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Bitar, Dina Mousa

THE SUPPRESSIVE EFFECTS OF ORAL MYELIN BASIC PROTEIN ON EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

The Ohio State University

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THE SUPPRESSIVE EFFECTS OF ORAL MYELIN BASIC PROTEIN
ON EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Dina M. Bitar, B.Sc., M.Sc.

The Ohio State University
1986

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DEDICATION

To my husband "Ahed" and son "Omar"
ACKNOWLEDGMENTS

I would like to express my sincere appreciation and gratitude to my adviser, Dr. Caroline Whitacre, who has provided guidance and encouragement through the course of this study. I wish to extend my thanks to the members of my advisory committee, Drs. Ronald Glaser, Norman Somerson and Raymond Lang for their interest and suggestions.

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Finally, I would like to thank my husband Dr. Ahed Abdulkhalilq and family for their support and encouragement during this study.
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Major Field: Immunology.
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<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine</td>
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<td>IFA</td>
<td>Incomplete Freund's adjuvant</td>
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<td>LNC</td>
<td>Lymph node cells</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<td>Myelin basic protein-splenic leukocytes</td>
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<td>MLN</td>
<td>Mesenteric lymph nodes</td>
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<td>Multiple sclerosis</td>
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<td>Purified protein derivative</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>STI</td>
<td>Soybean trypsin inhibitor</td>
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INTRODUCTION

Multiple sclerosis (MS) is a demyelinating central nervous system (CNS) disorder that was initially described as early as 1838 (Carswell, 1838), but it was Charcot who recognized the characteristic clinical and pathologic features of the disease (Charcot, 1877). There are 250,000 persons afflicted with MS in the U.S.A. alone (Rose, 1979; Davison and Cuzner, 1980; Morell and Norton 1980). The disease usually begins between the second and fifth decade of life (McFarlin and McFarland, 1982). It is more prevalent among whites, rare in Japanese and Chinese and absent in pure Bantu and Eskimo populations (Waksman, 1981). In northern Europe and the U.S.A., MS is more prevalent in people bearing the HLA haplotypes A3, B7, and DW2 or DR2 (Waksman, 1981).

Although much has been learned about MS, the cause and pathogenesis of this disease remain unknown. Currently there are two theories regarding the etiology of MS: 1. MS is an autoimmune disorder directed against an antigen of myelin or the oligodendrocyte, the myelin producing cell in the CNS. 2. MS represents an abnormal response to infection by one or more viruses. The possibility that MS is an autoimmune disease triggered by a viral infection in genetically predisposed hosts links the two hypotheses (Waksman, 1981). The autoimmune theory is supported by the animal model experimental allergic encephalomyelitis (EAE), which shares clinical, immunologic, biochemical, physiologic, and pathologic features with MS (Alvord et al., 1984; Raine, 1984; Pender and Sears, 1984).
I. EAE: General Features

EAE is an autoimmune demyelinating disease of the CNS, which can be induced in a variety of common laboratory animals, e.g., rats, mice, guinea pigs, rabbits, monkeys and sheep (Paterson, 1959; 1966). To induce EAE, animals are injected with whole homogenized CNS tissue, purified myelin basic protein (MBP) or encephalitogenic peptides, prepared by enzymatic cleavage of MBP or synthesis, emulsified in complete Freund's adjuvant (CFA) (Paterson, 1966; 1971a; 1976; Rauch 1974).

The Lewis rat is highly susceptible to EAE and serves as the prototype animal model in EAE research (Levine and Wenk, 1963). Ten to fourteen days after sensitization with encephalitogenic antigen and CFA, rats begin to show clinical neurologic signs including weight loss, loss of tail tonus, ataxic gait, paraparesis, and paralysis of both hind legs usually accompanied by incontinence and fecal impaction (Paterson, 1959; 1966; 1971a; 1976). The histopathologic changes characteristic of EAE consist of perivascular mononuclear infiltrates in the brain and the spinal cord, affecting the white matter more than the grey matter (Paterson, 1959; 1966; 1976; Alvord, 1970). The inflammatory infiltrates consist of histiocytes, lymphocytes, and a few plasma cells. One of the earliest immunopathologic changes in EAE in the Lewis rat is perivascular fibrin deposition (Oldestone and Dixon, 1968). Demyelination is also observed in the vicinity of the perivascular mononuclear infiltrates (Paterson, 1976).
EAE has been shown to be mediated by a cellular immune process (Paterson, 1960; Stone, 1961; Ortiz-Ortiz et al., 1976) and thus can be transferred to a naive recipient by means of lymphoid cells, but not serum (Chase, 1959). Lymph node cells (LNC) taken from Lewis rat donors, sensitized 9 days previously with MBP-CFA, can transfer EAE to normal syngeneic recipients (Paterson, 1960; Swierkosz and Swanborg, 1975), while spleen cells are ineffective in directly transferring the disease (Paterson, 1966). In 1977, Panitch et al., reported that spleen cells from MBP sensitized donors, in contrast to LNC, were able to transfer EAE following in vitro culture with concanavalin A (Con A). In 1979, Richert et al., showed that spleen cells following in vitro culture with the specific antigen MBP, were also capable of transferring EAE. T lymphocytes were shown to be responsible for transfer since anti-thyl.1 serum plus complement treatment abrogates transfer of disease (Bernard et al., 1976; Ortiz-Ortiz et al., 1976). Recently, it has been shown that T cells capable of causing murine EAE belong to the helper (Ly1, L3T4) subset (Pettinelli and McFarlin, 1981; Dialynas et al., 1983; Lando and Ben-Nun, 1984).

MBP is the major constituent in CNS tissue which contributes to its encephalitogenic activity (Kies, 1965; 1973). MBP represents about 30% of the total protein content of myelin and about 1% of the weight of whole nervous tissue. It has been characterized chemically and biochemically and its complete amino acid sequence is known (Carnegie et al., 1967; Eylar and Thompson, 1969). MBP is 18,000-20,000 daltons in size (Rauch and Einstein, 1974;) and is heat
stable. Its ability to induce disease or combine with antibodies is not impaired when heated with urea or treated to 100°C in aqueous solution. MBP is cationic mainly due to the high content of arginine, lysine and histidine, and it has no sulfhydryl groups (Eylar and Thompson, 1969). In aqueous solution, MBP has an open double chain structure with little internal folding (Braun and Brostoff, 1977). This conformation is thought to explain its susceptibility to proteolytic degradation by such enzymes as trypsin, chymotrypsin, thermolysin, aminopeptidase, carboxypeptidase A and B and pronase (Smith, 1977). In the Lewis rat host, there is a range of encephalitogenicity of MBP depending on the species from which the MBP was derived. MBP purified from guinea pig CNS is the most encephalitogenic of all MBP for the Lewis rat, and on a per weight basis, is twenty-five times more encephalitogenic than bovine, human or rabbit MBP and ten times more active than rat MBP (Dunkley, et al., 1973; McFarlin et al., 1973).

II. Suppression of EAE

One of the primary goals in EAE research has been to suppress or prevent the disease (Ferraro and Cazzullo, 1949). EAE has usually served as the model of choice to test MS therapeutic modalities e.g. cyclophosphamide, cyclosporine, MBP (Davison and Cuzner, 1980), hyperbaric oxygen (Warren et al., 1978), and interferon (Abreu, 1982) and all of the previously mentioned therapeutic approaches have been tested in EAE first prior to MS clinical trials. The term prevention is generally used when the experimental manipulations are
performed prior to EAE sensitization, whereas the term *suppression* is generally used when the manipulations follow sensitization. The term *therapy* or *reversal* of EAE are used when the manipulations are initiated after the onset of clinical signs of EAE (Alvord et al., 1965).

Several suppressive regimens for EAE have been described (reviewed in Paterson, 1976). Because of the autoimmune mechanisms operative in EAE, the use of immunosuppressive drugs has proven important. For example, Bolton and Cuzner (1980), found that Cyclosporin-A is effective in both prevention and therapy of EAE in guinea pigs. Furthermore, treatment of rats with Cyclosporin-A resulted in suppression of clinical EAE (Borel et al., 1976). However, Cammisuli et al., (1984) found that treatment with Cyclosporin-A (for 14 days starting from day of sensitization) can delay but not prevent EAE in rats. The authors attribute the discrepancy to the fact that in the former studies, rats were treated for a longer time (18-26 days) and were then observed only for short periods after Cyclosporin-A withdrawal. The immunosuppressive drugs cyclophosphamide and azothioprine are both effective in EAE when used prophylactically but are of little therapeutic benefit (Paterson et al., 1969; Paterson, 1971b). Other immunosuppressive drugs, e.g., purine and pyrimidine analogs, folic acid antagonists and alkylating agents have been utilized to suppress EAE (Paterson, 1976).

Specific antiserum has also been used to alter the course of EAE. First, the ability of anti-brain antibody to affect EAE was investigated. When rats were sensitized to nervous tissue-adjuvant
and injected daily or every other day with rat immune serum derived from animals developing EAE and containing high titers of complement fixing antibrain antibodies, these recipient animals developed a milder disease (Paterson and Harwin, 1963; Paterson et al., 1965). Second, injections of antilymphocyte serum also have been shown to inhibit (Waksman et al., 1961) or suppress (Leibowitz et al., 1968) EAE in guinea pigs. Recently, a great deal of interest has centered upon the therapeutic potential of antisera directed against cell surface antigens encoded by the major histocompatibility complex (MHC). In vivo administration of monoclonal antibodies directed against the I-A subregion of the MHC has been found to prevent and reverse EAE in mice (Steinman et al., 1981). Furthermore, the influx of lymphocytes into the CNS in EAE can be inhibited by anti-I-A administration (Steinman et al., 1983). Waldor et al., (1985) studied the reversal of EAE using a monoclonal antibody specific for the L3T4 marker found on mouse helper T cells. When administered before the onset of clinical EAE, it prevented the development of EAE in mice. Furthermore, administration of the monoclonal antibody after the first disease symptoms appeared, reversed the clinical course of EAE.

Proteinase inhibitors have been used by some investigators to suppress EAE. Brosnan et al. (1980) found that aminomethylcyclohexane carboxylic acid, \( \Sigma \)-amino caproic acid and \( p \)-nitrophenylguadino benzoate, which are inhibitors of plasminogen activator, suppressed clinical EAE in Lewis rats. In the same study, rats
treated with Trasylol (a trypsin inhibitor from bovine lung) were not protected, and, in fact, had an earlier onset of clinical signs and more severe paralysis than control animals. Furthermore, leupeptin (Osanai and Nagai, 1984), aminopeptidase B and esterase (Aoyagi et al., 1984) were shown to be effective in suppressing EAE in guinea pigs.

Total body X-irradiation prior to sensitization with CNS tissue-adjuvant prevents the development of EAE in rats (Paterson et al., 1963). However, if given 17-19 hours after sensitization, total body irradiation is observed to exert no inhibitory influence (Paterson and Beisaw, 1963).

There are several reports concerning the immunosuppressive effects of corticosteroids on EAE (reviewed in Komarek et al., 1971). In general, these drugs are known to be very effective if given within a few days after CNS sensitization and well before the appearance of clinical signs of disease, but are not remarkably effective if administered after the onset of clinical EAE. An exception has been reported by Greig et al. (1970), who found that both melengestrol and hydrocortisone not only prevented the development of EAE in Wistar rats, but also reversed the established disease. Arnason et al., (1969) studied the effect of estrogen and progesterone on the development of EAE in rats. Estrogen inhibited EAE, but progesterone had no suppressive activity and even appeared to increase the disease severity.

The course of EAE can also be affected by dietary manipulations. Guinea pigs deprived of vitamin C are inhibited from developing EAE
(Mueller et al., 1962). High doses of vitamin A have also been shown to suppress EAE in guinea pigs (Vladutiu et al., 1968).

Even though injection of animals with CNS tissue and CFA induces EAE, injection of CNS tissue homogenates alone rarely results in the complete picture of EAE (Paterson, 1976). Furthermore, adjuvant alone has been used to suppress EAE (Kies et al., 1958; Svet-Moldavskaya et al., 1958; Lumsden, 1964; Lisak et al., 1974; Englert et al., 1981).

III. Suppression of EAE with MBP

EAE can also be suppressed by immunologically specific means using whole CNS tissue or MBP or even MBP peptides. In 1949, Ferraro and Cazzulo were the first to observe that repeated injections of nervous tissue homogenates and incomplete Freund's adjuvant (IFA) in guinea pigs diminished their capacity to develop EAE following sensitization to CNS-CFA. In later studies, MBP rather than whole CNS tissue has been used to inhibit EAE in guinea pigs, rats and monkeys (Raine et al., 1980; Arnon et al., 1980; Hashim, 1980; Salk et al., 1980; Eylar et al., 1980). MBP emulsified with IFA has been quite effective in preventing the development of clinical signs and histopathologic changes of EAE in Lewis rats (Swierkosz and Swanborg, 1975). Specifically, pre-treatment with eight intradermal injections of MBP-IFA (100 μg each) rendered Lewis rats refractory to EAE. It was shown that LNC from pre-treated (MBP-IFA) and challenged (MBP-CFA) rats were incapable of transferring clinical or histopathologic EAE. LNC from these MBP-IFA tolerized rats, when transferred to
normal syngeneic recipients, suppressed clinical but not histologic EAE when these recipients were later challenged with an encephalito-
genic emulsion. Adoptive transfer of unresponsiveness was abrogated if LNC were treated in-vitro with anti-thymocyte serum and complement prior to transfer. Suppressor cells were found in the glass wool nonadherent LNC population, and coupled with the previous observation suggested that suppression was mediated by T cells (Welch and Swanborg, 1976). Further work provided evidence that there is also a macrophage-like suppressor cell present in the spleen of these MBP-IFA tolerized rats (Welch et al., 1978). When rats were pre-
treated by an intravenous (i.v.) injection of MBP (800 µg) in aqueous solution before challenge, they were also protected from EAE. However, rats tolerized in this manner failed to transfer unrespon-
siveness (Swierkosz and Swanborg, 1977). Pretreatment with MBP-IFA resulted in long-lasting unresponsiveness (8 weeks), whereas MBP administered intravenously in soluble form produced a more transient unresponsiveness (3 weeks). MBP-IFA has also been used to suppress acute (Raine et al., 1977) and chronic (Raine et al., 1977; 1978) EAE in guinea pigs. A model of chronic relapsing EAE in strain 13 guinea pigs was utilized, and it could be shown that disease was suppressed by a series of injections of MBP-IFA (1.4 mg MBP total) given 1 to 5 weeks post sensitization with spinal cord-CFA. This suppression appeared to be quite long-lasting, since guinea pigs rechallenged 1 to 2 years later were refractory to EAE.
Not only was the encephalitogenic protein MBP found to be effective in preventing or suppressing EAE, but other nonencephalitogenic proteins and/or peptides had similar protective effects. Hashim (1981) showed that a nonencephalitogenic synthetic peptide (S42) prevented the development of EAE in strain 2 guinea pigs. S42 consists of four repeating linear sequences of a modified encephalitogenic determinant for the guinea pig. S42 failed to induce EAE directly, but when 100 μg of the peptide in CFA was injected 40 days prior to EAE sensitization, there was a reduction in clinically evident disease as well as a marked reduction in histologic lesions in the brains and spinal cords of treated guinea pigs. However, a single injection of 100 μg of peptide S42 given together with MBP-CFA did not prevent the development of EAE.

