISO and THEO blocking the induction or down regulating the activity of MBP reactive T cells.

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TABLE 1. Effects of Isoproterenol and Theophylline on adoptively transferred EAE[a]

Treatment	Sick/Group	Disease Severity[b] of rats with EAE
Control	5/5	4.0 ± 0.0
Isoproterenol (.75 mg/kg - twice daily)	3/3	2.7 ± 1.2
Isoproterenol (2.5 mg/kg - twice daily)	3/3	4.0 ± 0.0
Theophylline (10 mg/kg - twice daily)	3/3	3.3 ± 1.2
Theophylline (50 mg/kg - twice daily)	2/7	1.0 ± 0.0
Theophylline - Isoproterenol (.75 mg/kg isoproterenol, 10 mg/kg theophylline - twice daily)	2/7	1.5 ± 0.0
Theophylline - Isoproterenol (.75 mg/kg isoproterenol, 50 mg/kg theophylline - twice daily)	0/4	0.0 ± 0.0

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 µg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean % one std deviation.

TABLE 2. Effects of Isoproterenol and Theophylline on cell line mediated EAE

Group	Sick/Group	Disease Severity[a] of rats with EAE
Recipients of 2 x 10^6 activated BP-3 cells		
Control	6/6	4.0 ± 0.0
Isoproterenol/Theophylline treated	1/5	2.0 ± 0.0
Recipients of 3 x 10^6 activated BP-3 cells		
Control	6/6	4.0 ± 0.0
Isoproterenol/Theophylline treated[b]	5/5	1.6 ± 0.6

[a]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

[b]Treated rats were given 0.75 mg/kg isoproterenol and 50 mg/kg of theophylline once daily.

TABLE 3. Time dependent influence of isoproterenol and theophylline on passive EAE.

Treatment	Sick/Group[a]	Disease Severity[b]
Untreated Controls	9/9	3.1 ± 0.7
Isoproterenol/Theophylline Treated[c]		
Group 1		
Days 0 to 7 following cell transfer	0/9	0.0 ± 0.0
Group 2		
Days 3 to 7 following cell transfer	0/4	0.0 ± 0.0
Group 3		
Days 0 to 2 following cell transfer	4/4	3.0 ± 0.0
Group 4		
Days 0 to 3 following cell transfer	8/8	3.4 ± 0.5

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 µg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

[c]Treated with 0.75 mg/kg isoproterenol and 50 mg/kg theophylline twice daily.

Figure 1

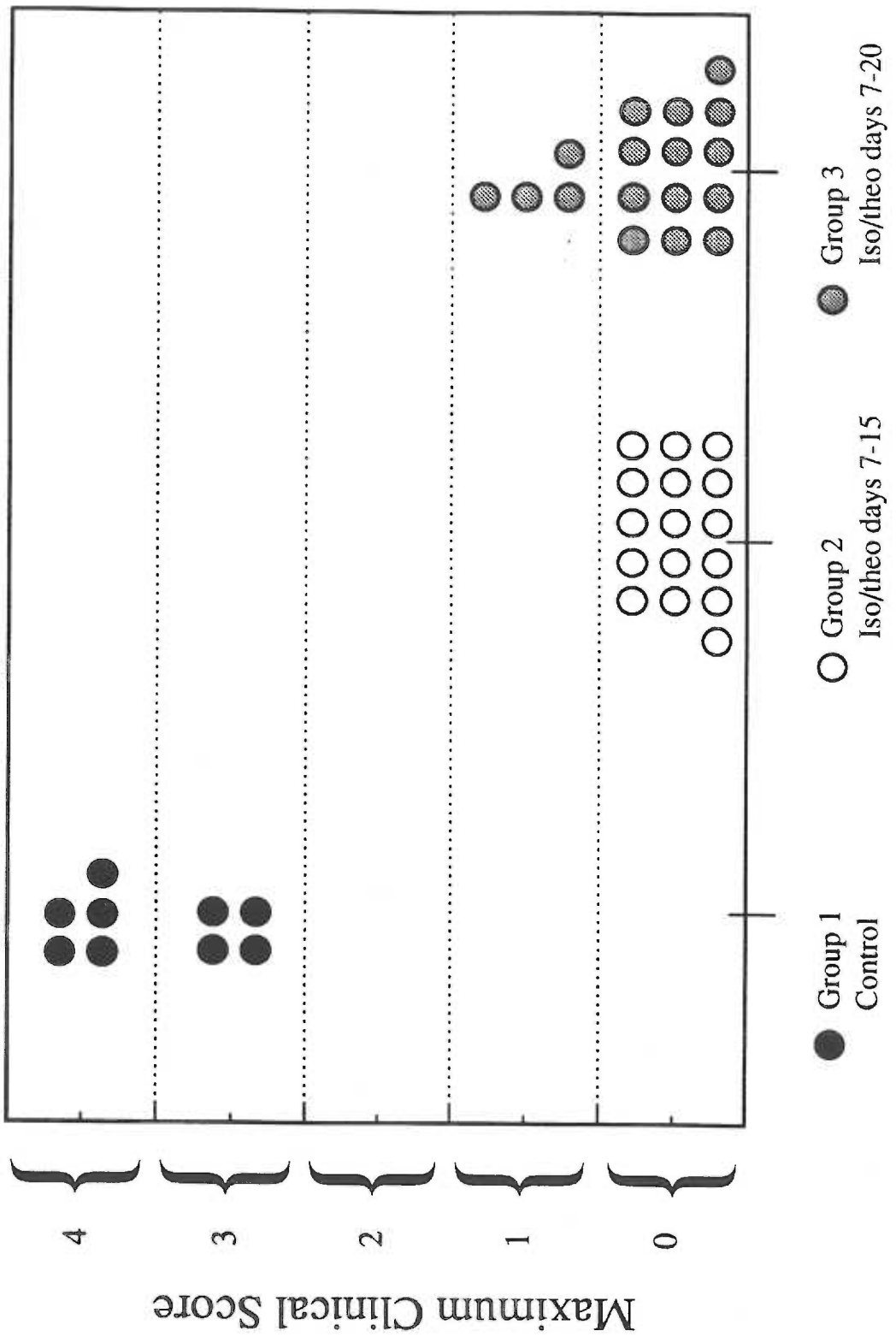
Maximum Clinical Score
4
3
2
1
0
Group 1
Control
Group 2
Iso/theo days 7-15
Group 3
Iso/theo days 7-20

TABLE 4. Adoptive transfer with isoproterenol and theophylline treated donors

Recipients[a]	Sick/Group	Disease Severity[b] of rats with EAE
Control Donors	5/5	2.8 ± 0.47
Treated Donors[c,d]	5/5	2.2 ± 0.47

[a]Recipients received 2 x 10^7 spleen cells cultured for 72 hours with 1 μg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

[c]Treated rats were given 0.75 mg/kg isoproterenol and 50 mg/kg of theophylline twice daily for days 7-9, and once daily for days 10-11.

[d]Treated rats had failed to express symptoms of clinical EAE when they were used as spleen cell donors on day 12.

TABLE 5. Effects of Isoproterenol/Theophylline on cell line proliferation[a].

Theophylline µg/ml	Isoproterenol µg/ml	Exp #1[b]	Exp #2[b]	Exp #3[b]
100	0.0	24.4	24.3	36.7
10	0.0	43.1	38.6	65.1
1	0.0	41.1	47.9	82.6
0	0.0	40.1	36.3	73.8
100	2.0	18.6	20.8	32.2
10	2.0	38.2	32.2	58.8
1	2.0	40.2	40.0	68.2
0	2.0	40.5	32.7	65.4
100	20.0	12.2	18.3	26.1
10	20.0	16.2	35.6	80.1
1	20.0	18.2	42.6	86.0
0	20.0	15.5	34.1	72.4

[a] Proliferation assay was done with BP-3 cells suspended at 5×10^5 cells/ml with 1×10^7 irradiated thymocytes and 10 µg/ml of MBP in the presence of the indicated concentrations of isoproterenol and theophylline.

[b] Results are expressed as stimulation index, based on the proliferative response of BP-3 cells cultured in the absence of MBP.

TABLE 6. Effects of Isoproterenol/Theophylline cell line activation

Treatment	Sick/Group[a]	Disease Severity[b]
Control	9/9	4.0 ± 0.0
Treated[c]	6/6	3.9 ± 0.3

[a]Recipients received 3 x 10^6 BP-3 cells activated under standard cell line culture conditions for 72 hours with 10 μg/ml BP.

[b]Disease severity of rats which show symptoms of EAE graded on a 1-4 scale and presented as mean ± one std deviation.

[c]Cultured in the presence of 100 μg/ml theophylline and 2 μg/ml isoproterenol which was added at the beginning of culture activation.

TABLE 7. Effects of Isoproterenol/Theophylline immunity to *Listeria Monocytogenes*[a]

Group	Rats/Group	Logs Protection[b]
Experiment 1		
Immunized	4	4.57
Immunized and treated[c]	4	4.69
Treated[d]	3	1.50
Experiment 2		
Immunized	5	4.10
Immunized and treated[c]	5	4.09
Treated[d]	3	0.00

[a]Immunized groups were given a 0.1 LD_{50} dosage of *Listeria Monocytogenes* stain 10403. twelve days later all groups were challenged with dosage of 10 LD_{50} of *L. monocytogenes* 10403.