Arnon and Teitelbaum (1980) used a synthetic peptide, designated copolymer 1 (Cop 1) composed of L-alanine, L-glutamic acid, L-lysine and L-tyrosine (M.W. 23,000 daltons) for EAE suppression studies. Cop 1 is nonencephalitogenic, but can suppress EAE in guinea pigs when administered in IFA or in aqueous saline solution after the encephalitogenic challenge. Cop 1 was ineffective in preventing the disease if injected in aqueous solution prior to the encephalitogenic challenge. Guinea pigs receiving a suppressive regimen of Cop 1, remained protected for at least six months in response to repeated challenge with MBP. Cop 1 was also effective in the suppression of EAE in rabbits, mice, rhesus monkeys and baboons. Cop 1 and IFA were also used successfully in the prevention or therapy of chronic
relapsing EAE in juvenile strain 13 guinea pigs.

In 1981, Strejan and coworkers investigated the capacity of MBP incorporated into liposomes to prevent EAE. Adult Hartley guinea pigs pretreated with a single intracardiac (i.c.) injection of 75 μg human MBP inserted into phosphatidyl-serine liposomes were protected from an MBP-CFA challenge. These animals demonstrated no clinical manifestations of acute EAE and exhibited reduced histopathologic changes in comparison with controls. In a later report (Strejan et al., 1984), the authors showed a significant reduction in both clinical and histopathological aspects of the chronic relapsing form of EAE in strain 13 guinea pigs, following i.c. administration of 3 weekly doses of 50 μg MBP-liposomes.

Sriram et al. (1983) investigated the ability of MBP coupled to syngeneic spleen cells to prevent the development of EAE in SJL/J mice. MBP or control ovalbumin coupled spleen cells (1 x 10^8) were injected i.v., 7 days prior to sensitization with mouse spinal cord and CFA. MBP coupled spleen cells prevented the induction of clinical EAE, while ovalbumin coupled spleen cells had no protective effect. McKenna et al. (1983) reported that guinea pig MBP coupled to syngeneic spleen leukocytes suppressed the subsequent induction of EAE in Lewis rats. Investigation into the mechanism of suppression induced by MBP coupled to spleen cells provided evidence consistent with a suppressor T-cell (McKenna, 1984).

The application of specific MBP-reactive T cells to treatment of CNS autoimmune disease was first demonstrated by Ben-Nun et al. (1981). These investigators isolated MBP specific T lymphocyte lines
from LNC of MBP-CFA sensitized Lewis rats. These cells proliferated in vitro only in response to MBP and were able to transfer severe acute EAE to normal recipients (Ben-Nun et al., 1981). When these T line cells were inactivated by irradiation or mitomycin C treatment before transfer, the recipients failed to develop EAE but were protected against subsequent EAE induction by MBA-CFA. Irradiated T line cells had a prophylactic effect, but were ineffective if administered simultaneously with sensitization or later. Furthermore, prophylaxis was restricted to active disease induction, since inactivated T line cells did not alter disease induced by the passive transfer of non-inactivated MBP-specific T line cells.

IV. Application to MS

EAE is thought to be a valid model system for studying the human demyelinating diseases, particularly MS (Alvord, 1970; 1984; Paterson 1977; 1978; 1979). Chronic relapsing EAE, an experimental model that can be induced in strain 13 guinea pigs and SJL mice, resembles MS in terms of its clinical and pathologic courses. After injection of whole spinal cord homogenate and adjuvant, guinea pigs and mice exhibit a remitting-relapsing clinical course with unpredictable time periods between relapses (Stone et al., 1965; Raine et al., 1974; Wisniewski et al., 1977; Lublin et al., 1981). MBP has been studied as a possible antigen in the autoimmune response to myelin observed in MS. MBP or fragments of MBP have been identified by radioimmuno-assay in cerebrospinal fluid (CSF) of persons with active MS (Whitaker, 1977; Whitaker et al., 1980). Anti-MBP antibodies are
present in the serum or CSF of MS patients (Panitch et al., 1980; Paterson et al., 1981). Also CSF or peripheral blood lymphocytes from MS patients demonstrate proliferative responses to MBP (Sheremata et al., 1974; Lisak et al., 1977). The lesions of EAE are similar to those of MS with respect to their perivascular pattern of inflammation and primary demyelination (Kabat et al., 1951; Tourtellotte, 1971). The majority of MS patients demonstrate increased levels of CNS IgG synthesized locally as well as oligoclonal bands in their CSF (Chu et al., 1983). Oligoclonal bands have been reported to occur in EAE in rabbits (Whitacre et al., 1981; 1982; Petrasek et al., 1981), in guinea pigs (Mehta et al., 1981; Olsson et al., 1982), and in monkeys (Chu et al., 1983).

In MS patients, IgG seems to be deposited within the sclerotic plaque (Tourtellotte and Parker, 1966; 1967; Tourtellotte, 1970). However, only a few animals with EAE have IgG deposits in their CNS lesions (Oldestone and Dixon, 1968). The production of complement-dependent demyelinating antibodies by animals with EAE and patients with MS provides a strong piece of evidence that immunologic events play an important role in the development of both diseases. When EAE or MS sera are added to cultures of myelinating cerebellum, myelin breakdown and swelling of glial cells is observed (Bornstein, 1973). Animals developing EAE have delayed-type hypersensitivity (DTH) reactions to a variety of neuroantigens (Shaw et al., 1965). In contrast, there is no evidence of DTH specific for CNS tissue in MS patients (Lisak et al., 1968), although the
Peripheral blood cells of some patients generated migration inhibitory factor (MIF) in response to CNS tissue antigens, a response considered to be an in vitro correlate to DTH (Bartfeld et al., 1972; Sheramata et al., 1976).

Prevention, suppression or treatment of acute EAE has provided much useful information on several possible therapeutic regimens for MS, particularly those involving MBP (Raine, 1983). Three clinical trials testing the efficacy of MBP in MS have been conducted to date. Campbell et al. (1973) and Gonsette et al. (1977) reported the results of intramuscular administration of MBP in MS patients. Small doses of human MBP (5mg/dose) were injected at weekly intervals. Neither beneficial nor harmful effects were observed in either study. Recently, Romine and Salk (1983) reported the use of porcine MBP in MS patients. Daily subcutaneous injections of 75 mg MBP where employed, a regimen which had previously been shown to suppress acute EAE in experimental animals. Of eleven patients who entered the treatment, nine completed 200 days or more of MBP injections. The results suggested improvement in five patients and none in four, but there was evidence of continued disease activity in all but two patients. Thus all 3 clinical trials testing the parenteral administration of MBP in MS patients provided no evidence for long-term improvement.
V. Oral Tolerance

The oral administration of antigen is a very effective route for the induction of tolerance and suppression of both antibody and cell mediated immunity (CMI). Exposure to erythrocyte, soluble protein, reactive chemical and bacterial antigens via the oral route can decrease the systemic immune responses to the same antigen administered parenterally (Wells, 1911; Well and Osbourne, 1911; Chase, 1946; Andre et al., 1975; Kagnoff, 1978a,b; Asherson et al., 1977; Mattingly and Waksman, 1978; Chiller et al., 1979; Richman et al., 1981; MacDonald, 1982).

Antigens introduced by the oral route may be absorbed to varying degrees across the intestine either intact or as fragments. Studies on the enteric absorption of human serum albumin (Andre et al., 1974) and bovine serum albumin in rats (Thomas et al., 1974) show that only about 0.1% of an intragastric dose of these antigens reaches the circulation as intact macromolecules. It seems advantageous to the host to have stimulation of intestinal mucosal IgA antibody, and diminished or absent systemic immune responsiveness to antigens encountered by the gastrointestinal route. The local IgA antibody aids in preventing the absorption of antigenic material from the intestinal tract and provides local immunity to viruses and bacteria. Otherwise, nonpathogenic organisms or food antigens absorbed from the intestine might initiate harmful allergic responses or cross-react with self components and result in autoimmune disease (Kagnoff, 1974).
The phenomenon of oral tolerance has been known since the 1800's. In 1829, Dakin reported that American Indians ingested Rhus leaves of poison ivy. This common practice reduced the incidence and severity of contact dermatitis. In 1911, Wells and Osborne reported that guinea pigs which were fed diets containing corn, developed specific immunological tolerance to zein, a major protein constituent of corn. In 1946, Chase showed that the oral administration of dinitrochlorobenzene to guinea pigs prevented subsequent antibody and delayed hypersensitivity responses induced by intracutaneous injection of the contact irritant. Asherson et al. (1977) showed that multiple feedings of contact irritants to mice abrogated contact sensitivity responses and antibody production induced by skin painting. In these studies, two types of suppressor cells were found after feeding the sensitizing agents oxazolone or picryl chloride. Suppressor cells were found in the Peyer's patches (PP) and mesenteric lymph nodes (MLN) after a single feeding of picryl chloride. After three feedings of either agent, suppressor cells were detected in the spleen. These suppressor cells were presumably B lymphocytes as shown by their ability to form rosettes with erythrocytes coated with antibody and complement and their resistance to anti-<i>o</i> serum and complement. The spleens and peripheral lymph nodes of mice fed contact sensitizing agents and then skin painted also had T cells capable of interfering with lymphocyte proliferative responses when mixed with LNC from painted only mice. This was also shown by injecting cells from fed and sensitized mice into normal recipients, which were then painted with contact
agent and DNA synthesis measured 4 days later in regional lymph
nodes. Kagnoff (1978a) showed that feeding mice sheep red blood
cells (SRBC) resulted in decreased DTH responses specific for SRBC,
and the observed suppression was shown to be due to suppressor cells.
Adoptive transfer of spleen cells from erythrocyte-fed mice to normal
mice inhibited the induction of DTH in the recipients. Miller et
al. (1979) reported that mice fed 20 mg ovalbumin (OVA) 7 days before
subcutaneous sensitization with OVA-CFA, had a significant decrease
in both humoral (antibody titers) and CMI responses specific for
OVA, when assayed in vivo by ear swelling or in vitro by antigen-
induced T cell proliferation. Either spleen cells (1.6 x 10^8) or
LNC (1 x 10^8 peripheral and MLN) but not serum from OVA-fed donors
transferred suppression to normal recipients as measured in vivo by
ear swelling.

Oral administration of antigen also results in a decrease in
humoral immunity. Thomas and Parott (1974) found that multiple
intragastric feedings of 25 mg bovine serum albumin (BSA) to rats
resulted in tolerance. A very small amount of antibody was detected
in the serum but anti-BSA producing cells could not be demonstrated
in the lamina propria, in PP, MLN or spleen of tolerized rats.
Kagnoff (1978b) reported that mice fed multiple doses of SRBC did not
respond to a subsequent SRBC intraperitoneal challenge as measured by
splenic IgM plaque forming cell (PFC) responses. Suppression
appeared to be due to a soluble factor in the serum of mice fed the
antigen, and not due to T suppressor cells, since suppression of the
PFC response could be transferred to normal mice by the injection of
serum from SRBC-fed mice. Andre et al. (1975) also described a serum suppressor factor in SRBC-fed mice which was thought to be an immune complex containing antigen and IgA. Mattingly and coworkers (1978, 1980) showed that rats fed $10^{10}$ SRBC had detectable antigen-specific suppressor cells in the PP and MLN after 2 daily feedings. After 4 days of feeding, suppressor cells were found in the thymus and spleen but not in the PP or MLN. These suppressor cells blocked IgM and IgG PFC responses to SRBC in in-vitro cultures and suppressed DTH responses to SRBC when transferred to syngeneic recipients. Furthermore, these authors have shown that spleen cells from SRBC-fed mice can produce two factors in vitro that influence the induction of the in-vitro anti-SRBC PFC response, a helper factor $T_H F$, M.W. 30,000 - 45,000 daltons and a suppressor factor $T_S F$, M.W. 60,000 - 75,000 daltons. These factors are distinguished by their kinetics of action, their ability to function across MHC barriers and by the cell types responsible for their production. Vaz et al. (1977) and Hanson et al. (1977) demonstrated that the oral administration of a single dose of OVA resulted in carrier-specific, long-lasting (8 weeks) inhibition of the IgE and IgG1 anti-OVA serum antibody response following primary and secondary immunization with OVA-CFA. Production of suppressor T cells was reported as one possible mechanism to explain this unresponsivness (Ngan et al., 1978; Richman et al., 1978).

In summary, various mechanisms have been shown to be involved in the induction of oral tolerance. For example, antigen-specific
suppressor T cells are demonstrable after feeding soluble protein antigens (Ngan et al., 1978; Miller et al., 1979), erythrocyte antigens (Mattingly et al., 1978) and contact sensitizing agents (Asherson et al., 1977). Suppressor B cells are also formed after feeding contact sensitizing agents (Asherson et al., 1977). A non-immunoglobulin serum suppressor factor (Kagnoff, 1978) and antigen-antibody complexes (Andre et al., 1975; 1977) have been reported to mediate unresponsiveness after SRBC feeding. No single laboratory has studied all the known mechanisms of suppression. It is quite possible that more than one mechanism may be operative in a single study.

From the literature cited, administration of an antigen by the oral route has a powerful tolerizing capacity. However, it is surprising that no work has appeared in the immunological literature involving attempts to suppress EAE by the oral administration of MBP. The purpose of the present study was to look at the effect of oral MBP administration on subsequent EAE induction in Lewis rats. I addressed the following questions: Can the oral administration of MBP result in tolerance as measured by a) inhibition of EAE and b) suppression of lymphocyte proliferation to MBP? Is oral tolerance to MBP antigen-specific? What are the kinetics of induction of oral tolerance to MBP? Can unresponsiveness to MBP be transferred from MBP-fed rats to normal Lewis recipients?
MATERIALS AND METHODS

Rats

Male Lewis rats (6-10 weeks old) were purchased from Harlan Sprague-Dawley, Inc., Indianapolis, Indiana. They weighed 175-200 gm at the time the experiments were begun. In one experiment only, four week old (100-125 gm) male Lewis rats were used. All rats were maintained on laboratory chow and tap water.