[b]Two days following challenge dosage of L. monocytogenes, the number of viable bacteria in the spleens of each rat were determined by a standard spleen clearance assay. Logs of protection and expressed as logs protection were: Average number of bacteria in spleen (log10) of the different groups was subtracted from the average number of bacteria (log10) in a group of non-immunized rats challenged with 10 LD_{50}.

[c]Rats were treated with 0.75 mg/kg isoproterenol and 50 mg/kg theophylline twice daily beginning on 7 days following immunization with *L. monocytogenes* and continuing until spleen clearance assay on day 14.

[d]Rats were treated with 0.75 mg/kg isoproterenol and 100 mg/kg theophylline twice daily beginning 5 days prior to challenge with 10 LD_{50} of *L. monocytogenes* and continuing until the spleen clearance assay was done.

MANUSCRIPT #6

TITLE: Mast Cell Activation by Myelin Basic Protein Specific T cell Factors: A Mechanism for Lymphocyte Trafficking into the Central Nervous System

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RUNNING TITLE: Antigen Specific T cell Factors and Mast cells

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[3]Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; CNS, central nervous system; TCF, T cell factor; AA, arachidonic acid

ABSTRACT

During the induction of experimental allergic encephalomyelitis (EAE), the endothelial barrier surrounding the central nervous system (CNS) is significantly compromised. This increase in vascular permeability may facilitate the movement of serum proteins and myelin basic protein (MBP) specific T lymphocytes out of circulation and into the CNS. In other systems, changes in vascular permeability is dependent on mediators released from mast cells which have been previously sensitized by antigen specific T cell factors (TCF). We report that spleen and lymph node cells from MBP immunized rats produce a T cell factor which binds to the surface of peritoneal mast cells and mediates their activation in the presence of specific antigen. Mast cells sensitized with supernatants from MBP reactive lymphocytes were found to bind MBP antigen as demonstrated by the use of FITC-labeled MBP. Furthermore, both a rat basophil leukemia cell line (RBL-2H3) and peritoneal mast cells sensitized with T cell supernatants and exposed to either whole MBP or an encephalitogenic peptide were found to metabolize arachidonic acid resulting in the production of a metabolite which may be a peptido-leukotriene.

INTRODUCTION

The induction of cutaneous delayed-type immune responses is dependent on antigen specific T cells of the CD4+ phenotype (1,2). Recognition of specific antigen in the tissues by T cells results in the subsequent release of lymphokines which recruit bone marrow derived mononuclear cells from circulation into the extravascular region. Cutaneous delayed type responses are also dependent on the release of vasoactive amines, particularly serotonin, which is stored in mast cells (3). When appropriately sensitized mice are exposed to antigen in the form of a skin test challenge, mast cells near the site are activated and subsequently release vasoactive amines (4). These mediators act on nearby endothelial cells to increase in vascular permeability and facilitate the movement of antigen specific T cells, mononuclear cells, and soluble proteins out of circulation and into the tissues where antigen is present (5,6).

Many experimental autoimmune diseases are the result of immune responses similar to cutaneous delayed type reactions. Antigen specific CD4+ lymphocytes recognize and respond to autoantigens, resulting in inflammation and mononuclear cell infiltration in the target tissues during the disease. Mast cell degranulation has been found to accompany may of these T cell mediated autoimmune responses. During murine chronic graft-vs-host disease (GVHD), mast cell degranulation accompanies the mononuclear cell infiltration and fibrosis that occurs in the skin (7). In experimental allergic neuritis (EAN), an autoimmune response to components of the peripheral nerves, mast cell degranulation is also believed to contribute to the disease process. Histologic studies

have found that mast cell degranulation accompanies the mononuclear cell infiltration seen in the peripheral nervous system (8). Moreover, pharmacologic agents which alter normal mast cell function have been found to delay or prevent EAN (9). Mast cells also been implicated in other T cell mediated autoimmune diseases including collagen type II arthritis (10), adjuvant arthritis (11), experimental allergic uveoretinitis (EAU) (12) and most recently experimental allergic encephalomyelitis (EAE) (13).

In the contact dermatitis system, mast cell degranulation is mediated through an antigen specific T cell factor (TCF). This factor has a molecular weight of 70 kd and does not cross react with IgE or other immunoglobulin subtypes (14). In contrast to the explosive fusion of storage granules with the cell membrane mediated by IgE, TCF-mediated degranulation proceeds by a slow release of stored mediators which may take place over a number of hours (15).

A TCF, similar to that described in the contact dermatitis system, has also been reported in both the rat collagen type II and adjuvant arthritis models (10,16). Cell free supernatants from either collagen or mycobacterium specific T cells injected directly into rat knees produced mast cell degranulation, inflammation and cellular infiltration in the joint. Biochemical characterization of the supernatants from both models has determined that an antigen specific TCF, with a molecular weight of 65 kd is responsible for the activity (10,16).

In previous reports we have demonstrated that mast cell degranulation is an integral component of EAE. Inhibition of normal mast cell function by pharmacologic agents which block mast cell degranulation or antagonize released vasoactive amines, prevents the

MATERIALS AND METHODS

Animals. Inbred Lewis female rats obtained from Charles River were used for immunization with MBP. Retired breeders also obtained from Charles River were used as a source of peritoneal mast cells. All animals were housed under standard conditions in the Animal research facility at the Veterans Administration Hospital (Portland, OR).

Materials. Tyrode's buffer, phosphatidyl-L-serine, Arachidonic acid (AA), calcium ionophore A23187, fluorescein isothiocyanate (FITC), bovine serum albumin (BSA), and FITC labeled goat anti-rabbit antiserum were obtained from Sigma Chemical Co. (St Louis, MO). [^{3}H] AA, NET-298, was purchased from New England Nuclear (Boston, MA). RPMI, MEM, L-glutamine, sodium pyruvate, penicillin, and streptomycin were obtained from Gibco Laboratories (Grand Island, NY). Fetal calf serum was obtained from Tissue Culture Biologicals (Tulare, CA)

Purification of MBP. Guinea pig brains were purchased from Pel-Freez Biologicals (Rogers, AK) and stored at -70 prior to extracting MBP. Extraction of MBP was carried out by previously reported methods (18). MBP was further purified from co-extracted contaminants by ion exchange column chromatography, as previously reported (13). Purity of the MBP preparation was assessed by SDS-PAGE, and determined to be greater that 95%.

Production of encephalitogenic peptide. A peptide of MBP sequence 68-86 (YGSLPQKSQRSQDENPV), previously reported to be encephalitogenic (19), was synthesized by the Merrifield solid phase method on a manual synthesizer. Purification of the peptide was accomplished by gel filtration with Sephadex G-50 (Pharamacia,

Piscataway, NJ), followed by C_{18} reverse phase (RP) chromatography. The amino acid sequence of the synthesized peptide was confirmed by Edman degradation with an Applied Biosystems 470A protein sequencer (Foster City, CA).

Immunization with MBP. Purified MBP was dissolved at 1 mg/ml in saline and emulsified in an equal volume of complete Freund's adjuvant containing 10 mg/ml of heat killed *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI). Eight to twelve week old Lewis rats were immunized with 100 µl of the BP/CFA preparation, distributed equally between the two front footpads.

Cells. RBL-2H3 cells were a gift from P.W. Askenase. These cells were grown in 75 cm^2 Falcon (Lincoln Park, NJ) tissue culture flasks in MEM supplemented with 20% FCS, 2 mM glutamate, and 120 µg/ml gentamicin. Peritoneal mast cells were collected from retired Lewis breeders by lavaging the peritoneal cavities with Tyrode's buffer supplemented with 10 mM HEPES, 0.1% BSA, and 35 ng/ml heparin, pH 7.4. Mast cells were isolated from the resident peritoneal cell population by centrifugation through 22.5% metrizamide as previously reported (20).

Generation of TCF containing supernatants. Supernatants were generated by two different methods. Using the method described by P.W. Askenase, spleen and draining lymph node cells from rats immunized with MBP were placed into a single cell suspension at 5 x 10^7 cells/ml and cultured for 48 hours in RPMI supplemented with 1 mM glutamine (14). Where indicated supernatants were concentrated in a Micro-ProDicon (Biomolecular Dynamic, Beaverton, OR). Supernatants were also derived by placing only draining lymph node cells into culture at 1 x

10^8 cells/ml overnight in RPMI supplemented with 1 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. All cell cultures were incubated at 37° in humidified incubator with 7% CO_2-93% air.

FITC-MBP conjugates. FITC was coupled to purified MBP using a previously reported method (21). In brief 10 mg of MBP was suspended at 5 mg/ml in 0.1 M $NaCO_3$, pH 9.0 to which 25.0 μg of fluorescein isothiocyanate was added. The reaction was allowed to proceed for 15 min at 4°C at which time the pH of the solution was readjusted to pH 9.0. The reaction mixture was then allowed to go overnight at 4°C, protected from light. The FITC-MBP conjugate was purified from the excess FITC by gel filtration using Sephadex G-50.

Flow cytometry. Samples were analyzed for FITC, using a Ortho Diagnostics flow cytometer which was set to analyze FITC.

Arachidonic acid metabolism. To evaluate cell activation with RBL-2H3 cells, monolayers of the cells in 75cm^2 flasks were exposed to T cell supernatants for 2 hours. Following sensitization, the monolayers were washed with Tyrode's buffer and the medium in each flask was replaced with 15 ml of Tyrode's buffer supplemented with 30 μM AA. Phosphatidyl-L-serine was sonicated in Tyrode's buffer and added to the flasks at a final concentration of 10 μg/ml. To the appropriate flasks 10 μg/ml of MBP was added. The cell monolayers were allowed to incubate for 30 min, with gentle agitation every 10 minutes. Following the incubation, supernatants were centrifuged to remove free cells and acidified to pH 3.0 with phosphoric acid. Arachidonic acid metabolites were than extracted from the supernatants by C_{18} solid phase extraction (SPE) columns (Baker Phillipsburg, NJ). To compensate for differences in

extraction efficiencies, 10 μM of PGE_2 was added as an internal standard to each sample just prior to the extraction step. Once RBL-2H3 supernatants were drawn through the SPE columns, the columns were washed 2 ml dH_2O (pH 3.0) and eluted with 2 ml of 100% methanol. Samples were concentrated to near dryness using a Speedvac concentrator (Savant, Farmingdale, NY) and analyzed by HPLC.

The assay was modified for use with peritoneal mast cells. Purified mast cells were suspended at 1 x 10^5 cells/ml in either RPMI or lymph node supernatants in Falcon 2070 tubes (Mountain View, CA), and incubated for 2 hours at 37°. Following the incubation cells were washed with Tyrode's buffer. 1 x 10^6 mast cells were suspended in 5 ml of Tyrode's buffer with 10 μM sonicated phosphatidyl-L-serine and 10 μl of [^{3}H]-AA, in Falcon 3033 tubes. 50 μg of the MBP fragment 68-84 was added to the appropriate tubes for a final concentration of 10 μg/ml, and the mast cell suspensions were incubated for 30 min at 37°C, with gentile agitation ever 10 minutes. Following the incubation, AA metabolites were extracted as previously described.

Sample analysis. Samples were analyzed by HPLC using a Hewlett Packard 1090 HPLC, equipped with a diode array detector. Sample separation was achieved with a Hypersil C_{18}, microbore (2.1 x 100 mm) column (Hewlett Packard, Avondale, PE) using a tertiary gradient consisting of: a linear gradient from 100% A to 100% B over 20 minutes, 100% B from 20 min to 25 minutes, linear gradient from 100% B to 100% C from 25 minutes to 29 minutes, and finally 100% C from 29 minutes to 39 minutes. Buffers used for the separation consisted of A; acetonitrile-methanol-H_2O (15:15:70) pH 3.0, B; acetonitrile-methanol-H_2O (60:30:10), pH 3.0, C; acetonitrile-H_2O (65:35), pH 5.0 (22).

Samples were monitored at both 280 and 229 nm and 1 minute fractions were collected when [^{3}H]-AA was used in the assay. Samples which contained [^{3}H]-AA, were evaluated for AA metabolites by scintillation counting, where individual fractions were mixed with 4 ml of Scintiverse II (Fisher Scientific, Fair Lawn, NJ).

RESULTS

Staining of mast cells. Purified peritoneal mast cells were incubated for 1 hour with T cell supernatants. Cells were washed extensively and incubated with 10 µl of FITC-MBP conjugate for 30 min at 4°. Mast cells were then washed and analyzed by flow cytometery. A small percentage of unsensitized mast cells were found to stain with the FITC-MBP shown in figure 1. However, a considerably larger percentage of the population stained when the mast cells had been sensitized with T cell supernatants, 38% compared to 18%. When T cell supernatants concentrated 25 fold were used to sensitize the mast cells, the percentage of cells staining with the FITC-BP increased to 68%. A second set of mast cells was used to determine if the staining with FITC-MBP could be attributed to IgE on the mast cell surface. Mast cells were incubated with rabbit anti-rat anti-serum for 30 min, washed 3X, and stained with FITC labeled goat anti-rabbit anti-serum. The percent of cells which stained with goat anti-rabbit anti-serum was found to increase only slightly following the exposure to the T cell supernatants, figure 1.

Arachidonic acid metabolism in RBL-2H3 cells. To determine if sensitization with T cell supernatants activates mast cell in an antigen dependent manner, AA metabolism by RBL-2H3 cells was evaluated. In the first control, RBL-2H3 monolayers incubated with only RPMI were exposed to MBP. Arachidonic acid metabolites were extracted from the supernatants and analyzed by HPLC to give the chromatograph shown in figure 2a. Relatively few peaks of significance were detected in the chromatograph from the RPMI control. (The large peak at 15 minutes

represents PGE_2 which was added as an internal control). As a second control RBL-2H3 cells were sensitized with 48 hour spleen/lymph node cell supernatants but not exposed to MBP. When these mast cell supernatants were analyzed for AA products the resulting chromatograph was similar to the RPMI controls, figure 2b. However, some differences, including a small peak at 24 minutes, were seen when the chromatographs in figures 2a and 2b were compared, suggesting that some non-specific activation had occurred.

Finally, analysis of AA metabolites in the supernatants of RBL-2H3 monolayers sensitized with T cell supernatants and exposed to MBP, resulted in the chromatograph shown in figure 2c. The peak at 24 minutes, originally identified in the supernatant control was again present. However, the addition of MBP had resulted in a significant increase in the amount of the product which eluted at 24 minutes. In similar experiments, [^{3}H] AA was added to the cultures. The peak eluting at 24 minutes was labeled with [^{3}H], demonstrating that this peak was a AA metabolite (data not shown).

Arachidonic acid metabolism in peritoneal mast cell. To establish whether other mast cell types could also be activated by the combination of TCF and antigen, peritoneal mast cells were tested in the AA metabolism assay. Purified peritoneal mast cells were incubated with either RPMI or supernatants from lymph node cells, washed and the appropriate samples were exposed to MBP fragment 68-86 in the presence of [^{3}H]-AA. Arachidonic acid metabolites were isolated from the mast cell supernatants by solid phase extraction and analyzed by HPLC. To quantitate the production of AA metabolites, one minute fractions

were collected during the HPLC separation and evaluated for [^{3}H] content. Control cultures sensitized with only RPMI showed little activation even with the addition of MBP 68-86, figure 3a. When mast cells were sensitized with T cell supernatants, a change was seen in the HPLC profile, figure 3b. A peak of activity eluting at approximately 24 minutes was seen in when the mast cells were exposed to TCF and the addition of MBP to the culture significantly augmented the production of this AA metabolite.

DISCUSSION

Previous reports have clearly demonstrated a role of mast cells in the induction of cell mediated immune responses. In the contact dermatitis system, elicitation of a skin test reaction is dependent on vasoactive amines which are released by tissue mast cells (3). In this system it has been demonstrated that mast cell degranulation is mediated through an antigen specific, non-immunoglobulin TCF produced by a subset of activated CD4+ lymphocytes (5). Mast cells "armed" with TCF release serotonin on subsequent exposure to the sensitizing antigen. Serotonin release increases vascular permeability which facilitates lymphocyte egress from circulation into the tissues at the site of antigen (23). When antigen is recognized by MHC-restricted CD4+ T cells moving through the tissue, these cells become activated and respond by producing lymphokines which recruit newly formed mononuclear cells to the site (5).

Evidence to support the role of mast cells in cutaneous delayed-type responses has come primarily from evaluating skin test responses elicited in appropriately sensitized mice. Careful monitoring of skin test sites in the ear has revealed that the response to antigen is biphasic. Shortly after challenge, antigen specific inflammation and edema occurs at the skin test site, then subsides several hours later. By 24 hours, a second swelling is seen at the site of antigen injection. The second phase of the response is characterized by extensive mononuclear cell infiltration and fibrin deposition, characteristic of cutaneous delayed type reactions.

The early phase of the skin test response is though to be mediated by mast cells and is required for the development of the late response.

Normal skin test responses cannot be elicited in either W/W^v or Sl/Sl^d mouse strains (24), which essentially devoid of mature mast cells (25,26). Furthermore, both the early and late phases of the skin test response can be abrogated by pharmacologic agents which alter mast cell function including; reserpine an agent which depletes mast cell of serotonin stores (23); proxicromil an agent which prevents mast cells degranulation (23); and serotonin antagonists such as methysergide and ketanserin, which block the effects of serotonin released by activated mast cells (23,27). These findings imply that the establishment of cutaneous delayed-type responses is dependent on the release of vasoactive amines from mast cells. Presumably the release of these inflammatory mediators causes local changes in vascular permeability which enables MHC-restricted lymphocytes to gain access to antigen deposited in the tissues.

It is well established that vasoactive amines also play a significant role in the development of EAE. In studies evaluating the induction of EAE in different inbred mice strains it was apparent that susceptible stains were generally of the $H\text{-}2^s$ or $H\text{-}2^q$ haplotype (28-30). However, EAE could not be induced in all strains with the $H\text{-}2^s$ and $H\text{-}2^q$ haplotypes, leading investigators to believe that other non-MHC encoded genes were also important in the induction of the disease. Later, Linthicum et al. reported that genes controlling sensitivity to vasoactive amines determined which of the strains with $H\text{-}2^s$ and $H\text{-}2^q$ haplotypes were susceptible to the induction of EAE (31). While these studies support a significant role for vasoactive amines in the induction of EAE, the source of these mediators was not determined. More recently it has been suggested that mast cells located in or near the CNS may be the source of these vasoactive amines. Pharmacologic agents which interfere

with mast cell activity and block delayed type responses in the murine contact dermatitis system, also prevent the induction of EAE (13).