Preparation of myelin basic protein

Myelin basic protein was prepared from whole CNS tissue by the method of Swanborg et al. (1974). Guinea pig or rat spinal cords or brains (Rockland Inc., Gilbertsville, Pa.) were subjected to lipid extraction using chloroform-methanol (2:1, v/v) and the residue then washed and acid extracted using 0.01 N HCl. The acid extract was precipitated using 50% saturated ammonium sulfate, resuspended in 0.01 N HCl, dialyzed against deionized water and then lyophilized. The MBP preparation was further purified by passage over a Sephadex G-50 superfine (Pharmacia, Uppsala, Sweden) column in 0.01 N HCl. The individual protein-containing fractions were monitored by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE), to test for the presence of the major MBP band (18,500 daltons). Appropriate fractions were pooled, dialyzed against water and lyophilized to obtain column-purified MBP.
Iodination of myelin basic protein

Labelling of MBP with $^{125}$I was performed by a modification of the technique described by Lamoureux et al. (1967). Twenty microliters of 0.5 M phosphate buffered saline (PBS), pH 7.3, was added to 1mCi of $^{125}$I sodium iodide (Amersham, Arlington Heights, IL # IMS-30). Ten micrograms of guinea pig MBP in 20 μl of 0.5 M PBS was added to the above mixture. Five microliters of chloramine T (Aldrich Chem. Co., Milwaukee, WI. #85-731-9), (5mg/ml in water) was then added and allowed to react for two minutes. To stop the reaction, 50 μl of sodium metabisulfite (1.25 mg/ml in water) was added. The $^{125}$I-MBP was separated from unreacted iodide by chromatography of the mixture on a column (25 ml pipette) of Sephadex G-25 fine (Pharmacia). The $^{125}$I-MBP was eluted with 0.1 N HCl and collected in 0.5 ml fractions. The radioactivity in each fraction was determined using a Beckman Gamma 7000 counter. A volume of 0.5 M phosphate buffer equal to the fraction volume was added to each fraction to adjust the pH to 7. Fractions containing $^{125}$I-MBP (specific activity of 2.4 μCi/μg) were pooled, dialyzed against saline, aliquoted into 3 ml fractions, and stored at -20°C.

Preparation of liposomes

To prepare MBP-liposomes, a modified procedure of Patel and Ryman (1976) was used. Briefly, 200 mg dipalmitoyl phosphatidylcholine (DPPC) (Sigma Chemical Company, St. Louis, MO. #P6267) and 32 mg cholesterol (Sigma, #CH-S) (7:2 molar ratio) were dissolved in 15 ml chloroform, and dried down on a rotary evaporator at 52°C.
under vacuum. The lipid film was dispersed in 5 ml of 50 mM Tris/NaCl buffer, pH 7.4, containing various amounts of column purified MBP. At 2 minute intervals, the flask was vortexed vigorously for 30 seconds. The liposomes formed were allowed to stand at room temperature for one hour, then sonicated for 2 min. at 4°C using a probe sonic dismembrator set at 50 (Artek Systems Corp. Farmingdale, N.Y.). The suspension was kept at 4°C for one hour, and then the liposomes were pelleted by centrifugation at 146,000 x g for 60 min. The liposomes were washed once with 5 ml of 50 mM Tris/NaCl buffer and then resuspended in 5 ml of buffer. Control liposomes were similarly prepared but in the absence of MBP. Each rat was fed 1 ml of MBP-liposomes or control liposomes. The amount of MBP incorporated into MBP-liposomes was determined using trace labelling with $^{125}$I-MBP according to the following formula:

$$\frac{\text{cpm in }^{125}\text{I-MBP-liposomes} \times 100}{\text{cpm of }^{125}\text{I-MBP added}}$$

The maximum amount of MBP incorporated into liposomes was found to be 30%. Therefore, control rats fed soluble MBP in these experiments received ≤30% of the MBP concentration used to prepare the liposomes.

In order to determine the fate of MBP-liposomes in vivo, $^{125}$I-MBP liposomes were administered by gastric intubation after light ether anesthesia. Each rat was fed 1 ml of $^{125}$I-MBP liposomes (20,194 cpm of $^{125}$I-MBP liposomes/rat in experiment I, and 12,278 cpm of $^{125}$I-MBP-liposomes/rat in experiment II). At 1 hour, 2 hours, 3 hours, and 24 hours post feeding, one rat was sacrificed, blood collected and
Intragastric administration of antigen

In the first few experiments, the following protocol was used. Male Lewis rats were lightly etherized and then fed by gastric intubation 1 ml of the desired concentration of column-purified MBP dissolved in sterile water or sterile saline or 1 ml of the desired concentration of column-purified MBP emulsified in an equal volume of IFA. In two experiments, male Lewis rats were fed various amounts of column-purified MBP incorporated into DPPC-cholesterol liposomes.

In later experiments, the following changes were made in the feeding protocol. Rats were starved for 24 hours prior to each feeding. Experimental rats were lightly etherized then fed by gastric intubation 1 ml of the appropriate dilution of MBP or ovalbumin (OVA) grade V (Sigma, #A5503) dissolved in 0.15 M sodium bicarbonate and 10 mg soybean trypsin inhibitor (STI) (Sigma, # T9003) dissolved in 0.5 ml of 0.15 M sodium bicarbonate. Two syringes were filled with the solutions to be fed, MBP or OVA in one syringe and STI in the other syringe. The two syringes were attached by means of a 3 way stopcock which had the feeding tube
attached to the third outlet. STI was administered intragastrically followed by MBP or OVA. Control rats were fed 10 mg STI in 0.5 ml 0.15 M sodium bicarbonate only (referred to in the text as vehicle). The schedule of feeding varied from one experiment to another and is detailed in the specific figure or table legends.

Induction of EAE

Rats were sensitized by intradermal injection into one or both hind footpads with 10 μg column-purified guinea pig or 50 μg rat MBP emulsified in CFA containing 4 mg/ml killed Mycobacterium tuberculosis, Jamaica strain. Sensitized rats were divided into two groups: 1) one group was sacrificed under ether anesthesia 12 days after sensitization in order to perform a lymphocyte proliferation assay using draining LNC; 2) the second group was monitored daily for clinical neurologic signs. Rats exhibiting clinical signs were scored as follows: 1, limpness of the distal portion of the tail; 2, complete loss of tail tonicity; 3, definite hind limb weakness; 4, complete hind limb paralysis usually accompanied by incontinence and fecal impaction. All rats were sacrificed by exsanguination at the time of severe paralysis or 28 days after sensitization if no clinical signs appeared. Brains and spinal cords were fixed in 10% formalin, dehydrated and embedded in paraffin. Seven micron transverse sections of the thalamus, mesencephalon, cerebellum-pons as well as longitudinal sections of the entire spinal cord were processed for hematoxylin and eosin staining. Duplicate histologic slides from each animal were assessed for the presence of peri-vascular mononuclear infiltrates and scored as follows: 1-10 lesions
1+, 11-30 lesions 2+, and greater than 30 lesions 3+.

Lymphocyte proliferation assay

Twelve days after MBP-CFA sensitization, rats were sacrificed under ether anesthesia and lymph nodes draining the site of footpad injection (inguinal, popliteal and periaortic) were removed, trimmed of fat, and expressed through a stainless steel screen (120 mesh) resulting in a single cell suspension. LNC were suspended in Hanks' balanced salt solution (HBSS) (M.A. Bioproducts, Walkersville, MD. #10-508Y) washed two times by centrifugation (140 x g), and viability determined by exclusion of trypan blue (Gibco, Grand Island, N.Y. #630-5250). LNC were finally suspended in RPMI 1640(M.A. Bioproducts,#12-167Y) supplemented with 2 mM L-glutamine (M.A. Bioproducts,#17-605A), 5 x 10^{-5} M 2-mercaptoethanol (2-M.E.) (Sigma, #6250), 25 mM Hepes (Gibco, #845-1344), 50 units/ml penicillin, 50 μg/ml streptomycin (M.A. Bioproducts, #17-603A) and 2% autologous rat serum. LNC were cultured at 37°C and 5% CO_2 in flat-bottomed 96 well tissue culture plates (Costar, Cambridge, MA. #3596) at 4 x 10^5 cells/well in the presence of various concentrations of column-purified MBP (0-100 μg/well), OVA (0-500 μg/well), purified protein derivative (PPD)(0-20 μg/well)(Parke-Davis, Morris Plains, NJ.#N0071-9526-10) or Con A (0-0.5 μg/well)(Miles, Yeda Ltd. Israel.#79-003). After 48 hours for Con A and 72 hours for MBP, OVA or PPD stimulations, the cultures were pulsed for 18 hours with 1 μCi/well ^3H-thymidine (Amersham #TRA-120). Cells were harvested using a multiple automated sample harvester (M.A. Bioproducts) and proliferation was measured by scintillation counting using a Beckman
model LS 9000 scintillation counter. Results were expressed as counts per minute (cpm) or stimulation index (S.I.) cpm with antigen cpm w/o antigen.

Proteolytic degradation of MBP and sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE).

In order to investigate the degradation of MBP by enzymes contained within the gastrointestinal tract, washings of various organs were incubated with MBP and analyzed by SDS-PAGE. Lewis rats were starved for 24 hours, then the stomach, pancreas, and small intestines excised, and each organ washed with 2 ml saline. The stomach, pancreatic and intestinal washes were centrifuged at 140 xg for 10 minutes at 4°C. Intestinal or pancreatic wash (0.5 ml) was incubated with 0.5 ml MBP (2mg/ml) suspended in PBS or sodium bicarbonate buffer and with or without 0.5 ml STI (1-2 mg/ml) for 30 minutes at 37°C. Stomach wash (0.5 ml) was incubated with 0.5 ml MBP (2mg/ml) suspended in PBS or in sodium bicarbonate buffer for 30 minutes at 37°C. At 0, 5, 10, 15, 20, and 30 minutes, samples were removed and immediately frozen at -20°C. Frozen samples were thawed and reduced by boiling in the presence of 2-M.E. Electrophoresis was carried out using a Bio-Rad Protean gel system according to the procedure of Laemml (1970) employing a running gel (15% T, 2.6% C) and stacking gel (4% T, 2.4% C). Gels were electrophoresed (25 ma/slab) in electrode buffer pH 8.3 (containing 0.025 M Tris and 0.192 M glycine and 0.1% SDS) and stained with Coomassie brilliant blue (Sigma, #B-0630).
Adoptive Transfer

Three adoptive transfer protocols were used. First, a modified procedure described by Swierkosz and Swanborg (1975) was used. Donor rats were fed four 5 mg doses of MBP over an 8 day period and donor animals sacrificed three days after the last feeding. Serum was collected and pooled. Single cell suspensions of spleen, thymus, Peyer's patches and MLN prepared. These cell suspensions were washed two times in HBSS and viability determined by dye exclusion. Cell populations were injected i.v., into groups of normal syngeneic recipients, and 24 hours later, the recipients were sensitized intradermally in one hind footpad with 10μg MBP-CFA and then monitored daily for clinical neurologic signs. Control rats were sensitized only.

Secondly, a modified procedure described by Asherson et al. (1977) was used. Donor rats were fed four 5 mg doses of MBP over an 8 day period and sensitized with 10 μg column-purified MBP-CFA 7 days after the last feeding. Two days after sensitization, donor rats were sacrificed by exsanguination. Serum and plasma were collected and pooled. Single cell suspensions of spleen, thymus, Peyer's patches, MLN and lymph nodes draining the site of footpad injection were prepared. These cell suspensions were washed two times in HBSS and viability determined by dye exclusion. Cell populations were injected i.v., into groups of normal syngeneic Lewis recipients, and 24 hours later, the recipients were sensitized intradermally in one hind footpad with 10 μg column-purified MBP-CFA and then monitored daily for clinical neurologic signs.
In the third protocol, a modified procedure described by Swierkosz and Swanborg (1975) was used. Donor rats were either fed four 5 mg doses of MBP or vehicle alone over an 8 day period and sensitized with 10 μg column-purified MBP-CFA three days after the last feeding. Nine days after sensitization, the majority of the rats were sacrificed and their spleens as well as LNC draining the site of footpad injection were used for adoptive transfer. The remaining rats were kept for observation of clinical neurologic signs. For adoptive transfer, single cell suspensions of draining LNC and spleens were prepared. These cell suspensions were washed two times in HBSS and viability determined by dye exclusion. These lymphoid cell populations were either transferred directly or subjected to in vitro activation prior to transfer. For direct transfer, draining LNC (221-500 x 10^6) were injected i.v. into normal recipients which were monitored daily for clinical neurologic signs (12-15 days). To obtain MBP-activated cells, LNC and spleen cells were separately cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 5 x 10^{-5} M 2-M.E., 25 mM Hepes, 50 units/ml penicillin 50 μg/ml streptomycin and 5% fetal bovine serum (M.A. Bioproducts # 14-501B). Cells (2 x 10^6/ml) were cultured in 75 cm^2 tissue culture flasks (Falcon, McGraw Park, Il. #T4162-75) in the presence of MBP (10μg/ml) at 37°C and 5% CO₂ for 8 hours. Following culture, MBP-activated LNC and spleen cells were washed twice with HBSS and viability determined. MBP-activated cells (32-112 x 10^6) were injected i.v. into normal recipients, which were monitored daily for clinical neurologic signs.
Statistical Analysis

Two way analysis of variance was used to test the significance of the proliferation assay data, employing dose-response determinations. The Student's t-test was used to test the significance of mean day of onset of clinical signs, mean clinical score and mean histologic score. Chi-square analysis was used for the statistical determination of paralytic incidence.
RESULTS

Proliferative response of LNC from OVA-fed and vehicle-fed rats to ovalbumin.

Since ovalbumin has been used in many oral tolerance studies (Wells 1911; Thomas and Parrot, 1977; Richman et al., 1978; Miller and Hanson, 1979), it was first necessary to establish, in my own hands, the induction of oral tolerance in the Lewis rat using ovalbumin and a reported oral tolerance regimen (Miller and Hanson, 1979). Genetic differences do exist in the immune response to ingested antigens and interstrain differences have been reported in rats (Mota, 1964; Jarret and Stewart, 1974; Jarret et al., 1976). In my first series of experiments, rats were fed 25 mg OVA or saline alone, 7 days prior to footpad sensitization with OVA-CFA. Seven days after sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with OVA. Figure 1 shows significant suppression of $^{3}$H-thymidine incorporation to OVA by LNC from OVA-fed rats compared to vehicle-fed controls ($P \leq 0.001$). These results demonstrated that the Lewis rat is susceptible to the induction of oral tolerance.