How mast cells are activated to release vasoactive amines during the induction of EAE remains an issue of considerable interest. In previous studies, Paterson et al. found that supernatants from MBP reactive T cells could transfer histologic evidence of EAE (32,33). Spinal cords of rats receiving the supernatants had extensive lesions consisting of mononuclear cell infiltrates along the post capillary venules. These lesions were essentially indistinguishable from lesions seen in rats with clinical EAE induced by an injection of MBP/CFA. Although these studies did not explore the mechanism responsible for the cellular infiltration, these results may be explained by the presence of a mast cell binding TCF in the supernatants. In the contact dermatitis system mast cells sensitized with TCF become responsive to nanogram concentrations of specific antigen (34). A similar factor in the supernatants of MBP specific T cells could make mast cells in or near the CNS responsive to sub-microgram concentrations MBP which may be shed from the myelin sheath (6). The ensuing release of vasoactive amines would act on brain capillary endothelial cells, causing the otherwise highly restrictive blood-brain-barrier (BBB) to become leaky. The increase in vascular permeability would allow soluble serum proteins to diffuse into the CNS, and facilitate cellular infiltration into the region.

There is substantial evidence that a similar TCF acts in other T cell mediated responses. In both the collagen and adjuvant arthritis models, antigen specific, soluble T cell factors have been described (10,16). These antigen specific T cell factors, referred to as arthritogenic factors, have been found to cause significant inflammation and cellular

infiltration when injected into the joints of normal rats (35,36). Substantial mast cell degranulation accompanies cellular infiltration of the joint, alluding to the possibility that arthritogenic factor is the equivalent of the mast cell sensitizing TCF described in the contact dermatitis system.

In light of these previous findings, we have attempted to demonstrate that mast cells can become responsive to MBP following exposure to supernatants from MBP reactive T cells. Initially, purified mast cells were incubated with T cell supernatants and then stained with FITC labeled MBP. A higher percentage of mast cells sensitized with T cell supernatants stained above background levels, 38% compared to 18%, Figure 1. To demonstrate that staining increases with higher levels of TCF, mast cells were incubated with supernatants that were concentrated 25 fold. When analyzed, the concentrated supernatants had sensitized a significantly higher percentage of the mast cell population than non-concentrated supernatants, where the percentage of positively staining cells increased from 38% to 64%, shown in Figure 1. Control staining experiments were also conducted to determine whether the binding of FITC-MBP could be attributed to immunoglobulin on the mast cell surface. Following exposure to the primary antibody, rabbit anti-rat, mast cells were stained with FITC-labeled goat anti-rabbit anti-serum. While some mast cells did stain positive with the goat anti-rabbit anti-serum, these levels were considerably less than what was seen with supernatant sensitized mast cells. Furthermore, staining levels of sensitized and unsensitized mast cells populations remained relatively constant. Therefore, the binding of FITC-MBP to mast cells can not be attributed to MBP specific immunoglobulin in the supernatants.

Although relatively little is known about mast cell activation during delayed type immune responses, mast cells exposed to TCF should undergo metabolic changes when exposed to the sensitizing antigen. Previous studies have demonstrated that mast cells activated with anti-IgE, metabolize AA through either the cyclooxygenase or lipoxygenase pathway to producing prostaglandins or leukotrienes respectively. In an attempt to demonstrate that supernatant sensitized mast cells become activated when exposed to MBP, the production of AA metabolites was monitored. Initially, the rat basophil leukemia cell line RBL-2H3 was incubated with T cell supernatants and then exposed to MBP in the presence of exogenous AA. When the supernatants were analyzed for AA metabolites, a peak absent or present at low levels in control cultures was produced at significant levels by sensitized RBL-2H3 cells exposed to MBP. To confirm that the peak was an AA metabolite, [^{3}H]-AA was added to the cultures. When fractions were collected and analyzed for [^{3}H] content, the peak at 24 minutes was found to contain the label, positively identifying it as an AA metabolite.

Studies have found that RBL cells have biochemical and functional properties which are more representative of "atypical" or mucosal mast cells than "typical" or serosal mast cells (37). To determine if the supernatants could also sensitize serosal mast cells, the assay was repeated with peritoneal mast cells which are thought to be similar to mast cells found in the CNS. When the supernatants from peritoneal mast cells were analyzed for AA metabolites, a peak absent or present at low levels in control cultures was enhanced when T cell supernatant sensitized mast cells were exposed to MBP. Interestingly, the peak had the same retention time as the AA metabolite produced by RBL-2H3 cells

sensitized with similar supernatants. This finding was unexpected since mucosal mast cells activated with anti-IgE have been reported to metabolize AA differently than activated serosal mast cells. Mucosal mast cells generally metabolize AA through the lipoxygenase pathway, producing leukotrienes, while serosal mast cells preferentially utilize the cyclooxygenase pathway and produce prostaglandins (37). Therefore, it is very interesting that both sensitized RBL-2H3 cells and peritoneal mast cells appeared to metabolize AA through the same pathway when exposed to MBP. While the identity of this AA metabolite remains unknown at this time, its UV spectrum and retention time during HPLC analysis has lead us to suspect it is a peptido-leukotriene. When a leukotriene C (LTC_4) standard was analyzed by HPLC under the same conditions as the samples, it was found to elute earlier than the AA metabolite peak. This has lead us to believe that the mast cell product may be LTD_4 or LTE_4.

Although leukotriene are potent mediators of inflammation, we had not previously considered their possible role in the elicitation of EAE. However, work done from other investigators suggests that leukotriene release does play a significant role in EAE. DiMartino et al reported that the dual lipoxygenase and cyclooxygenase inhibitor SK&F 86002, [6-94-fluoropheny)2,3-Dihydro-5(4-Pyridinyl)imidazo(2,1-b)thiazole], which blocks both immune and non-immune triggered mediator release from mast cells, effectively suppresses the development of clinical EAE (38). These finding indicate that the release of lipoxygenase products may act in concert with vasoactive amines to increase vascular permeability during EAE.

The increase in vascular permeability caused by mediators released from activated mast cells provides an explanation for the breakdown of the BBB which is seen at the onset of EAE. This generalized increase in vascular permeability would facilitate the movement of fluid, and cells from circulation. The net effect of this would be edema and mononuclear cell infiltration in the CNS, two of the hallmarks of EAE.

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Figure 1

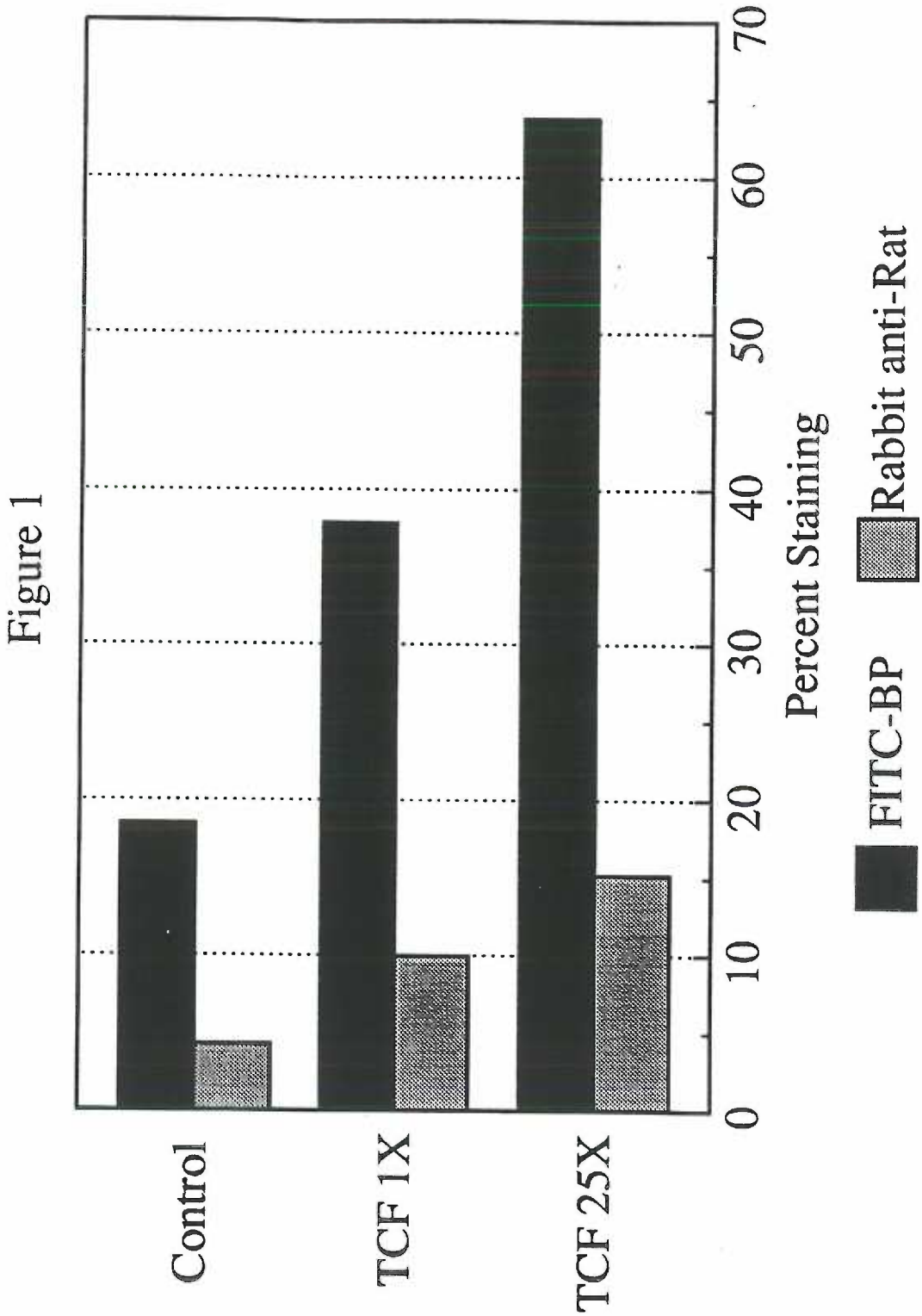

Control
TCF 1X
TCF 25X
0
10
20
30
40
50
60
70
Percent Staining
FITC-BP
Rabbit anti-Rat

Figure 1

Purified mast cells were incubated with either Tyrode's buffer, spleen/lymph node supernatants or concentrated spleen/lymph node supernatants for 1 hour at 37°C. Following the incubation cells were washed with RPMI, resuspended in 500 µl and incubated for 30 min with either 5 µl of rabbit anti-rat Ig or 40 µl of FITC-MBP (1 mg/ml). The FITC-MBP stained cells were washed and fixed with formaldehyde. Mast cells incubated with rabbit anti-rat Ig were also washed and stained for 30 min with 10 µl of goat anti-rabbit anti-serum, washed and then fixed with formaldehyde. Samples were analyzed on an Ortho flow cytometer setup to read FITC. Values are reported as percentage of cells staining above the cutoff which was arbitrably set at channel 250.

Figure 2a

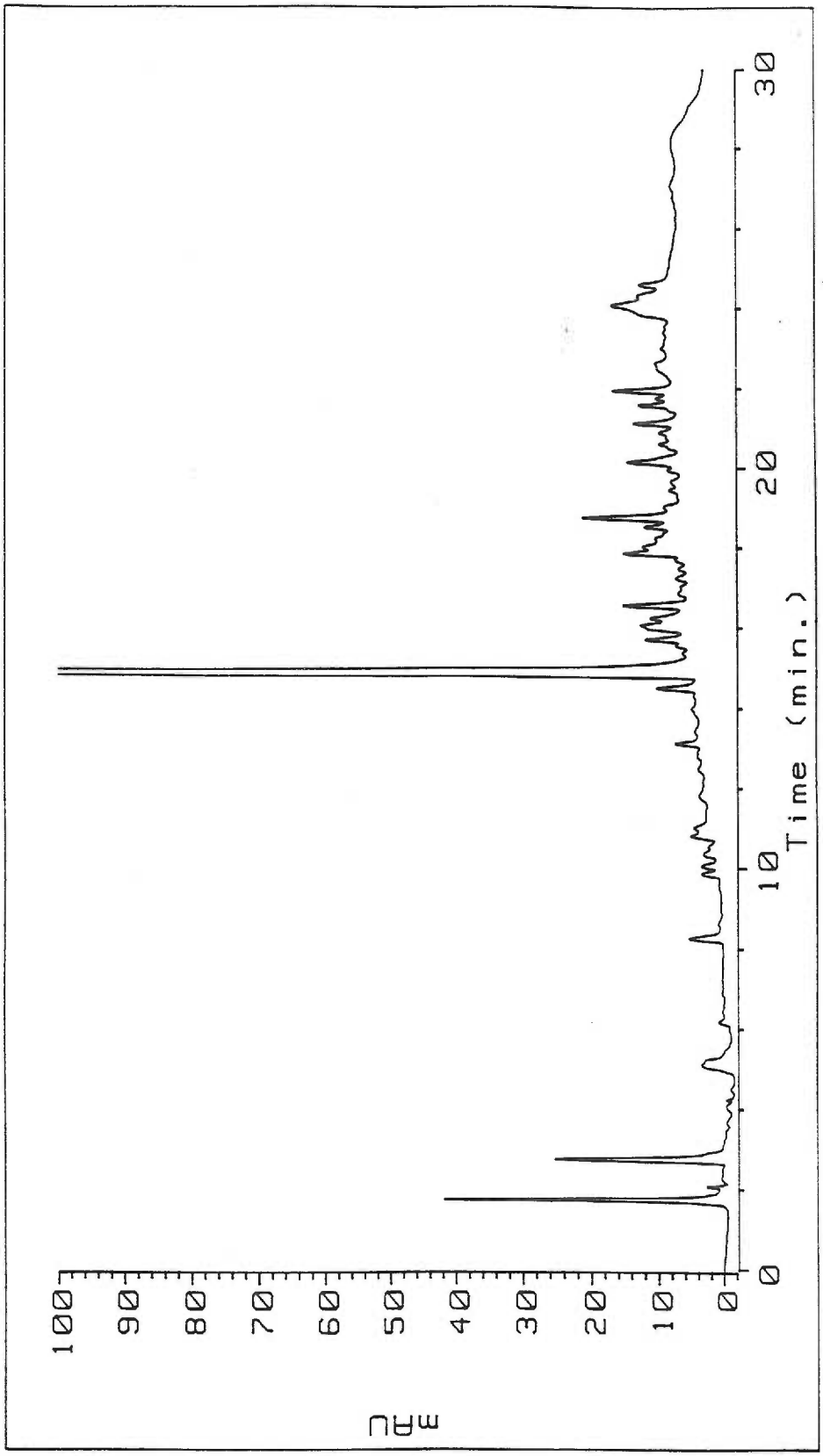


Figure 2b

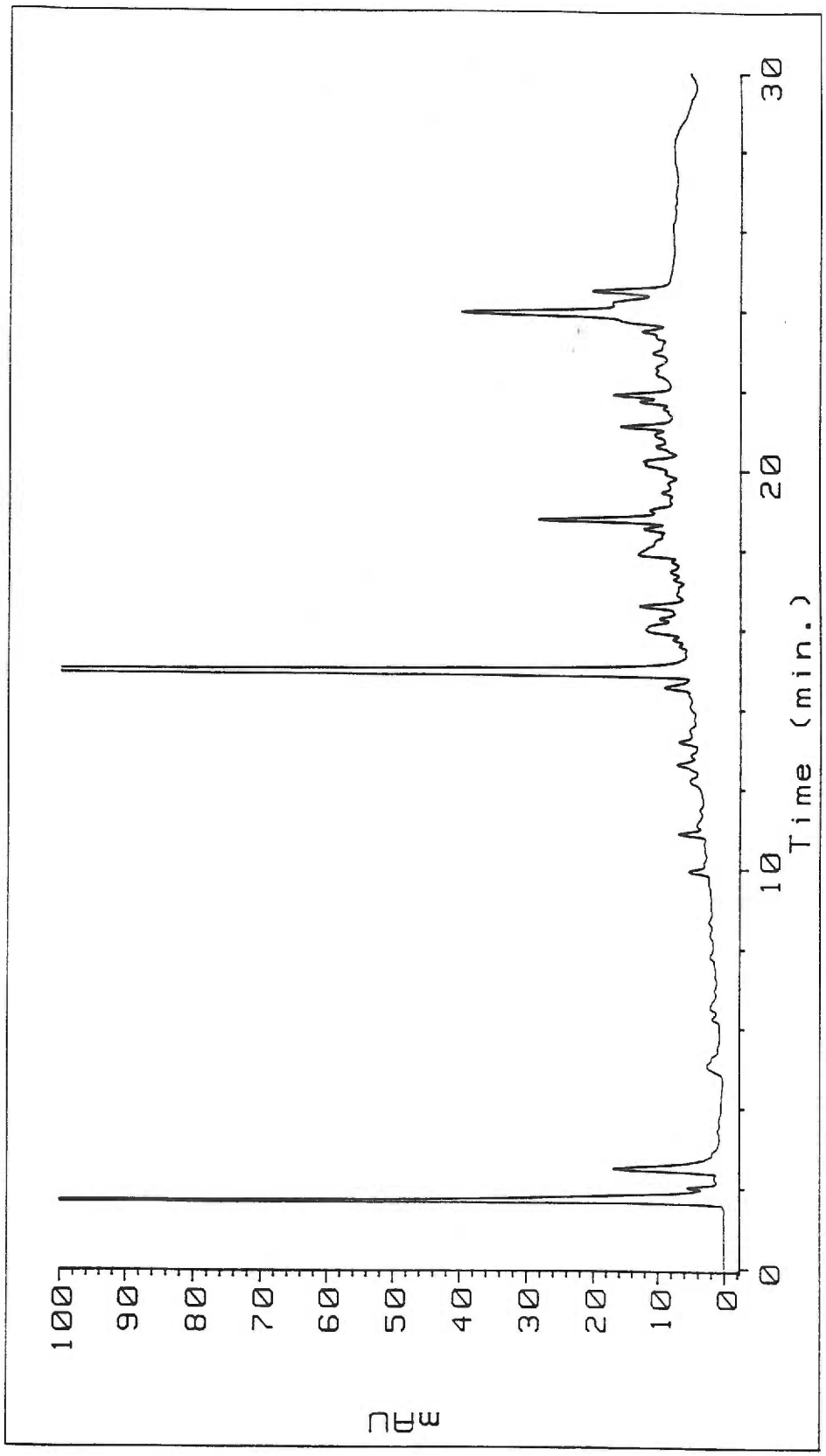


Figure 2c

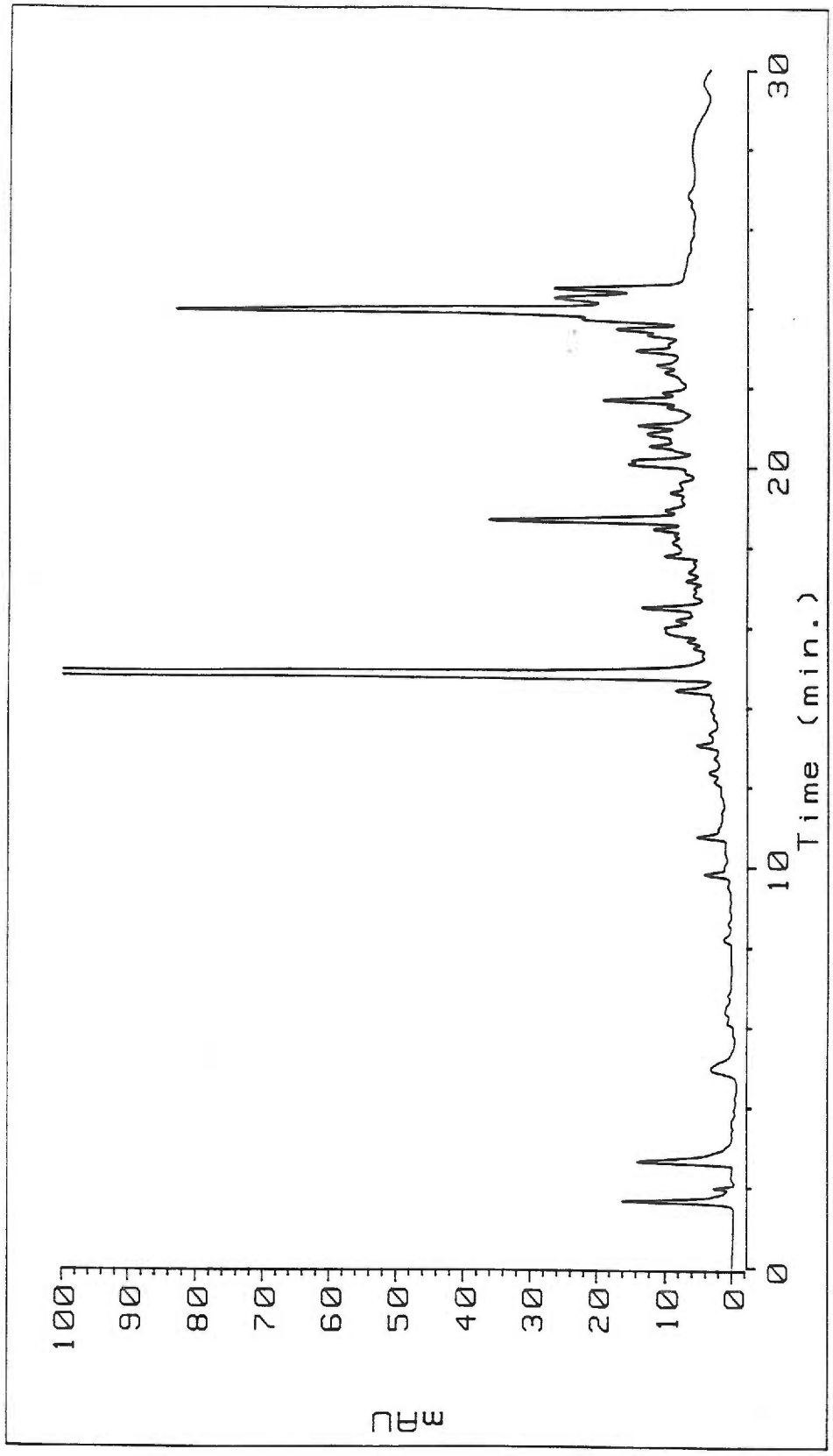
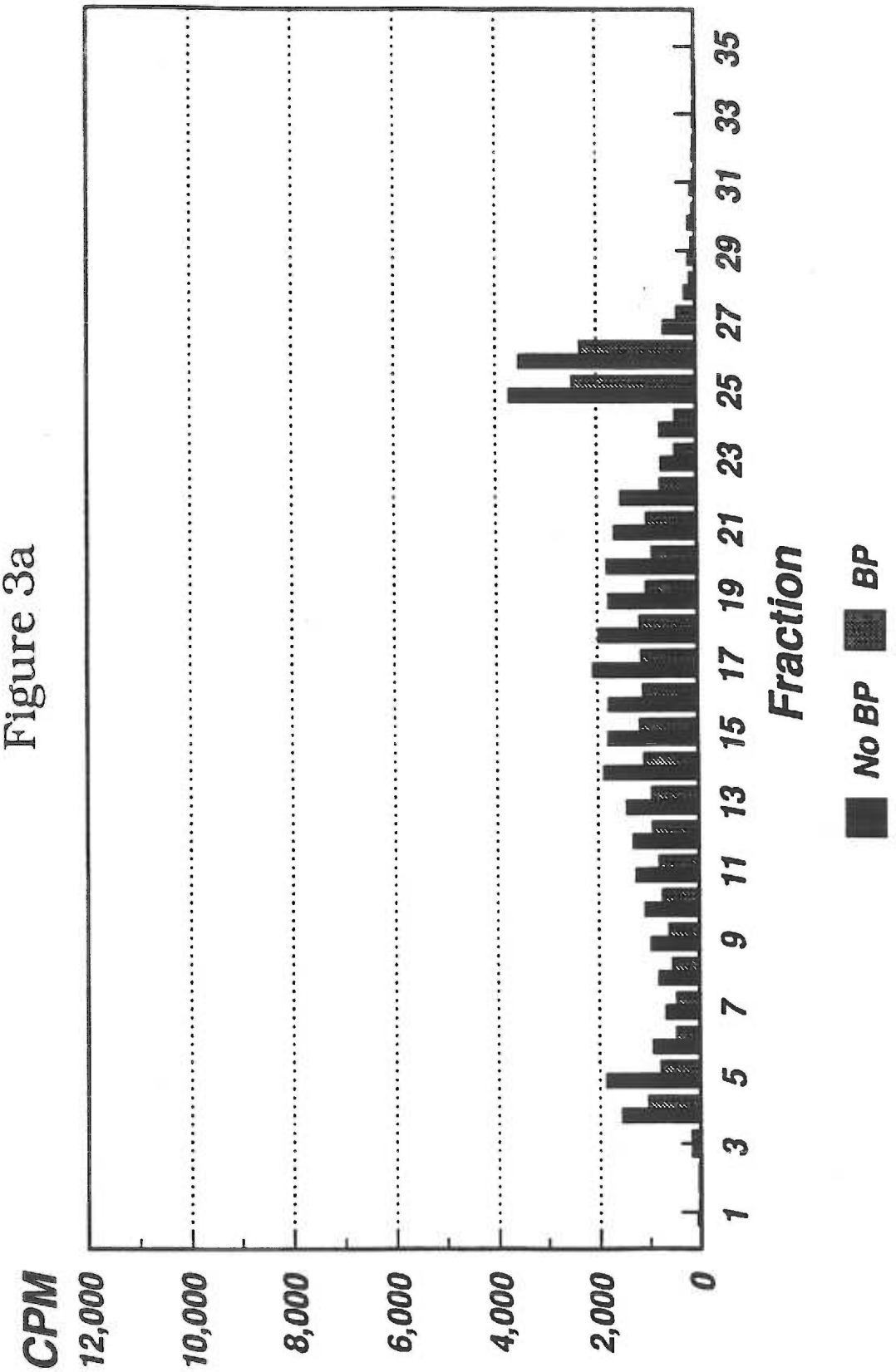


Figure 2.

Confluent monolayers of RBL-2H3 cells were incubated with either 20 ml of RPMI or 20 ml of spleen/lymph node cell supernatants from rats immunized with MBP/CFA. Following sensitization monolayers were washed and covered with 15 ml of Tyrode's buffer supplemented with 30 μM AA, and 10 μg/ml phosphatidyl-L-serine. 10 μg/ml of MBP was added to appropriate flasks, and all the monolayers were incubated for an additional 30 min. Following the incubation, AA metabolites were purified from the supernatants and analyzed by RP C_{18} HPLC. The chromatograph for RBL-2H3 cells sensitized with only RPMI is shown in Figure 1a, while the AA metabolites produced by RBL-2H3 cells sensitized with supernatants resulted in the chromatograph shown in Figure 2b. RBL-2H3 cells sensitized with supernatants and subsequently exposed to MBP resulted in the chromatograph shown in Figure 2c.