Assessment of EAE in rats fed MBP in solution or MBP in IFA.

Rats were fed varying amounts of column-purified MBP (100µg-20 mg) suspended in saline or water, or MBP (1-5mg) emulsified in IFA. Control rats were not fed and were sensitized only. All rats received a 10 µg MBP-CFA challenge and were monitored daily for clinical neurologic signs. As shown in table 1, rats fed 100 µg MBP (group A), 3 x 100 µg MBP (group C), 3 x 500 µg MBP (group D),
Figure 1. Proliferative response of LNC from OVA-fed and vehicle-fed rats to ovalbumin

Values shown represent the mean cpm of triplicate determinations from 4 rats/group. Rats were fed 25 mg ovalbumin in 1 ml of saline or 1 ml saline alone, 7 days prior to footpad sensitization with 100 µg OVA-CFA. Seven days after sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with OVA.

P < 0.001 for cpm of OVA-fed rats compared to vehicle-fed rats.
Table 1. Assessment of EAE in rats fed MBP in solution or MBP in IFA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Feeding regimen</th>
<th>Day of onset of EAE</th>
<th>Incidence of clinical paralysis</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1x100 ug MBP</td>
<td>13.7 ±0.6</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>B</td>
<td>1x500 ug MBP</td>
<td>10.7 ±0.6</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>C</td>
<td>3x100 ug MBP</td>
<td>14.0 ±2.0</td>
<td>2/3</td>
<td>3.7 ±0.6</td>
</tr>
<tr>
<td>D</td>
<td>3x500 ug MBP</td>
<td>14.0 ±2.6</td>
<td>2/3</td>
<td>3.3 ±1.2</td>
</tr>
<tr>
<td>E</td>
<td>1x1 mg MBP</td>
<td>11.7 ±2.1</td>
<td>10/11</td>
<td>3.8 ±0.6</td>
</tr>
<tr>
<td>F</td>
<td>1x5 mg MBP</td>
<td>12.0</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>G</td>
<td>3x1 mg MBP</td>
<td>14.0</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>H</td>
<td>3x5 mg MBP</td>
<td>13.7 ±0.6</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>I</td>
<td>1x20 mg MBP</td>
<td>12.3 ±0.5</td>
<td>3/4</td>
<td>3.5 ±1.0</td>
</tr>
<tr>
<td>J</td>
<td>1x1 mg MBP-IFA</td>
<td>11.5 ±0.6</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>K</td>
<td>1x5 mg MBP-IFA</td>
<td>10.0 ±1.2</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>L</td>
<td>---</td>
<td>10.6 ±1.4</td>
<td>11/11</td>
<td>4.0</td>
</tr>
</tbody>
</table>

a. Rats were fed column-purified guinea pig MBP suspended in saline or water, or column-purified guinea pig MBP emulsified in IFA. Single feedings were administered 7 days prior to sensitization, multiple feedings were administered every other day with the last feeding on day 7 prior to sensitization. Group L was not fed and sensitized only. All rats were sensitized with 10ug column-purified guinea pig MBP-CFA and monitored daily for clinical neurologic signs.

b. Mean ±S.D.

c. $P \leq 0.01$ compared to group L.

d. $P \leq 0.05$ compared to group L.
3 x 5 mg MBP (group H) and 20 mg MBP (group I) had a significant delay (mean of 2.9 days) in the day of onset of EAE, compared to control rats, group L. No protective effect of MBP-IFA was observed in terms of day of onset of EAE. However, nearly all rats, whether they were fed MBP in solution or incorporated in adjuvant, in single or multiple doses, progressed to paralysis as did the control rats, group L.

Assessment of EAE in rats fed MBP-liposomes.

Because I was able to detect some small effect (2.9 day delay in the onset of EAE), resulting from the oral administration of MBP in solution, I next sought a means for delivering the protein to the gastrointestinal (G.I.) tract so that it was protected from enzymatic degradation. Several studies have used liposomes to administer proteins such as insulin, factor VIII and factor IX to the G.I. tract (Patel and Ryman, 1976; 1977; Dapergolas and Gregoriadis, 1976; Hemker et al., 1980; Sakuragawa et al., 1983). In these experiments, rats were fed MBP (15µg - 1500µg) incorporated into DPPC-cholesterol liposomes, MBP (150µg - 500µg) suspended in saline or control liposomes prepared without MBP. Control rats (group K) were not fed and were sensitized only. All rats received a 10 µg MBP-CFA challenge and were monitored daily for clinical neurological signs. Table 2 shows that, in all cases, whether rats were fed MBP-liposomes, MBP suspended in saline or control liposomes, animals started exhibiting clinical signs day 11-12 post sensitization with no significant differences observed between
Table 2. Assessment of EAE in rats fed MBP-liposomes.a.

<table>
<thead>
<tr>
<th>Group</th>
<th>Feeding regimen</th>
<th>Day of onset of EAE</th>
<th>Incidence of paralysis</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1x MBP-liposomes (150ug MBP)</td>
<td>11.5 ±1.7</td>
<td>3/4</td>
<td>3.8 ±0.5</td>
</tr>
<tr>
<td>B</td>
<td>2x MBP-liposomes (2x150 ug MBP)</td>
<td>12.7 ±0.6</td>
<td>2/3</td>
<td>3.3 ±1.2</td>
</tr>
<tr>
<td>C</td>
<td>1x MBP-liposomes (850 ug MBP)</td>
<td>11.5 ±0.6</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>D</td>
<td>2x MBP-liposomes (2x750 ug MBP)</td>
<td>12.8 ±1.0</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>E</td>
<td>1x150 ug MBP</td>
<td>12.3 ±1.5</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>F</td>
<td>2x150 ug MBP</td>
<td>11.3 ±1.0</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>G</td>
<td>1x1 mg MBP</td>
<td>11.5 ±1.9</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>H</td>
<td>2x750 ug MBP</td>
<td>11.5 ±0.7</td>
<td>2/4c</td>
<td>2.0 ±2.3c</td>
</tr>
<tr>
<td>I</td>
<td>1x control liposomes</td>
<td>11.0 ±1.2</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>J</td>
<td>2x control liposomes</td>
<td>12.3 ±1.5</td>
<td>3/4</td>
<td>3.8 ±0.5</td>
</tr>
<tr>
<td>K</td>
<td>---</td>
<td>11.7 ±1.4</td>
<td>7/7</td>
<td>4.0</td>
</tr>
</tbody>
</table>

a. Rats were fed column-purified guinea pig MBP incorporated into liposomes, column-purified guinea pig MBP suspended in saline or water, or control liposomes which were prepared in saline. Liposomes were prepared from DPPC and cholesterol (7:2) molar ratio. Single feedings were administered 7 days prior to sensitization. Multiple feedings were administered 10 and 7 days prior to sensitization. Group K was not fed and sensitized only. All rats were sensitized with 10ug column-purified guinea pig MBP-CFA and monitored daily for clinical neurological signs.

b. Mean ±S.D.

c. P <0.05 compared to group K.
groups. Nearly all rats exhibited paralysis with the exception of group H where only two rats fed 1500 µg MBP in solution did not show any clinical signs. This group alone was significantly different from the control (group K) with respect to incidence of paralysis and clinical score. Thus, feeding MBP-liposomes did not provide a protective effect from EAE.

Information on the fate of MBP-liposomes post feeding was obtained by orally administering MBP-liposomes prepared with $^{125}$I-labelled MBP, and assessing the radioactivity contained in various organs with time. Results in table 3 show that starving the rats prior to feeding did not seem to dramatically affect the distribution of $^{125}$I-MBP liposomes, since similar distribution of radioactivity was seen in both experiments I and II. The radioactivity contained in the blood was not above background counts at any time point tested except for 1 hour post feeding in experiment I. The liver, but not the spleen, contained higher than background counts at all time points tested. In both experiments, the radioactivity in the lungs was above background in the first 2 hours after feeding, but declined at 3 and 24 hours after feeding. Most of the cpm's in the first three hours in both experiments I and II were contained in the stomach and intestines but had declined by 24 hours when the majority of the counts were found in the urine and feces. Peyer's patches and MLN counts remained the same at background levels at all time points tested. In experiment II the thyroid had higher than background counts, at 3 and 24 hours after feeding. The kidneys exhibited somewhat increased counts in the first 3 hours after feeding in only
Table 3. The fate of $^{125}$I-MBP-liposomes at various times after feeding$^a$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Experiment I$^b$</th>
<th></th>
<th>Experiment II$^b$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>1ml Blood</td>
<td>190</td>
<td>84</td>
<td>65</td>
<td>28</td>
</tr>
<tr>
<td>Liver</td>
<td>300</td>
<td>210</td>
<td>180</td>
<td>330</td>
</tr>
<tr>
<td>Spleen</td>
<td>90</td>
<td>50</td>
<td>120</td>
<td>50</td>
</tr>
<tr>
<td>Lungs</td>
<td>230</td>
<td>550</td>
<td>ND</td>
<td>60</td>
</tr>
<tr>
<td>Stomach</td>
<td>2320</td>
<td>4650</td>
<td>2560</td>
<td>120</td>
</tr>
<tr>
<td>Intestines</td>
<td>4470</td>
<td>3180</td>
<td>2040</td>
<td>120</td>
</tr>
<tr>
<td>Peyer's patches</td>
<td>50</td>
<td>50</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>Mesenteric lymph nodes</td>
<td>ND</td>
<td>80</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Thyroid</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>300</td>
<td>200</td>
<td>210</td>
<td>70</td>
</tr>
<tr>
<td>Urine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Feces</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3500</td>
</tr>
<tr>
<td>Background</td>
<td>60</td>
<td>58</td>
<td>61</td>
<td>94</td>
</tr>
</tbody>
</table>

$^a$ Rats were fed $^{125}$I-MBP liposomes (150ug MBP). At 1, 2, 3, or 24 hours post feeding, one rat was bled, sacrificed, and the following organs excised: liver, spleen, lungs, stomach, small intestines, Peyer's patches, mesenteric lymph nodes, thyroid and kidneys. Urine and feces were collected. Results shown represent cpm obtained from counting entire organs. The radioactivity in each sample was counted on a gamma counter. Background counts represent counts of empty tube.

$^b$ In experiment I, rats were starved 24 hours prior to feeding, 20,194 cpm of $^{125}$I-MBP-liposomes was fed/rat.
In experiment II, rats were not starved prior to feeding, 12,278 cpm of $^{125}$I-MBP-liposomes was fed/rat.
ND : not done.
one of the two experiments. Feeding 125I-MBP liposomes showed that our intragastric intubation was indeed delivering the protein to the stomach, and that the majority of the MBP was remaining within the G.I. tract. The radioactive counts contained in the blood, liver, and lungs probably represent MBP which gained access to the circulation. However, the radioactivity localized in the thyroid may represent dissociation of the iodine label. We were not able to detect uptake of labelled antigen by the gut lymphoid tissue.

Proteolytic Degradation of MBP.

Knowing the extreme susceptibility of MBP to proteolytic degradation, particularly pepsin and trypsin digestion (Hashim and Eylar, 1969; Benuck et al., 1975; Brostoff et al., 1974), it was not surprising to encounter difficulties in inducing oral tolerance by feeding MBP in saline solution. To find the optimum conditions for preventing the breakdown of MBP in the G.I. milieu, I first determined the length of time that MBP suspended in PBS could remain intact in the G.I. environment. MBP was incubated in the presence of lavage fluids from Lewis rat stomach, pancreas, and small intestine for varying periods of time (0-30 min). Plate Ic. shows that when MBP is suspended in PBS in the presence of stomach lavage fluid, the MBP is degraded in 15 minutes, since the major band (arrow) cannot be detected at 15, 20, or 30 minutes after the initiation of incubation. However, when MBP is first suspended in 0.15 M sodium bicarbonate buffer, and then subjected to stomach lavage fluid, the major band can still be seen even up to 30 minutes later (plate I-d). In the presence of intestinal lavage fluid (plate IIb), MBP suspended in PBS
Plate I. Proteolytic degradation of MBP by stomach lavage fluid.

a. Rat MBP (arrow pointing to major band).

b. Stomach lavage fluid.

c. Rat MBP suspended in PBS with stomach lavage fluid at 0, 5, 10, 15, 20 and 30 minute time points.

d. Rat MBP suspended in 0.15 M sodium bicarbonate buffer with stomach lavage fluid at 0, 5, 10, 15, 20 and 30 minute time points.
Plate I. Proteolytic degradation of MBP by stomach lavage fluid.
Plate II. Proteolytic degradation of MBP by intestinal and pancreatic lavage fluids.

a. Rat MBP (arrow pointing to major band).

b. Rat MBP suspended in PBS with intestinal lavage fluid at 0 and 30 minute time points.

c. Pancreatic lavage fluid.

d. Rat MBP suspended in PBS with pancreatic lavage fluid at 0 and 30 minute time points.

e. Rat MBP in the presence of STI (2 mg/ml) with pancreatic lavage fluid at 0, 5, 15 and 30 minute time points.

f. Rat MBP in the presence of STI (1 mg/ml) with pancreatic lavage fluid at 0, 10, 15 and 30 minute time points.

g. STI
Plate II. Proteolytic degradation of MBP by intestinal and pancreatic lavage fluids.
is degraded instantaneously, since the major band cannot be seen even at the 0 time point. In the presence of pancreatic lavage fluid (Plate II-d), MBP suspended in PBS appears to be degraded slowly, since after a 30 minute incubation, the major MBP band can still be seen. Incubation of MBP with pancreatic lavage fluid in the presence of 2 mg/ml STI (Plate II-e) or 1 mg/ml STI (Plate II-f), revealed neither protection nor degradation of MBP. Interpretation of these gels was complicated by the fact that the relative size of the major band of MBP (M.W. 18,000) (plate II-a) and STI (M.W. 21,500) (plate II-g) are similar and migrated very closely on the gels. For the same reason, analysis of SDS-PAGE gels of MBP incubated with intestinal lavage fluids in the presence of STI was also complicated (results not shown). When guinea pig MBP was used instead of rat MBP in similar SDS-PAGE gel analyses, similar results were obtained (results not shown). Since there was protection from proteolytic degradation of MBP in vitro, by suspending the protein in sodium bicarbonate buffer, the feeding protocol, from this point onward, involved suspending MBP in 0.15 M sodium bicarbonate buffer in the presence of 10 mg STI. Andre et al. (1978) used 0.15 M sodium bicarbonate containing pancreatic protease inhibitor for the oral administration of human serum albumin in rats. Sakuragawa et al. (1983) used a trypsin inhibitor Trasylol in the oral administration of factor IX concentrate in dogs.
Proliferative response of LNC from rat MBP-fed and control rats to rat MBP.