Figure 3a


CPM
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10,000
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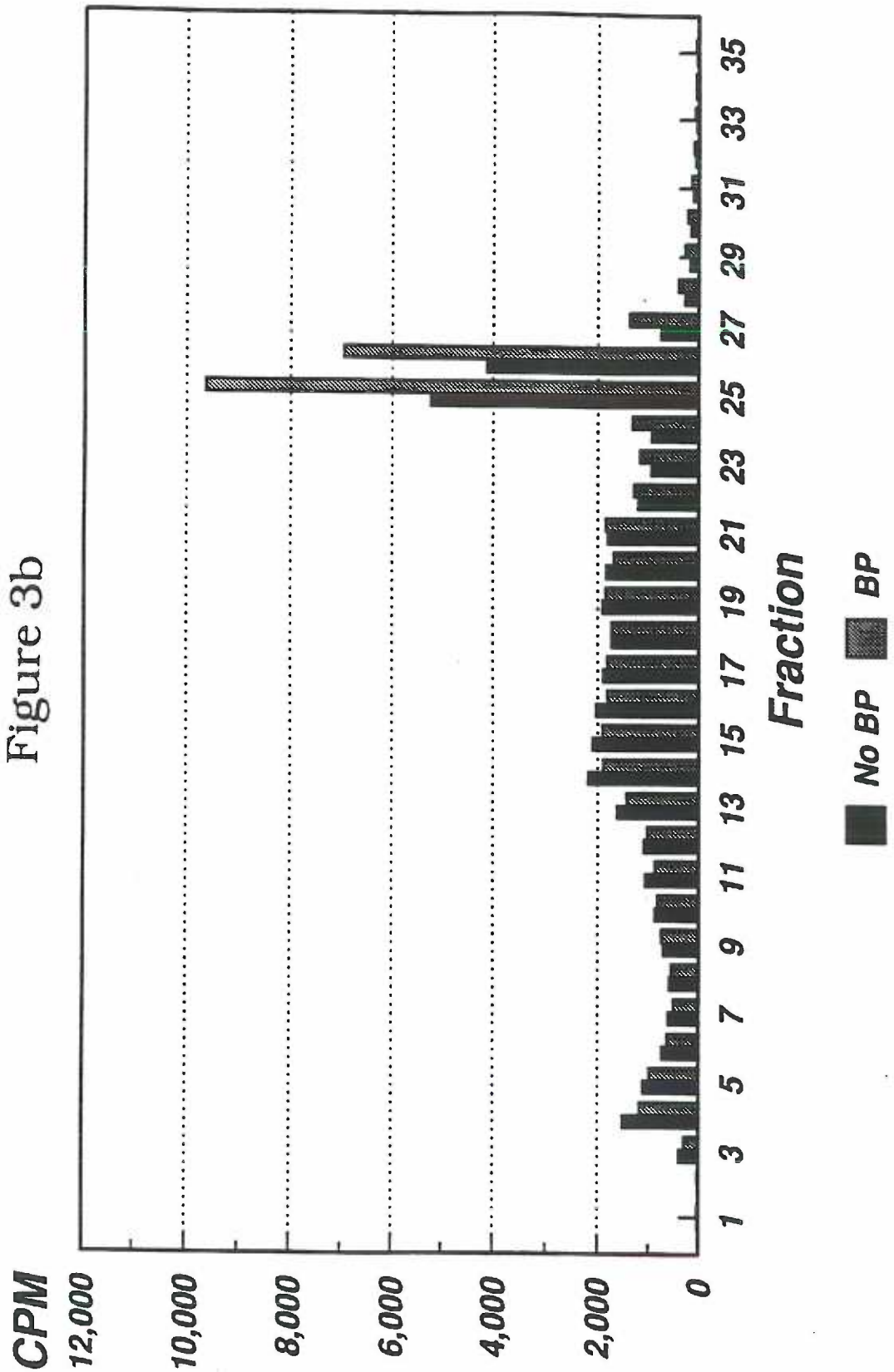
6,000
4,000
2,000
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
Fraction
No BP
BP

Figure 3b


CPM
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No BP
BP

Figure 3

Purified peritoneal mast cells were sensitized by incubating the cells at 1 x 10^5 cells/ml with either RPMI or supernatants derived from the lymph nodes of rats immunized with MBP/CFA. Following sensitization the mast cells were washed and 1 X 10^6 mast cells were resuspended in 5 ml of Tyrode's buffer supplemented with 10 µl of [^{3}H]-AA, and 10 µg/ml phosphatidyl-L-serine. 10 µg/ml of MBP fragment 68-86 was added to appropriate flasks, and the cultures were incubated for an additional 30 min. Following the incubation, AA metabolites were purified from the supernatants separated by RP C_{18} HPLC, and analyzed by scintillation counting. The activity of fractions from mast cells sensitized with RPMI and exposed or not exposed to the MBP fragments are shown in Figure 3a. Mast cells sensitized with lymph node supernatants and exposed or not exposed to the MBP fragments are shown in Figure 3b.

CONCLUSIONS

Relatively little is known about the events which allow lymphocytes to infiltrate the CNS during the course of EAE. One proposal is that capillary endothelial cells pinocytose MBP shed from the myelin sheath and present it on the lumen side in association with MHC class II antigens (1). Interactions between antigen-presenting endothelial cells and MBP reactive T cells moving through capillary beds could result in lymphocyte activation and subsequent lymphocyte infiltration of the CNS. In support of this model, endothelial cells isolated from the cerebral vasculature of SJL mice with EAE, express Ia (2) and can be used to activate lymphocytes to adoptively transfer clinical EAE. Furthermore, histologic studies have identified Ia positive cerebral vascular endothelial cells at sites where cellular infiltration has taken place (3).

To further explore the antigen presenting role of endothelial cells in EAE, adoptive transfer studies were designed to determine if MHC-compatibility between donor endothelial cells and transferred lymphocytes was required for the induction of EAE. For these studies F1-to-parent bone marrow chimeras were used. Activated MBP reactive cells from one parental strain were adoptively transferred into the chimera recipients. In this particular transfer, the MBP reactive cell population was semi-syngeneic with respect to the transferred bone marrow, but allogeneic with respect to the host vascular endothelium. Successful adoptive transfer into these recipients demonstrates that MHC compatibility between vascular endothelial cells and lymphocytes is not a requirement for successful induction of EAE. However, these

findings also raise the issue of how activated MBP reactive lymphocytes are able to cross the highly restrictive BBB as they infiltrate the CNS.

We have attempted to explain lymphocyte trafficking into the CNS by using the model proposed by Askenase and Van Loveren, which resulted from their work in the murine contact dermatitis system (4,5). In cutaneous delayed-type responses to antigens such as picryl chloride and oxazalone, responsive T cells are stimulated to produce an antigen specific TCF which sensitizes mast cells in a manner analogous to IgE (6). On exposure to the sensitizing antigen, TCF "armed" mast cells are stimulated to release potent mediators (5,7). The release of vasoactive amines, particularly serotonin in murine models, acts on nearby endothelial cells. The resulting increase vascular permeability allows antigen specific T cells to infiltrate the region where antigen has been deposited (8,9).

In numerous reports, investigators in the EAE system have made observations which are consistent with the model proposed in the contact dermatitis system. Linthicum et al., demonstrated that sensitivity to vasoactive amines was important in the induction of murine EAE (10), and that the serotonin antagonists could alter the course of clinical disease (11). Brosnan et al., reported that a generalized increase in vascular permeability occurs in the CNS of rats with EAE. Serum proteins normally excluded from the CNS were readily detected in the spinal cords of rats with EAE (12,13). Furthermore, this change in vascular permeability coincided spatially with the ensuing clinical paralysis, occurring first in the lumbar section of the spinal cord and progressing proximally.

Lymphocyte trafficking mediated through the release of vasoactive amines predicts that activated lymphocytes, regardless of antigen specificity, should cross into the CNS of rats with EAE. Therefore, the population of T cells infiltrating the CNS at the onset of EAE should not be limited to those that recognize neuroantigens, but represent all activated lymphocyte populations in the animal at the time of disease induction. This prediction is in agreement with the findings of Lisak et al. Rats were co-immunized with tetanus toxoid (TT) and MBP and allowed to develop EAE (14). At the height of clinical disease, the rats were sacrificed and the antigen specificity of lymphocytes infiltrating the spinal cord was evaluated in a limiting dilution assay. In these studies the frequency of TT reactive cells found in the CNS was comparable to that of MBP reactive cells. These studies concluded that lymphocytes can infiltrate the CNS during the onset of clinical EAE, regardless of their antigen specificity.

Finally, reports of antigen specific TCFs are not unique to the contact dermatitis system. In both the collagen type II arthritis and adjuvant arthritis models, TCFs analogous to those described in the contact dermatitis system, have been reported (15-17). Injection of supernatants from either collagen type II or mycobacterium responsive T cell lines into the joints of Lewis rats results in inflammation similar to what is seen with adoptive transfer of antigen specific T cells. Histologic studies have found that extensive mast cell degranulation occurs with the transfer of the T cell supernatants. Purification of "arthritogenic factor" has yielded an antigen specific TCF with a molecular weight comparable to the TCF described in the contact dermatitis model.

A similar activity has also been derived by culturing lymph node cells from rats immunized with MBP/CFA (18,19). Paterson et al, found naive rats developed extensive mononuclear infiltrates around post capillary venules in the CNS when they were injected with cell free, lymph node cell culture supernatants. Although the basis for histologic EAE mediated by these T cell supernatants was not pursued, an antigen specific TCF similar to arthritogenic factor may have been responsible for these findings.

Collectively, these findings of other investigators and in work presented in this thesis make a strong case for mast cells playing a significant role in the pathology of EAE. Therefore, I would like to conclude by proposing a model which attempts to explain lymphocyte trafficking and other inflammatory events which are important in the induction of EAE.

Proposed Model for the Induction of EAE.

Following immunization with MBP/CFA populations of MBP-reactive lymphocytes develop and can be easily identified in the spleen and draining lymph nodes of the sensitized animals. In the model proposed by Askenase et al., two populations of MBP reactive cells would develop in response to the sensitization (5). Both cell populations express the CD4 surface antigen, although it is possible to separate the two cell populations on adherent properties using of nylon wool (20). One cell population, referred to as the "early" T cell population responds to antigen stimulation by secreting an antigen specific, mast cell tropic, TCF. The second or "late" cell population is the classic "helper" T cell which recognizes antigen in the context of Ia, and once activated

mediates a delayed type hypersensitive response though the release of various lymphokines.

In the rat model of EAE it is unclear whether two unique cell populations actually exist. The observation that cloned rat MBP reactive T cells are capable of mediating EAE contradicts the two cell model in EAE. One explanation for this paradox is that the immune system is a complicated series of overlapping effector pathways, and perhaps in some cases cell mediated immune responses can proceed in the absence of the TCF. The second possibility is that in the rat system, a single cell population is responsible for secreting the antigen specific TCF and recognizing MBP presented by antigen presenting cells. This is supported by findings in the collagen arthritis system, where arthritogenic factor is made by antigen specific, T cell lines (16). Perhaps the TCF molecule is a secreted form of the T cell receptor (TCR), although this proposal is at odds with current models for antigen recognition by T cells.

Current dogma is that T cells do not recognize free antigen, but only antigens that are presented in association with self Ia molecules. However, there is compelling evidence indicating that the TCR may be capable of binding free antigen (21,22). Perhaps association of antigen with Ia is more important for signaling T cell activation than for the actual recognition of antigen. In light of these findings it is possible that a single molecule could serve as a secreted factor which activates mast cells, and a membrane structure which is important in T cell activation. This proposal is of course purely speculative and a molecular approach will be required to fully elucidate the origin of the mast cell binding TCF.

Following the production of TCF, mast cells in the CNS would become responsive to MBP. In numerous histologic studies involving a variety of different species, mast cells have been identified in the brain and spinal cord (23-26). In the CNS, mast cells are prevalent along the blood vessels of calvarial dura mater, and the spinal dura mater (27). Large numbers of mast cells have also been observed as periganglionic rings around dorsal root ganglia (DRG) and on or within the dorsal and ventral roots proximal to the DRG. Moreover, the largest numbers of spinal root mast cells are associated with the lumbar DRG, while mast cells are less prevalent in the thoracic DRG (27). It is interesting that the region distribution of mast cells in the DRG is consistent with the pattern of edema and inflammation seen in EAE (28).

How TCF sensitization of mast cells occurs remains unclear. If TCF is secreted by T cells in circulation then it must transverse the endothelial layers to sensitize mast cells in the tissue. One possibility is that TCF is actively transported across endothelial layers. This is consistent with the observations of Askenase, where purified factor injected i.v. sensitizes tissue mast cells (5). An alterative possibility is that TCF does not need to cross the endothelium. Activated T cells have endoglycosidases which may allow them to degrade the extracellular matrix and move through tissues as part of a immune surveillance mechanism (29,30). T cells moving through the tissues could produce TCF and sensitize nearby tissue mast cells.

Mast cells degranulate in response to basic proteins (31), including MBP (32). However, it seems unlikely that under normal physiologic conditions that MBP shed from the myelin sheath would initiate mast cell degranulation independent of other events. In a report by H. Weiner

et al., concentrations of 50 μg/ml of MBP caused extensive mast cell degranulation as assayed by the release of B-hexosaminidase (30). We have also observed that purified MBP at concentrations as low as 10 μg/ml can stimulate purified mast cells to release [^{3}H] serotonin. However, it is unlikely that these levels of MBP are ever seen in the CNS. Furthermore, native MBP is inactivated by serum proteins (33) and quickly degraded by proteases in both the serum and CNS (34,35). Our studies have found that any proteolytic degradation of native MBP significantly reduces this molecules capacity to stimulate mast cell degranulation, although the encephalitogenic activity may remain intact.

In the murine contact dermatitis system, TCF sensitized MC become sensitive to nanogram concentrations of antigen (7). Sensitization of MC in the CNS by an analogous TCF would make then sensitive to small amounts of MBP shed from the myelin. Activation of the mast cells would result in the gradual release of serotonin and histamine from storage granules within the cells. This release probably does not proceed by fusion of storage granules with the cell membrane but occurs through a slow shuttling of the granule contents to produce a sustained release of vasoactive amines (36). Previous findings by Linthicum et al., have demonstrated that sensitivity to vasoactive amines is a requirement for successful induction of EAE in the mouse (10,11), however, the source of these mediators was not fully evaluated. We have provided evidence in the rat model that mast cells are the source of these vasoactive amines. Agents which impair normal mast cell function by either depleting vasoactive amines, inhibiting MC degranulation, or acting as vasoactive amine antagonists, inhibit or significantly reduce both clinical and histologic EAE.

Arachidonic acid metabolites are also released by activated MC (37) and may act in concert with the vasoactive amines to increase vascular permeability (38). We report that culture supernatants from lymph node cells of rats immunized with MBP, sensitize purified MC so that they metabolized arachidonic acid when exposed to MBP. While the identity of the arachidonic acid product(s) is not known at this time, it is probably a peptido-leukotriene, a family of molecules which can mediate dramatic changes in vascular permeability.

Interestingly, alterations in vascular permeability resulting from leukotriene release are consistent with previous reports in the EAE system. The dual lipoxygenase and cyclooxygenase inhibitor SK&F 86002, [6-94-fluoropheny)2,3-Dihydro-5(4-Pyridinyl)imidazo(2,1-b)thiazole], which blocks both immune and non-immune triggered mediator release from mast cells, effectively suppresses the development of clinical EAE (39). In contrast, inhibiting only prostaglandin production with the cyclooxygenase inhibitor indomethacin does not prevent EAE (39), and actually exacerbates clinical disease (40). One possible explanation for the more severe clinical disease is that indomethacin treatments shunt arachidonic acid through the lipoxygenase pathway which upregulates leukotriene production.

Mast cell degranulation could also produce the edema which has been shown to contribute to the pathology of EAE. Loss of integrity of the BBB allows serum proteins and fluid to leave the circulation and enter the CNS (12,13). This would account for the edema while activation of the clotting cascade, perhaps mediated in part by mast cell proteases (41), would produce fibrin deposition which correlates closely with the progression of clinical disease (42-44).

It is interesting that supernatants from MBP reactive T cells described by Paterson, et al. produced histologic but not clinical EAE. Normally during the induction of either actively induced and adoptively transferred EAE, TCF produced by activated MBP responsive cells, would sensitize mast cells in the CNS. When exposed to MBP, perhaps shed from the myelin sheath, sensitized mast cells would release mediators which increase vascular permeability and facilitate lymphocyte egress from circulation into the CNS. The subsequent recognition of MBP by responsive cells infiltrating the CNS would trigger the release of lymphokines, which mediate an ill-defined series of events and ultimately cause expression of clinical EAE. When cell free supernatants were transferred, mast cell degranulation and alterations in the blood brain barrier may have still occurred. But the MBP reactive cell population was absent in these recipients and the cascade of events leading to expression of clinical disease could not occur. However, circulating lymphocytes responsive to other antigens were able to infiltrate the CNS, and accumulate around the post capillary venules.

Mast cell granules contain large amounts of neutral serine proteases which are released during mast cell activation. In addition to facilitating additional mast cell degranulation, neutral serine proteases may damage the myelin sheath. Previous studies have found a selective loss of MBP from the myelin sheath of rats with EAE (45). Other investigators have found that supernatants from activated MC have myelinolytic activity which degrades proteins contained in the myelin sheath, with MBP being particularly sensitive to the degradation (32).

All indications are that MBP must be released from the sheath, pinocytosed by antigen presenting cells and expressed in association

with MHC class II antigens to stimulate CD4+ lymphocytes which mediate EAE. However, it is unclear why MBP, a protein which is intimately associated with the myelin sheath, would normally be available stimulate these autoaggressive lymphocytes. Degradation of MBP by mast cell enzymes could make portions of the MBP molecule available to stimulate MBP reactive T cells as they infiltrate the CNS at the onset of EAE.

In our studies we have found that an encephalitogenic region of the MBP molecule is released from the myelin by the action of mast cell proteases. Mast cell chymase, a chymotrypsin "like" enzyme with specificity for aromatic amino acid residues, cleaves MBP between glycine and tyrosine at positions 68-69 and between the two phenylalanines at positions 89-90 thereby liberating the 69-89 fragment (GSLPQKSQRTQDENPVVHF) from the myelin sheath. Subsequent degradation of 69-89 at the carboxy-terminus by carboxypeptidase A, results in the immediate loss of phenylalanine reside 89, followed by the gradual loss of histidine at position 88. The resulting fragment contains the entire encephalitogenic region of the MBP molecule and is resistant to subsequent degradation. Immunization with the fragment results in active EAE, while MBP reactive cell lines stimulated with the peptides adoptively transfer EAE.

Recently, activated MC have been found to produce a TNF "like" activity (46). TNF has been found to increase the expression of adhesion molecules on capillary endothelial cells, an event required for lymphocyte extravasiation (47). The release of TNF by activated CNS mast cells may act on nearby endothelial cells, upregulating the expression of adhesion molecules. This allows circulating lymphocytes to attach to the

endothelium and facilitate their movement from circulation into the nearby tissues. Furthermore, TNF has been implicated as a mediator in the damage to the myelin sheath during clinical EAE (48,49). The release of TNF by activated mast cells in the CNS would facilitate the mononuclear cell infiltration that is seen in EAE, and could mediate damage to the myelin sheath, producing clinical paralysis. The release of TNF by activated MC in the CNS is an area of research which deserves attention in the future.

SUMMARY

In many tissues mast cells act as "sentinels", which regulate lymphocyte egress out of the circulation and into the surrounding tissues, through the release of potent mediators. The focus of this thesis has been centered on mast cell function during EAE and the associated mechanisms through which such activity may occur. Activation and subsequent degranulation of mast cells in or near the CNS could compromise the integrity of the BBB and facilitate lymphocyte trafficking into the CNS. During EAE, mast cell degranulation may be mediated by an antigen specific TCF which triggers mast cell degranulation. Enzymes released by activated mast cells may contribute to the disease process by degrading MBP causing the release of encephalitogenic peptides which are capable of stimulating encephalitogenic T cells.

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