Since rat MBP is less encephalitogenic than guinea pig-MBP, I reasoned that by feeding and challenging with rat MBP, tolerance might be more easily demonstratable. Rats were fed 20 mg rat MBP suspended in 0.15 M bicarbonate buffer and in the presence of 10 mg STI suspended in 0.5 ml sodium bicarbonate buffer, in a single dose. Control rats were fed sodium bicarbonate buffer only. Seven days after feeding, rats were challenged with rat MBP-CFA. Half the rats were observed for clinical signs, and the other half were sacrificed twelve days after sensitization in order to perform a lymphocyte proliferation assay, using rat MBP as an in vitro stimulant.

Results in table 4 show that there was very little \(^{3}H\)-thymidine incorporation by control LNC in response to rat MBP (stimulation index < 2.0). Moreover there was no difference in the lymphocyte proliferative responses to rat MBP of LNC from rat MBP-fed and control rats. Moreover, there was no significant difference in day of onset or incidence of EAE in rats fed rat MBP compared to vehicle-fed controls (data not shown). Therefore, I did not pursue the use of rat MBP as an oral tolerogen, since proliferative responses to this homologous antigen were not demonstrable.

Proliferative response of LNC from guinea pig MBP-fed and control rats to guinea pig MBP.

The next step was to determine if it was possible to induce oral tolerance in response to feeding guinea pig MBP rather than rat MBP. In one experiment, rats were fed 20 mg guinea pig MBP suspended in
Table 4. Proliferative response of LNC from rat MBP-fed and control rats to rat MBP.

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP-fed</td>
<td>2.3 ±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 ±2.4</td>
<td>4.8 ±2.4</td>
<td>4.3 ±2.1</td>
<td>3.6 ±1.6</td>
</tr>
<tr>
<td>Control</td>
<td>2.7 ±0.6</td>
<td>4.9 ±0.2</td>
<td>5.0 ±0.1</td>
<td>3.8 ±0.5</td>
<td>2.9 ±0.8</td>
</tr>
</tbody>
</table>

- Experimental rats were fed 20 mg rat MBP in 1 ml 0.15 sodium bicarbonate buffer and 10 mg STI suspended in sodium bicarbonate buffer. Control rats were fed 1 ml 0.15M sodium bicarbonate buffer. Both groups were fed once, 7 days prior to sensitization with 50 ug column-purified rat MBP-CFA. Twelve days after sensitization rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay using column-purified rat MBP.

- Values shown represent mean cpm x 10<sup>-3</sup> ± S.D. of triplicate determinations from 3-4 rats.
1 ml 0.15 M sodium bicarbonate buffer and in the presence of 10 mg STI suspended in 0.5 ml sodium bicarbonate buffer i.e., vehicle only (10 mg STI in 0.5 ml sodium bicarbonate buffer). Eight days later, rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Twelve days following sensitization, the animals were sacrificed, and their draining LNC used in a lymphocyte proliferation assay to guinea pig MBP. The results shown in figure 2 indicated suppression of lymphocyte proliferation to MBP of LNC from MBP-fed rats compared to vehicle-fed controls. Thus, these results demonstrated that 1) in vitro proliferative responses to MBP were indeed detectable following challenge with guinea pig MBP-CFA, and 2) those proliferative responses could be depressed by prior MBP feeding of the donor animals.

Assessment of EAE in rats fed varying amounts of guinea pig MBP.

In order to determine the amount of MBP required for the induction of oral tolerance, groups of rats were fed 20 mg MBP (4 feedings of 5 mg each), 15 mg MBP (3 feedings of 5 mg each), 10 mg MBP (2 feedings of 5 mg each), 5 mg MBP (1 feeding of 5 mg) or no MBP (1 feeding of vehicle only) as detailed in figure 3 and table 5. Three days following the last feeding, all rats were sensitized with MBP-CFA. Half of the rats were observed daily for clinical neurologic signs of EAE. The other half were sacrificed 12 days post sensitization and their draining LNC used in a lymphocyte proliferation assay in response to MBP. Figure 3 shows that even feeding 5 mg MBP resulted in a decline in the LNC proliferative response to MBP when compared with vehicle-fed control rats. Feeding
Figure 2. Proliferative response of LNC from guinea pig MBP-fed and control rats to guinea pig MBP.

The values shown represent the mean cpm of quadruplicate determinations from 4-6 rats/group. Rats were fed 20 mg guinea pig MBP or vehicle only. Eight days later rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP.

P < 0.001 for cpm of MBP-fed compared to vehicle-fed rats.
Figure 3. Proliferative response of LNC from rats fed varying amounts of guinea pig MBP to MBP.

The values shown represent the mean cpm's of quadruplicate determinations from 2 rats/group. Rats were fed varying amounts of guinea pig MBP ranging from 5 to 20 mg. Control rats were fed vehicle once. The single feeding was administered 3 days prior to sensitization, multiple feedings were administered every other day with the last feeding given 3 days prior to sensitization. All rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP.

P < 0.001 for cpm's of MBP-fed 10, 15, or 20 mg compared to vehicle-fed rats.

P < 0.15 for cpm's of MBP-fed 5 mg compared to vehicle-fed rats.
Table 5. Assessment of EAE in rats fed varying amounts of guinea pig MBP.

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>Day of onset of EAE</th>
<th>Clinical signs</th>
<th>Clinical score</th>
<th>Histologic score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none LT EP P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4x5mg MBP</td>
<td>13.0</td>
<td>1/2 0/2 1/2 0/2</td>
<td>1.5 ±2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>3X5mg MBP</td>
<td>14.5 ±0.7</td>
<td>1/3 2/3 0/3 0/3</td>
<td>1.3 ±1.2</td>
<td>1.0 ±0.0</td>
</tr>
<tr>
<td>2X5mg MBP</td>
<td>11.3 ±1.2</td>
<td>0/3 0/3 0/3 3/3</td>
<td>4.0</td>
<td>1.3 ±0.6</td>
</tr>
<tr>
<td>1x5mg MBP</td>
<td>12.0 ±1.0</td>
<td>0/3 0/3 0/3 3/3</td>
<td>4.0</td>
<td>1.0 ±0.6</td>
</tr>
<tr>
<td>1x vehicle</td>
<td>10.0 ±1.0</td>
<td>0/3 0/3 0/3 3/3</td>
<td>4.0</td>
<td>2.5 ±0.7</td>
</tr>
</tbody>
</table>

Rats were fed various amounts of guinea pig MBP ranging from 5mg to 20mg. MBP (5mg) was dissolved in 1ml 0.15M sodium bicarbonate and was fed with 10mg STI suspended in sodium bicarbonate buffer. Control rats were fed vehicle only, consisting of 10mg STI suspended in sodium bicarbonate buffer. The single feeding was administered 3 days prior to sensitization, multiple feeding were administered every other day with the last feeding administered 3 days prior to sensitization. All rats were sensitized with 10ug column-purified guinea pig MBP-CFA and monitored daily for clinical neurological signs.

b. Mean ±S.D.

c. P ≤0.025 compared to vehicle-fed control.

d. P ≤0.05 compared to vehicle-fed control.
regimens consisting of 10, 15, and 20 mg MBP resulted in significant suppression of MBP-induced lymphocyte proliferation (P < 0.001).

The development of clinical signs and histopathologic changes of EAE in rats fed vehicle only or 5, 10, 15, or 20 mg MBP is shown in Table 5. A significant delay in the onset of EAE as well as significantly lower clinical and histologic scores were observed in the group receiving 15 mg oral MBP. The histologic score of rats fed 5 mg MBP was significantly different from that of the vehicle-fed group. None of the rats fed 15 or 20 mg MBP progressed to total paralysis. In contrast, all of the animals fed 5 mg or 10 mg MBP were paralyzed as was the vehicle-fed group. Even though the numbers of animals per group in this single experiment were small, the results indicated the necessity for a minimum of 15 mg MBP administered orally in order to induce tolerance.

In order to test the effect of feeding control rats vehicle (10 mg STI suspended in 0.5 ml of 0.15 M sodium bicarbonate buffer) once versus feeding vehicle four times, in a preliminary experiment, half the control rats were fed vehicle once, the other half were fed vehicle four times. Single feedings were administered 8 days prior to sensitization, multiple feedings were administered every other day with the last feeding given 8 days prior to sensitization. All rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Half the rats were observed for clinical signs, the other half were sacrificed 12 days post sensitization and their draining LNC used in a lymphocyte proliferation assay with guinea pig MBP. There was no difference in incidence of EAE of control rats fed vehicle once
(three rats began showing clinical signs on day 10-12 post sensitization and all progressed to paralysis), versus control rats fed vehicle four times (three rats began showing clinical signs on day 10-11 post sensitization and all progressed to paralysis). Furthermore, proliferative responses of LNC from the control group fed vehicle once to MBP was not statistically different from MBP induced proliferative responses of LNC of the control group fed vehicle four times (data not shown). Moreover, the data in table 7 (antigen specificity of oral tolerance) was pooled from 5 experiments. Control rats received four feedings of vehicle in one experiment (12 rats total); in the other four experiments, control rats received one feeding of vehicle (21 rats total). Comparison of incidence, mean day of onset of EAE, mean clinical or histologic scores in those two groups showed no significant differences between the two treatments.

**Kinetics of oral tolerance induction:** Assessment of EAE in rats fed guinea pig MBP and challenged 3 days, 8 days or 15 days after the last feeding.

The question of kinetics of oral tolerance induction to MBP was addressed by investigating different time periods between feeding and encephalitogenic challenge. Three groups of rats (4 weeks old) were fed four 5 mg doses of MBP and were subsequently challenged with MBP-CFA 3 days, 8 days, or 15 days after the last feeding. Figures 4, 5, and 6 show the lymphocyte proliferative responses to MBP of LNC derived from the day 3, day 8 and day 15 groups respectively, assessed 12 days following sensitization. Significant suppression of lymphocyte proliferation in response to MBP was evident at all
three of the time points examined ($P \leq 0.002$). Therefore, suppression of lymphocyte proliferation to MBP could be demonstrated as early as 3 days and is still evident at 15 days after the last feeding.

Even though there were small numbers of animals per group in this particular experiment, Table 6 shows an almost total inhibition of clinical EAE at all time points tested. None of the rats challenged 3 days after the last feeding showed any clinical signs, although they did exhibit mild lesions in the CNS. A significant delay in the onset of EAE was observed in rats fed MBP and challenged 8 days after the last feeding. Moreover, rats in the 8 and 15 day groups had significantly reduced clinical signs (1/2 LT or LT) and did not progress to paralysis. Rats which were challenged 3 or 8 days after the last feeding had significantly fewer lesions in the CNS than vehicle-fed controls.

In investigation of the kinetics of the lymphocyte proliferation assay to guinea pig MBP, LNC from MBP-fed and challenged rats were compared with LNC from vehicle-fed and challenged controls at different time points after challenge, i.e., 9 days, 12 days, 16 days and 23 days. Results (not shown) indicated significant suppression of lymphocyte proliferation of LNC derived from MBP-fed rats compared to vehicle-fed controls at all time points tested. I also investigated the time of incubation of lymphocyte cultures, i.e., 72 hours versus 96 hours versus 120 hours. The highest counts for both groups, MBP-fed and vehicle-fed, were obtained at 96 hours. Therefore, lymphocyte proliferation assays in all subsequent
Figure 4. Kinetics of oral tolerance induction: Proliferative response of LNC from MBP-fed and vehicle-fed rats, challenged 3 days after the last feeding to MBP.

The values shown represent the mean cpms of quadruplicate determinations from 3 rats/group. Rats were fed four 5 mg doses of guinea pig MBP or vehicle once. Single feedings were administered 3 days prior to sensitization. Multiple feedings were administered every other day over an eight day period. Three days after the last feeding, rats were challenged with 10 μg column-purified guinea pig MBP-CFA. Twelve days post challenge, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP.

P < 0.002 for cpms of MBP-fed rats compared to vehicle-fed rats.
Figure 5. Kinetics of oral tolerance induction: Proliferative response of LNC from MBP-fed and vehicle-fed rats, challenged 8 days after the last feeding to MBP.

The values shown represent the mean cpm of quadruplicate determinations from 3 rats/group. Rats were fed guinea pig MBP or vehicle and then challenged with column-purified guinea pig MBP-CFA as described in figure 4, except that challenge was performed 8 days after the last feeding.

P \leq 0.001 for cpm of MBP-fed rats compared to vehicle-fed rats.
Figure 6. Kinetics of oral tolerance induction: Proliferative response of LNC from MBP-fed and vehicle-fed rats, challenged 15 days after the last feeding to MBP.

The values shown represent the mean cpms of quadruplicate determinations from 3 rats/group. Rats were fed guinea pig MBP or vehicle and then challenged with column-purified guinea pig MBP-CFA as described in figure 4, except that challenge was performed 15 days after the last feeding.

P < 0.001 for cpms of MBP-fed rats compared to vehicle-fed rats.
Table 6. Kinetics of oral tolerance induction: Assessment of EAE in rats fed guinea pig MBP and sensitized 3 days, 8 days or 15 days after the last feeding.

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>Time of sensitization</th>
<th>Day of onset</th>
<th>Incidence of paralysis</th>
<th>Clinical score</th>
<th>Histologic score</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x5mg MBP</td>
<td>3 days</td>
<td>_</td>
<td>0/3^d</td>
<td>0.0^c</td>
<td>1.7 ±0.6^c</td>
</tr>
<tr>
<td>4x5mg MBP</td>
<td>8 days</td>
<td>14.0 ±1.7^c</td>
<td>0/3^d</td>
<td>1.3 ±0.6^c</td>
<td>1.3 ±0.6^c</td>
</tr>
<tr>
<td>4x5mg MBP</td>
<td>15 days</td>
<td>14.0</td>
<td>0/3^d</td>
<td>0.7 ±1.2^c</td>
<td>1.0 ±1.7</td>
</tr>
<tr>
<td>1x vehicle</td>
<td>3,8,15 days</td>
<td>11.3 ±1.2^c</td>
<td>8/9</td>
<td>3.7 ±1.0^c</td>
<td>2.5 ±0.7</td>
</tr>
</tbody>
</table>

a. Rats were fed four 5mg doses of guinea pig MBP or vehicle once. MBP was dissolved in 1ml 0.15M sodium bicarbonate buffer and was fed with 10mg STI in sodium bicarbonate buffer. Control rats were fed vehicle only, consisting of 10mg STI in sodium bicarbonate buffer. The single feeding was administered 3, 8 or 15 days prior to sensitization. Multiple feedings were administered every other day over an eight day period. Three, eight or fifteen days after the last feeding, rats were sensitized with 10ug column-purified guinea pig MBP-CFA. All rats were monitored daily for clinical neurological signs.

b. Mean ±S.D.
c. P < 0.05 compared to vehicle-fed control.
d. P < 0.005 compared to vehicle-fed control.
experiments were performed 12 days post sensitization, at a time when the rats start to exhibit clinical signs and were terminated at 96 hours.

**Antigen Specificity of oral tolerance.**

I next addressed the question of antigen specificity in the tolerance induced by intragastric feeding of MBP. Rats were fed 20 mg MBP, 20 mg OVA or vehicle alone and were challenged with MBP-CFA 3, 8 or 15 days later. Half of the rats were observed daily for clinical neurologic signs. The other half were sacrificed 12 days post challenge, and their draining LNC used in a lymphocyte proliferation assay in response to OVA or MBP. Figure 7 shows that LNC from MBP-fed animals were significantly suppressed in their proliferative responses to MBP when compared with LNC responses from vehicle-fed or OVA-fed animals (P \( \leq \) 0.0002). Figure 8 shows that none of the three groups of rats exhibited any proliferative response to OVA as expected, since none had received OVA-CFA sensitization.

Since both MBP-fed and control vehicle-fed rats had been sensitized with MBP-CFA containing mycobacterial antigens, a proliferative assay in response to PPD was performed. As seen in figure 9, the proliferative responses to PPD of both MBP-fed and vehicle-fed groups were nearly identical. Furthermore, in order to test whether the suppression observed in MBP-fed rats was the result of a generalized T-cell suppression, lymphocyte proliferative responses to the T-cell mitogen Con A was assessed. Figure 10 shows that the proliferative responses to Con A of both groups were very similar. Thus, the results shown in Figures 9 and 10 argue strongly
Figure 7. Antigen specificity of oral tolerance: Proliferative response of LNC from MBP-fed, OVA-fed and vehicle-fed rats to MBP.

The values shown represent the mean cpm's of quadruplicate determinations from 4-5 rats/group from a representative experiment. Rats were fed four 5 mg doses of guinea pig MBP or OVA. Control rats were fed vehicle once. Single feedings were administered 3 days prior to sensitization; multiple feedings were administered every other day with the last feeding given 3 days prior to sensitization. All rats were sensitized with 10 µg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP.

P < 0.0002 for cpm's of MBP-fed compared to OVA-fed or vehicle-fed rats.
Figure 8. Antigen specificity of oral tolerance: Proliferative response of LNC from MBP-fed, OVA-fed and vehicle-fed rats to OVA.

The values shown represent the mean cpms of quadruplicate determinations from 4-5 rats/group from a representative experiment. Rats were fed MBP, OVA or vehicle and sensitized with MBP-CFA according to the procedure described in Figure 7. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with OVA.
Figure 9: Antigen specificity of oral tolerance: Proliferative response of LNC from MBP-fed and vehicle-fed rats to PPD.

The values shown represent the mean cpm values of quadruplicate determinations from 3 rats/group from a representative experiment. Rats were fed four 5 mg doses of guinea pig MBP. Control rats were fed vehicle once. Single feedings were administered 8 days prior to sensitization; multiple feedings were administered every other day with the last feeding given 8 days prior to sensitization. All rats were sensitized with 10 µg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with PPD.
Figure 10. Antigen specificity of oral tolerance: Proliferative response of LNC from MBP-fed and vehicle-fed rats to Con-A.

Values shown represent the mean cpm's of quadruplicate determinations from 3 rats/group. Rats were fed four 5 mg doses of guinea pig MBP or vehicle four times, administered every other day over an eight day period. Three days after the last feeding, all rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with Con-A.
Table 7. Antigen specificity of oral tolerance: Assessment of EAE in rats fed MBP, OVA or vehicle.

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>Day of onset</th>
<th>Clinical signs</th>
<th>Clinical score</th>
<th>Histologic score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>none</td>
<td>1/2LT</td>
<td>LT</td>
<td>EP</td>
</tr>
<tr>
<td>MBP</td>
<td>13.4 ±1.0d</td>
<td>17/33</td>
<td>2/33</td>
<td>6/33</td>
</tr>
<tr>
<td>OVA</td>
<td>11.4 ±1.0c</td>
<td>2/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10.9 ±0.9a</td>
<td>0/33</td>
<td>1/33</td>
<td>0/33</td>
</tr>
</tbody>
</table>

a. Results shown represent data pooled from 5 experiments. Rats were fed four 5mg doses of guinea pig MBP or OVA administered every other day over an eight day period. Control rats received four feedings of vehicle in one experiment; in the other four experiments, control rats received one feeding of vehicle. MBP was dissolved in 1ml 0.15M sodium bicarbonate buffer and was fed with 10mg STI suspended in sodium bicarbonate buffer. Control rats were fed vehicle only. Three, eight or fifteen days after the last feeding, all rats were sensitized with 10ug column-purified guinea pig MBP-CFA. All rats were monitored daily for clinical neurological signs.

b. Mean ±S.D.
d. P <0.05 compared to OVA fed and vehicle-fed controls.
e. P <0.0001 compared to OVA-fed and vehicle-fed controls.
f. P <0.005 compared to OVA-fed and vehicle-fed controls.
g. P <0.05 compared to vehicle-fed control.
that the oral administration of MBP results in an antigen-specific suppression of LNC proliferative responses.

As seen in Table 7, of 33 rats given oral MBP, 17 (52%) exhibited no clinical signs whatsoever. Nine (27%) showed mild clinical signs and only 7 (21%) progressed to paralysis. The MBP-fed rats which did exhibit clinical signs of EAE were observed to be significantly delayed in the onset of these signs by 2.5 days, when compared with vehicle-fed control rats. In contrast, 32 of 33 (97%) vehicle-fed rats exhibited paralysis following encephalitogenic challenge. Oral feeding of the control protein, OVA, had no significant effect on subsequent EAE induction, since 18 of 20 (90%) rats demonstrated paralysis following MBP-CFA challenge. MBP-fed rats had significantly lower clinical scores as well as significantly lower histologic scores compared to OVA-fed or vehicle-fed control rats.

In all previous experiments, rats were sacrificed at the time of severe paralysis or 28 days post sensitization, when the experiments were terminated. In order to test the finite length of time that the rats remain tolerant to MBP, a group of four animals were fed MBP, challenged with MBP-CFA and monitored for 60 days after sensitization. One rat progressed to early paralysis, recovered and did not show recurrence of disease. Three of four rats (75%) showed no clinical evidence of EAE for the entire 60 day period of observation.
Assessment of EAE in rats fed MBP on the same day as sensitization versus rats fed MBP prior to sensitization.

Exposure to an antigen by parenteral immunization has been reported to alter the outcome of oral administration of the same antigen, such that mice fed 20 mg OVA were shown to be unresponsive to a subsequent parenteral challenge with OVA in adjuvant as determined by antibody titer measured by the Farr assay. However, in previously immunized mice, a single feeding of 1 to 20 mg of OVA resulted in a secondary antibody response to OVA (Hanson et al., 1979). To address this question in orally induced tolerance to MBP, I investigated the effects of feeding MBP on the same day as sensitization. Two groups of rats were fed MBP and two similar groups were fed vehicle. One group of each was sensitized with MBP-CFA on the first day of feeding, the other two groups were sensitized 3 days after the last feeding. Figure 11 shows significant suppression of LNC proliferation from rats fed MBP and sensitized on the same day as the first feeding, compared to vehicle-fed rats ($P < 0.005$). Similarly, figure 12 shows suppression of LNC proliferation from MBP-fed animals sensitized 3 days after the last feeding, compared to vehicle-fed controls ($P < 0.001$).

Table 8 shows the assessment of clinical signs in these animals. Rats fed MBP and sensitized on the same day as the first feeding (Group A) began to exhibit clinical signs at about the same time as the vehicle-fed controls (Group C), and 100% of the rats progressed to paralysis. In contrast, in Group B where rats were fed MBP and sensitized 3 days after the last feeding, only 3 out of 10 rats
Figure 11. Proliferative response to MBP of LNC from rats fed MBP on the same day as sensitization, expressed as stimulation index.

Values shown represent mean stimulation index of quadruplicate determinations from 3 rats/group. Rats were fed four 5 mg doses of guinea pig MBP or vehicle four times administered every other day over an eight day period. Rats were sensitized on the same day as the first feeding. Twelve days post sensitization rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP. Mean cpm without antigen was 1130 ± 11 for MBP-fed and 845 ± 185 for vehicle-fed rats.

\( P < 0.005 \) for S.I. from MBP-fed compared to vehicle-fed rats.
Figure 12. Proliferative response to MBP of LNC from rats fed MBP prior to sensitization.

Values shown represent mean stimulation index \( \text{cpm with antigen} / \text{cpm w/o antigen} \) of quadruplicate determinations from 3 rats. Rats were fed guinea pig MBP or vehicle as described in figure 11. Rats were sensitized 3 days after the last feeding. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP. Mean cpm without antigen was 2973 ± 591 for MBP-fed and 1664 ± 264 for vehicle-fed rats.

\( P < 0.001 \) for S.I. from MBP-fed rats compared to vehicle-fed rats.
Table 8. Assessment of EAE in rats fed MBP on the same day as sensitization versus rats fed MBP prior to sensitization.*

<table>
<thead>
<tr>
<th>Feeding regimen</th>
<th>Day of sensitization</th>
<th>Day of onset</th>
<th>Clinical signs</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 4x5mg MBP</td>
<td>day of first feeding</td>
<td>12.3 ±0.9</td>
<td>0/13 0/13 0/13 13/13</td>
<td>4.0</td>
</tr>
<tr>
<td>B 4x5mg MBP</td>
<td>3 days after last feeding</td>
<td>13.6 ±1.2^c</td>
<td>1/10 5/10 1/10 3/10^d</td>
<td>2.5 ±1.3^b</td>
</tr>
<tr>
<td>C 4xVehicle</td>
<td>day of first feeding</td>
<td>11.6 ±0.8</td>
<td>0/5 0/5 0/5 5/5</td>
<td>4.0</td>
</tr>
<tr>
<td>D 4xVehicle</td>
<td>3 days after last feeding</td>
<td>10.5 ±1.3</td>
<td>0/4 0/4 0/4 4/4</td>
<td>4.0</td>
</tr>
</tbody>
</table>

a. Rats were fed four 5mg doses of guinea pig MBP, or four doses of vehicle administered every other day over an eight day period. MBP was dissolved in ml 0.15M sodium bicarbonate buffer and was fed with 10mg STI suspended in sodium bicarbonate buffer. Control rats were fed vehicle only. Groups A and C were sensitized with 10ug column-purified guinea pig MBP-CFA on the same day as the first feeding. Groups B and D were sensitized 3 days after the last feeding. All rats were monitored daily for clinical neurological signs.

b. Mean ±S.D. c. P ≤0.005 compared to group D.
d. P ≤0.025 compared to group A and D.
e. P ≤0.05 compared to group D.
(30%) exhibited paralysis. Those rats that did show clinical signs in Group B had a delay of 3.1 days in the onset of EAE compared to vehicle fed controls (Group D). Thus, the oral administration of MBP prior to sensitization suppressed in vitro proliferative responses to MBP and caused significant inhibition of disease induction. However, oral MBP administered on the same day as sensitization suppressed in vitro proliferative responses, but did not suppress clinical disease activity.

Adoptive transfer I: Attempts to transfer unresponsiveness to MBP from MBP-fed donors to normal recipients.

I next addressed the issue of whether MBP-fed rats contained cells or serum factor(s) capable of transferring unresponsiveness to normal recipients. A modified procedure described by Swierkosz and Swanborg (1975) was used. To accomplish this, rats were first fed MBP, and three days after the last feeding, these animals were bled, sacrificed, and lymphoid cell suspensions prepared. Serum, thymocytes, spleen cells, mesenteric LNC and Peyer's patch lymphocytes were transferred by i.v. injection into normal recipients. The recipients were challenged 24 hours later with MBP-CFA. As illustrated in table 9, all control rats that were only sensitized exhibited paralysis. Recipients of serum, thymus, spleen, MLN, or Peyer's patches from MBP-fed donors began to exhibit clinical signs at about the same time as the controls (day 11-12 post sensitization), and all the rats progressed to paralysis except for one spleen cell recipient. Thus, no transfer of unresponsiveness was observed by transferring either serum, or lymphoid cells from MBP-fed rats to normal recipients.
Table 9. Adoptive transfer I: Attempt to transfer unresponsiveness to MBP from MBP-fed donors to normal recipients.

<table>
<thead>
<tr>
<th>Material transferred</th>
<th>No. cells</th>
<th>Day of onset</th>
<th>Incidence of EAE</th>
<th>Clinical paralysis</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (2ml)</td>
<td>---</td>
<td>12.3 ±1.5</td>
<td>4/4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>460x10⁶</td>
<td>12.3 ±1.5</td>
<td>4/4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>138x10⁶</td>
<td>12.0 ±1.4</td>
<td>3/4</td>
<td>3.5 ±1.0</td>
<td></td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>92x10⁶</td>
<td>11.8 ±2.0</td>
<td>4/4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Peyer's patches</td>
<td>6x10⁶</td>
<td>11.5 ±2.0</td>
<td>2/2</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>11.4 ±1.5</td>
<td>4/4</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

a. Donor rats were fed four 5mg doses of guinea pig MBP, administered every other day over an eight day period. MBP was dissolved in 1ml 0.15M sodium bicarbonate buffer and was fed with 10mg STI suspended in sodium bicarbonate buffer. Three days after the last feeding, rats were bled, sacrificed, lymphoid cell suspensions were prepared, washed and transferred by intravenous injection into normal recipients. The recipients were challenged 24 hours later with 10ug column-purified guinea pig MBP-CFA and monitored daily for clinical neurologic signs. Control rats were sensitized only.

b. Mean ±S.D.
### Table 10. Adoptive transfer II: Attempt to transfer unresponsiveness to MBP from MBP-fed and challenged donors to normal recipients

<table>
<thead>
<tr>
<th>Material transferred</th>
<th>No. cells injected</th>
<th>Day of onset of EAE</th>
<th>Incidence of paralysis</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (2ml)</td>
<td>---</td>
<td>12.5 ± 1.3</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>Plasma (2ml)</td>
<td>---</td>
<td>13.3 ± 1.2</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>Thymus</td>
<td>450x10^6</td>
<td>14.0 ± 1.4</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>470x10^6</td>
<td>13.3 ± 1.0</td>
<td>4/4</td>
<td>4.0</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>200x10^6</td>
<td>12.7 ± 0.6</td>
<td>3/3</td>
<td>4.0</td>
</tr>
<tr>
<td>Peyer's patches</td>
<td>16x10^6</td>
<td>13.0</td>
<td>2/2</td>
<td>4.0</td>
</tr>
<tr>
<td>Draining lymph node</td>
<td>100x10^6</td>
<td>12.5 ± 2.0</td>
<td>2/2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

---

a. Donor rats were fed four 5mg doses of guinea pig MBP, administered every other day over an eight day period as in table 9. Seven days after the last feeding, rats were sensitized with 10ug column-purified guinea pig MBP-CFA. Two days after sensitization, donor rats were bled, sacrificed, lymphoid cell suspensions were prepared, washed and transferred by intravenous injection into normal recipients. Recipients were challenged 24 hours later with 10ug column-purified guinea pig MBP-CFA and monitored daily for clinical neurologic signs.

b. Mean ± S.D.
Adoptive transfer II: Attempts to transfer unresponsiveness to MBP from MBP-fed and challenged donors to normal recipients.

Since I was unable to demonstrate transfer of unresponsiveness in the previous experiment where donors were only MBP-fed, I reasoned that the oral tolerance regimen may require an MBP-CFA challenge in order to allow proliferation of cells that are required for transfer of unresponsiveness. This approach was used by Asherson et al. (1977) in the demonstration of suppressor cell-mediated oral tolerance to the contact sensitizing agent oxazolone. As shown in table 10, donor rats were fed MBP and challenged with MBP-CFA seven days after the last feeding. Serum, plasma and various lymphoid cell populations were transferred to Lewis recipients, which were then challenged with MBP-CFA. All recipients exhibited paralysis with a maximum clinical score of 4.0, again indicating no transfer of unresponsiveness.

Next, I tested whether LNC from MBP-fed rats would exert in vitro suppressive influences on MBP-induced proliferation of LNC from vehicle-fed rats. As illustrated in Figure 13, LNC (4 x 10^5) from vehicle-fed rats exhibited proliferative responses to MBP, whereas LNC (4 x 10^5) from MBP-fed rats were inhibited from proliferating. When 2 x 10^5 cells from MBP-fed rats were co-cultured with an equal number of cells from vehicle-fed rats, no inhibition was observed. That is, the response of the co-cultured cells to MBP was greater than half of the response of the cultured cells from the vehicle-fed control group, rather than decreased to the level of the MBP-fed group, as would be expected if suppressor cells were operative.
Figure 13. Proliferative response to MBP of LNC from vehicle-fed control rats mixed with LNC from MBP-fed rats.

Values shown represent the mean stimulation index of quadruplicate determinations from 4 rats/group. Rats were fed four 5 mg doses of guinea pig MBP or vehicle four times administered every other day over an eight day period. Three days after the last feeding, some rats were sacrificed and used as cell donors for the adoptive transfer experiment in Table 10, the remaining rats were sensitized with 10 μg column-purified guinea pig MBP-CFA. Twelve days post sensitization, rats were sacrificed and their draining LNC used in a lymphocyte proliferation assay with column-purified guinea pig MBP. 4 x 10^5 LNC from MBP-fed or vehicle-fed rats were cultured separately with various concentrations of MBP, or 2 x 10^5 cells from MBP-fed and vehicle-fed rats were mixed together and cultured with MBP. Mean cpm without antigen was 2336 ± 720 for MBP-fed 2166 ± 711 for vehicle-fed and 1928 ± 608 for MBP-fed + vehicle-fed rats.

P < 0.001 for S.I. from MBP-fed compared to vehicle-fed or vehicle-fed and MBP-fed rats.

P < 0.002 for S.I. from vehicle-fed compared to vehicle-fed and MBP-fed rats.
Table II. Lack of adoptive transfer of EAE following MBP feeding of cell donors.

<table>
<thead>
<tr>
<th>Treatment of donor of donor</th>
<th>Treatment of recipient of recipient</th>
<th>EAE in recipients</th>
<th>No of cells</th>
<th>clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-fed</td>
<td>LNC 240-500x10^6</td>
<td>5/8</td>
<td>1.1 ±1.0</td>
<td></td>
</tr>
<tr>
<td>MBP actv. LNC</td>
<td>32-40x10^6</td>
<td>5/5</td>
<td>2.4 ±1.1</td>
<td></td>
</tr>
<tr>
<td>MBP actv. spleen</td>
<td>40-112x10^6</td>
<td>17/18</td>
<td>1.7 ±0.6</td>
<td></td>
</tr>
<tr>
<td>MBP-fed</td>
<td>LNC 221-500x10^6</td>
<td>0/5^d</td>
<td>0.0^g</td>
<td></td>
</tr>
<tr>
<td>MBP actv. LNC</td>
<td>36-40x10^6</td>
<td>0/5^e</td>
<td>0.0^e</td>
<td></td>
</tr>
<tr>
<td>MBP actv. spleen</td>
<td>40-110x10^6</td>
<td>3/16^f</td>
<td>0.3 ±0.6^f</td>
<td></td>
</tr>
</tbody>
</table>

a. Results shown represent data pooled from 3 experiments.
b. Donor rats were fed four 5mg doses of guinea pig MBP or four doses of vehicle administered every other day over an eight day period as detailed in table 8. Three days after the last feeding, rats were sensitized with 10ug column-purified guinea pig MBP-CFA.
c. Draining LNC and spleens were collected from donors 9 days after sensitization. LNC or spleen cells were cultured for 48 hours with MBP(10ug/ml). Transferred cells were injected intravenously into normal recipients which were monitored daily for clinical neurologic signs.
d. P < 0.025 compared to vehicle-fed LNC recipients.
e. P < 0.005 compared to vehicle-fed MBP actv. LNC recipients.
f. P < 0.005 compared to vehicle-fed MBP actv. spleen recipients.
g. P < 0.05 compared to vehicle-fed LNC recipients.
Lack of adoptive transfer of EAE following MBP feeding of cell donors.

In order to determine whether it is possible to adoptively transfer EAE from MBP-fed donors to normal recipients, donor rats were fed MBP or vehicle and subsequently sensitized with MBP-CFA. This approach had been previously reported by Swierkosz and Swanborg (1975) in the demonstration of suppressor cells in rats rendered unresponsive to EAE by MBP-IFA injection. In my studies, draining LNC and spleens were collected from donors 9 days after sensitization. A portion of the LNC (2.2 - 5 x 10^8/recipient) were transferred i.v. to normal rats. The remainder of the LNC and all of the spleen cells were cultured with MBP for 48 hours, as a means of in vitro amplification, prior to transfer (3-11 x 10^7 cells/recipient). Results in table 11 show that five of eight (63%) recipients of directly transferred LNC from vehicle-fed donors exhibited EAE clinical signs compared to none of the recipients of directly transferred LNC from MBP-fed donors. Moreover, five of five (100%) recipients of MBP activated LNC from vehicle-fed donors exhibited clinical signs, whereas none of the recipients of MBP-activated LNC from MBP-fed donors showed any evidence of clinical EAE. Seventeen of eighteen (94%) recipients of MBP activated spleen cells from vehicle-fed donors exhibited clinical signs compared to three of sixteen (19%) recipients of MBP activated spleen cells from MBP-fed donors. Thus, LNC from vehicle-fed and challenged rats transferred EAE to normal recipients. LNC and spleen cells cultured with MBP from vehicle-fed and challenged rats transferred EAE to
normal recipients. However, LNC from MBP-fed and challenged rats
did not transfer EAE, either directly or after in vitro culture
with MBP. Spleen cells cultured with MBP from MBP-fed rats,
transferred EAE to normal recipients at a significantly lower rate
\((P \leq 0.005)\) than spleen cells from vehicle-fed rats cultured with
MBP. All recipients showed mild histologic EAE.
DISCUSSION

The major findings of this study can be summarized in the following manner. The oral administration of MBP to Lewis rats prior to EAE induction results in total inhibition of disease or a significant delay in the onset of EAE clinical signs. The oral administration of MBP is also observed to significantly depress the in vitro proliferative response of LNC to MBP. Inhibition of EAE as well as suppression of in vitro proliferative responses are both antigen specific and occur as early as 3 days and as late as 15 days after the last feeding. The oral administration of MBP begun simultaneously with encephalitogenic challenge, resulted in significant inhibition of in vitro proliferative responses to MBP, but no inhibition of clinical disease activity. The results derived from the adoptive transfer experiments were twofold. First, MBP-specific unresponsiveness could not be transferred from MBP-fed to normal rats by means of lymphoid cells or humoral factors. Second, lymphoid cells from MBP-fed and challenged rats did not transfer EAE to normal syngeneic recipients, even after in vitro activation with specific antigen, MBP.

The administration of MBP by the oral route has several advantages over other previously reported tolerance regimens: 1) no need for adjuvants such as IFA, 2) ease of administration, 3) potential for orally introduced antigens to induce antigen-specific T suppressor cells (Asherson et al. 1977, Kagnoff, 1978a, Ngan and Kind, 1970), which are known to suppress EAE (Welch and Swanborg, 1977) and 4) MBP introduced by the oral route is safer than
parenterally introduced MBP, mainly because of its potential encephalitogenicity. When administered orally, MBP would be degraded by intestinal enzymes and thus the entire encephalitogenic molecule is not presented to the lymphoid system but rather fragments of MBP are presented. Since the suppressive determinant of the MBP molecule appears to be less labile than the encephalitogenic determinant for several species (Paterson, 1980), it could be predicted that the degraded MBP molecule would generate a suppressive response. This is of particular importance in testing this therapeutic regimen in MS patients. The unique nature of the MBP molecule, i.e., its small size, linear conformation and exquisite susceptibility to enzymatic degradation, made it initially difficult to induce oral tolerance by simply administering MBP suspended in water (Table 1). However, by suspending MBP in sodium bicarbonate buffer to retard pepsin digestion and including a trypsin inhibitor with each feeding, it was ultimately possible to orally tolerize Lewis rats to guinea pig MBP. Feeding soybean trypsin inhibitor did not have any apparent effect on the clinical signs or histopathologic changes of EAE.

In EAE, it has been suggested that the more severe the disease process, the more difficult it will be to overcome, i.e., there is a balance between protection and challenge (Coates et al., 1974; Kies, 1978). Based on this reasoning, my studies were initiated using rat MBP, a homologous antigen with weak encephalitogenic activity. Even though guinea pig MBP is markedly more encephalitogenic for Lewis rat MBP, it was possible to induce oral tolerance to guinea pig but not rat MBP. Feeding 20 mg rat MBP (in sodium bicarbonate buffer
and in the presence of STI) did not prevent the subsequent induction of EAE. However, despite the occurrence of EAE in both rat MBP-fed and control rats, the proliferative responses in both groups to rat MBP were extremely low (Table 4). In contrast, rats fed guinea pig MBP and challenged with guinea pig MBP-CFA were refractory to EAE induction (Table 7), and demonstrated significant suppression of MBP induced LNC proliferation (Figures 2, 3, 4, 5, 6, 7). Swierkosz and Swanborg (1977) reported a similar observation. In trying to tolerize rats to MBP, bovine MBP provided partial protection to Lewis rats against EAE when given subcutaneously in IFA but was not effective when given i.v. in soluble form. Guinea pig MBP was effective in suppressing EAE when given i.v. in soluble form or emulsified in IFA. Thus, guinea pig MBP which is more encephalitogenic for rats than bovine MBP, was also a better tolerogen. It may be that, since guinea pig MBP is more foreign to rats than rat MBP, when introduced orally, it induces the production of suppressor factors or cells. However, when rat MBP is fed, it is recognized by the gut associated lymphoid tissues as self and no suppressor factors or cells are produced.

Antigen-induced proliferation of LNC has previously been shown to be specifically inhibited in animals rendered tolerant by protein feeding using such antigens as OVA (Richman et al., 1978; Miller and Hanson, 1979). However, most of the oral tolerance work in the literature involves feeding soluble proteins such as OVA, bovine
serum albumin, human gamma-globulin, keyhole limpet hemocyanin, bacterial antigens such as cholera toxin, contact sensitizing agents such as picryl chloride or erythrocyte antigen such as SRBC. To my knowledge, my study is the first report which involves the extrapolation of oral tolerance principles to a disease state.

My results demonstrated that a minimum of 15 mg guinea pig MBP given orally was required to inhibit induction of EAE (Table 5). However, 10, 15, or 20 mg MBP administered orally caused significant inhibition of LNC proliferation in response to MBP (Figure 3). These results indicate that the LNC proliferative response is a more sensitive indicator of orally induced unresponsiveness to MBP, than in vivo EAE induction. In studies conducted by other investigators, a dosedependent decrease in both humoral and CMI responses was also observed after oral administration of antigen. For example, Miller and Hanson (1979) demonstrated that a single intragastric dose of 20 mg OVA gave the maximum decrease observed in humoral (anti-OVA antibody titer measured by the Farr assay) and CMI (ear-swelling) responses in mice. My results, in terms of relative quantities of antigen required for oral tolerance induction, also agree with Vaz et al. (1977) and Vives et al. (1980) who used 1.25 - 20 mg OVA and 2.5-30 mg human gammaglobulin, respectively. McKenna et al. (1984) showed that the degree of suppression of clinical EAE was greater in rats that were pretreated with multiple i.v. doses of MBP-coupled syngeneic spleen cells (MBP-SL) (13.2 - 290.4 μg MBP) versus animals receiving a single dose of MBP-SL (4.4 - 96.8 μg). In contrast,
multiple doses of MBP-SL did not further increase the suppression observed in the in vitro proliferative response to MBP.

Oral administration of MBP resulted in specific unresponsiveness to MBP. Feeding ovalbumin did not affect induction of EAE (table 7), nor MBP induced LNC proliferation (Figure 7). Whereas MBP-induced proliferative responses of LNC from MBP-fed rats were significantly suppressed compared to LNC from vehicle-fed control rats (Figure 7), proliferative responses of both LNC populations to PPD and Con A were almost superimposable (Figures 9, 10). These results indicate that cells sensitized to PPD by injection of MBP-CFA are not inhibited from proliferating by the oral administration of MBP. Identical proliferation of both LNC populations to the T-cell mitogen Con A indicates that it is not a generalized suppression of T cells. MBP-specific cells constitute a very low percentage of the T cells that respond to Con A stimulation.

The oral administration of MBP was shown to result in tolerance as early as three days after the last feeding and to persist for at least 15 days after the last feeding which was the last time point tested (Table 6, Figures 4, 5, 6). Challacombe and Tomasi (1980), have shown that mice challenged with OVA 2-60 days after intragastric administration of 20 mg OVA exhibited suppression of LNC proliferative responses to OVA. These results agree with kinetics of oral tolerance induction to MBP in the present study. However, my results differ from kinetic studies on the suppression of EAE induced by MBP-SL administration (McKenna et al., 1984). These workers showed that pretreatment of Lewis rats with MBP-SL inhibited EAE,
and suppressed in vitro LNC proliferative responses to MBP if injected 7 but not 3 days before the encephalitogenic challenge.

The question of whether oral tolerance to MBP could be induced following encephalitogenic challenge was addressed. When rats were sensitized with MBP-CFA and MBP feeding initiated on the same day, no inhibition of EAE was observed in MBP-fed rats (Table 8). However, LNC proliferative responses to MBP from fed rats were still significantly decreased in comparison with control responses (Figure 11). The oral administration of MBP, under these experimental circumstances, was sufficient to suppress lymphocyte proliferation to MBP, which was evident in vitro, but was not sufficient in inhibit in vivo disease activity. McKenna et al. (1984) also found that, in general, suppression of the in vitro response to MBP was more easily brought about than suppression of clinical EAE, following pretreatment of rats with MBP-SL. It has been reported that parenteral immunization may influence the absorption of specific antigen from the gut by increasing the level of specific antibodies in the intestinal lumen (Walker et al., 1973). This factor may contribute to the immune elimination of absorbed antigen resulting in a diminished tolerant state. This may have occurred in MBP-fed rats sensitized on the same day as the first feeding. Hanson et al. (1979) showed that feeding OVA to already OVA-sensitized mice resulted in significant secondary antibody titers. The increase in titers was directly proportional to the oral dose of antigen, whereas in unsensitized animals, antigen feeding reduced the antibody response following subsequent challenge. Oral tolerance regimens are unable
to inhibit preformed effector cells, and immunity rather than tolerance may follow the oral administration of an antigen to an animal that has had prior contact with the antigen (Chiller et al., 1978; Pierce and Koster, 1980). It appears from my results that the feeding of MBP results in suppression of the afferent limb of the EAE immune response, but not the efferent limb. This idea was pursued further in one preliminary experiment, in which preformed EAE effector cells were transferred into an orally tolerized recipient. MBP-specific T-line cells, prepared in our laboratory by the procedure of Vandenbark et al. (1985), capable of transferring severe paralytic signs of EAE, were the source of the effector cells. Following injection of T-line cells (5 x 10^6) into a single MBP-fed rat, the animal exhibited marked clinical signs of EAE, suggesting that MBP-induced oral tolerance was not effective against preformed effector T cells.

In order to address the question of the lymphoid element responsible for mediating the MBP unresponsiveness, adoptive transfer experiments were conducted where serum, plasma, thymus, spleen, MLN, PP or draining LNC were transferred from MBP-fed donors to normal recipients, which were challenged 24 hours later (Tables 9, 10). None of these cellular or humoral elements were observed to suppress EAE induction, similar to the findings observed by Swierkosz and Swanborg (1977). In their studies, i.v. injection of soluble MBP protected rats from subsequent EAE challenge. Donor cells from such protected rats failed to transfer unresponsiveness, regardless of whether the source was LNC (2 x 10^8), spleen (2 x 10^8) or thymus
(9 x 10^8). In contrast, Asherson et al. (1977) was able to show transfer of unresponsiveness with spleen cells (2.2 x 10^7) from donor mice fed the contact sensitizing agent oxazolone, thus demonstrating the involvement of suppressor cells. Furthermore, Challacombe and Tomasi (1980) have shown transfer of unresponsiveness with MLN cells (40 x 10^6) from donor mice fed OVA, also demonstrating the involvement of suppressor cells.

Several factors may explain the inability to demonstrate transfer of EAE unresponsiveness in my hands. First, the number of cells transferred may have been fewer than necessary especially for PP and MLN populations (Table 9). Richman et al. (1978) showed that spleen cells from mice fed OVA were capable of suppressing the OVA-specific PFC responses of recipient mice, when compared with spleen cells from control saline-fed mice. The degree of suppression was dependent on the number of cells transferred; 20 x 10^6 but not 1 x 10^6 cells provided significant suppression. Secondly, in my studies, the conditions for stimulating a detectable level of suppressor cells may not have been quite achieved. For example, Ngan and Kind (1978) found that although a single feeding of 20 mg OVA to mice resulted in tolerance as measured by decreased anti-OVA IgE titers, eight feedings of 20 mg were required in order to transfer the tolerant state from OVA-fed donors to normal recipients using 250 x 10^6 PP cells or 100 x 10^6 spleen cells. Thirdly, the possibility exists that the recipients require irradiation in order to show transfer of EAE unresponsiveness. Phanuphak et al. (1974) reported that tolerance induced in mice by i.v. injection of 15 mg
2, 4-dinitrobenzene-1-sulfonic acid sodium salt (DNBSO₃Na), could be transferred into lightly irradiated (250R) syngeneic recipients by i.v. injection of 160 x 10⁶ spleen cells. When nonirradiated recipients were used, tolerance could also be transferred, but the degree of suppression was not as marked as when cells were transferred into irradiated recipients. The authors speculated that light irradiation might make available "room" for homing of the transferred cells, or provide nonspecific factors from the damaged tissues of the irradiated recipients.

A fourth factor which may play a role in our findings involves the kinetics of transfer of donor cells. Miller and Claman (1977) investigated the kinetics of tolerance induction in mice tolerized by the i.v. injection of (DNP) hapten-modified spleen cells in terms of the ability to transfer unresponsiveness from tolerant donors to normal recipients (i.e., induction of suppressor T cells). The tolerant state was transferrable with 100 x 10⁶ LNC, when the cells were obtained 4 to 7 days after i.v. injection of DNP-SC. However, donor mice rendered tolerant one day or two weeks prior to transfer and demonstrated to be fully tolerant, could not transfer unresponsiveness to naive recipients. The authors explain their results on the basis of two possible mechanisms for tolerance induction. One mechanism, demonstrable for a finite period was shown to be due to suppressor T cell activity, while the other mechanism appeared to be inhibition of antigen-reactive T cell clones. Thus, the precedent exists in the immunological literature for the induction of oral tolerance in the absence of suppressor T cells. Furthermore,
orally induced tolerance could also be elicited in the absence of
demonstrable suppressor cells as shown in lymph nodes (inguinal and
periaortie) of adult mice rendered unresponsive to OVA (Richman,
et al., 1978) and in the spleens of neonatal mice tolerized to human
γ-globulin (Benjamin, 1977). Hanson and Miller (1982) also reported
that pretreatment of OVA-fed mice with cyclophosphamide (CY) had no
significant influence on the level of tolerance in donor mice as
measured by DTH, lymphocyte proliferation and serum antibody titer.
In contrast, CY pretreatment of donors before OVA feeding eliminated
the capacity of lymphoid cells from OVA-fed donors to transfer
suppression to normal recipients. The authors concluded that CY
sensitive T suppressor cells are not necessary for either induction
or maintenance of specific tolerance after OVA-feeding.

In light of the literature cited above, my results of the lack
of adoptive transfer of tolerance from 1) donors that were fed MBP
only prior to transfer and 2) donors that were fed and challenged
prior to transfer, are not consistent with a suppressor cell
mechanism.

Antigen-induced proliferation of LNC was specifically suppressed
in rats rendered tolerant by oral MBP. However, these same LNC from
MBP-fed rats did not exert suppressive influences on the MBP-induced
proliferative responses of LNC from vehicle-fed control rats in co-
culture experiments (Figure 13). There are several possible
explanations for these findings. 1) Suppressed lymphocyte
proliferation may not reflect the presence of specific suppressor
cells, but rather the absence or inactivation of specific
MBP-reactive lymphocytes. 2) Suppressed proliferative responses to MBP may be a reflection of a defect in the function of antigen presenting cells which prevents antigen priming. 3) The proliferative response of MBP-primed lymphocytes may not be easily suppressed.

The adoptive transfer of EAE with lymphoid cells has historically been used as a method of analyzing the mechanisms of disease induction and inhibition. We have shown that LNC from MBP-fed and challenged donor rats do not transfer EAE to normal syngeneic recipients either directly or following in vitro amplification with MBP (Table 11). Spleen cells from MBP-fed rats, cultured in vitro with MBP, transferred mild clinical signs of EAE in one of three experiments performed (19% of the recipients showed mild clinical signs versus 94% of control recipients) (Table 11). Swierkosz and Swanborg (1975) utilized this approach in analyzing the mechanism of EAE inhibition in Lewis rats pretreated with eight intradermal injections of MBA-IFA. In their studies, LNC from pretreated (MBP-IFA) and challenged (MBP-CFA) rats were incapable of directly transferring clinical EAE. These investigators have shown that suppressor T cells play a significant role in EAE suppression in this experimental system. In a later study, Killen and Swanborg (1982) showed that spleen cells or LNC, from rats tolerized by a single injection of MBP-IFA, transferred EAE only after in vitro amplification with MBP. A similar observation was made in another experimental system; rats that have recovered from EAE are resistant to subsequently induced disease, probably as a result of activation
of suppressor cells (Willenborg, 1979; Welch et al., 1980). It has been reported that LNC and spleen cells from EAE recovered rats are able to transfer EAE to naive recipients following in vitro culture with MBP. Thus, based on two independent observations, the in vitro amplification step appears to override the resistance to EAE mediated by T suppressor cells. It appears, therefore, that the mechanism mediating oral tolerance to MBP in the present study is different from the mechanism mediating tolerance in MBP-IFA treated or EAE recovered rats, since I observed no transfer of EAE from orally tolerized donors even after in vitro activation with MBP.

The mechanisms responsible for oral tolerance in MBP-fed rats could be due to mechanisms other than suppressor cells. It has been shown in other studies investigating suppression of CMI responses that clonal inhibition as well as suppressor cells may be responsible for the tolerant state. Richman (1979) reported that suppressor T cells could be demonstrated 1 week after OVA feeding, but could not be demonstrated 4 weeks after OVA feeding, even though the animals were still unresponsive when challenged with OVA-CFA. Furthermore, circulating serum factors have been reported to be responsible for tolerance after oral administration of SRBC (Kagnoff, 1978b).

The interposition of the liver between the gastrointestinal tract and the systemic circulation provides certain metabolic advantages to the host. This interposition appears to be immunologically important, since the liver functions to prevent sensitization to potential antigens contained in food and absorbed into the portal system. Cantor and Dumont (1967) found that the
establishment of a portacaval shunt in dogs abolished the unresponsiveness that normally follows the oral ingestion of dinitrochlorobenzene. These findings suggested that antigens going to the liver via the portal system induce specific unresponsiveness, but the mechanism of such unresponsiveness is unknown. Qian et al. (1985) demonstrated that portal venous inoculation of alloantigens in mice results in long-lasting specific immunologic tolerance. These workers concluded that tolerance induced in this fashion was due to elimination or functional impairment of DTH effector clones specific for the relevant alloantigens.

It is certainly possible that a variety of immunologic events occur at varying times and locations in an animal following enteric exposure to antigen. The unresponsiveness which follows the feeding of an antigen has been documented in the immunologic literature to involve several different mechanisms. Thus, the presence of one mechanism does not exclude the involvement of others. Furthermore, different mechanisms of unresponsiveness may exist simultaneously for B-cell-mediated (humoral) and T-cell-mediated (DTH) responses (Kagnoff, 1978 a.b.).

It is not entirely clear from the results obtained whether tolerance following oral administration of MBP is established through inactivation of MBP specific T cell clones or through the influence of suppressor cells. Additional experiments are required in order to finally resolve these questions. However, after compiling all of the relevant in vivo and in vitro observations, there is stronger evidence for the former mechanism. The following findings, taken together,
1) inhibition of EAE in MBP-fed rats; 2) lack of adoptive transfer of EAE even after an in vitro amplification step; 3) kinetics of induction of MBP-induced oral tolerance; 4) antigen-specific suppression of MBP-induced LNC proliferative responses; 5) no suppression of MBP-induced proliferation of LNC from control rats when mixed with LNC from MBP-fed rats, may be interpreted to show that functional inactivation of MBP-reactive cells may be one likely mechanism involved in this model of MBP induced tolerance.

Since we now know that oral administration of intact MBP prior to challenge can inhibit EAE, it would be interesting to know whether MBP-fed rats are also unresponsive to challenge with other neuro­antigens such as whole CNS tissue. Other questions are: to define the portion of the MBP molecule which is responsible for eliciting the suppressive effect; to investigate the effectiveness of orally induced tolerance to MBP in suppressing ongoing EAE, or influence the induction or relapses occurring in chronic relapsing EAE in SJL mice or strain 13 guinea pigs. The latter experiments would be important in considering oral MBP for therapeutic trials in MS. The real significance of the results of orally induced tolerance to MBP depends on its applicability to the MS disease process. To date, there is no treatment which alters the long term course of MS.
If MS is truly an autoimmune response to myelin, it should be possible to control the disease process by interfering with the continued production of sensitized cells. The currently available experimental treatments which strive to achieve those results make use of immunosuppressive drugs, e.g., cyclosporine, cyclophosphamide (Hauser et al., 1983). Suppression of specifically sensitized cells would potentially be more effective and safer.
REFERENCES


60. Jarrett, E., D. Haig, W. McDougall and E. McNulty. 1976. Rat IgE production. II. Primary and booster reaginic antibody responses following intradermal or oral administration. Immunology, 30: 671.


114. Paterson, P.Y. 1980. The immunopathology of EAE. In The
Suppression of EAE and MS, (Ed. A.N. Davison and M.L. Cuzner)

x-irradiation on induction of allergic encephalomyelitis in

on experimental allergic encephalomyelitis in Lewis rats.
Science 165: 191.

encephalomyelitis in rats by means of antibrain serum. J. Exp.

118. Paterson, P.Y., A.F. Jacobs and E.M. Coia. 1965. Complement-
fixing antibrain antibodies and allergic encephalomyelitis.
II. Further studies concerning their protective role. Ann. N.Y.

(MBP-SFs) and anti-MBP antibodies in humans.

120. Pender, M.P. and T.A. Sears. 1984. The pathophysiology of
acute experimental allergic encephalomyelitis in the rabbit.

oligoclonal antibody in experimental allergic

experimental allergic encephalomyelitis in SJL/J mice after in
vitro activation of lymph node cells by myelin basic protein:

and contact sensitivity to DNFB in mice. III. Transfer of

the intestinal immune response to Cholera toxoid/toxin by


