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Genetic resistance in experimental autoimmune encephalomyelitis: Analysis of the resistance mechanisms in the Lewis Resistant or LeR rat

Pelfrey, Clara Mackenzie, Ph.D.
The Ohio State University, 1988

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GENETIC RESISTANCE IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS:
ANALYSIS OF THE RESISTANCE MECHANISMS IN THE
LEWIS RESISTANT OR LeR RAT
DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of the Ohio State University
By
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The Ohio State University
1988

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Dedicated to my husband, Scott Thomas Pelfrey,

and to my parents,

Dr. Allen Hugh Mackenzie and Dr. Clara Childs Mackenzie
ACKNOWLEDGMENTS

I gratefully acknowledge the guidance, encouragement, and assistance of my advisor, Dr. Caroline Whitacre, whose limitless energy has been a great motivation and inspiration to me. I would like to extend my thanks to the members of my advisory committee, Drs. John Hughes, Marshall Williams and John Sheridan for their suggestions and support. My gratitude goes to Dr. Frank Waxman, whose grant proposal provided much inspiration for my research.

I wish to extend my thanks to Ingrid Gienapp and Karen Cox for their expert technical assistance, and to Kathy Fuller for her help and encouragement through the course of our studies together.

I would like to thank my parents, whose encouragement and love have enabled me to strive for excellence throughout my life. Finally, to my husband, Scott Pelfrey, I offer sincere thanks for your enduring faith in me and your willingness to help me achieve my goals.
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<tr>
<td>BM</td>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>BMC</td>
<td>Bone marrow cells</td>
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</tr>
<tr>
<td>BN</td>
<td>Brown Norway (rat)</td>
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<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
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<tr>
<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>CR-EAE</td>
<td>Chronic relapsing EAE</td>
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<td>Cyclophosphamide</td>
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<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>EF</td>
<td>Encephalitogenic fragment (of MBP)</td>
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<td>GPMBP</td>
<td>Guinea pig myelin basic protein</td>
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<td>HuMBP</td>
<td>Human myelin basic protein</td>
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<td>IL-2</td>
<td>Interleukin-2</td>
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<tr>
<td>Le</td>
<td>Lewis (rat)</td>
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<td>LeR</td>
<td>Lewis Resistant (rat)</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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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
<td></td>
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<tr>
<td>NS</td>
<td>Natural suppressor cells</td>
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<td>PHA</td>
<td>Phytohemagglutinin</td>
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<td>PLNA</td>
<td>Popliteal lymph node assay</td>
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<td>PPD</td>
<td>Purified protein derivative</td>
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<td>TCGF</td>
<td>T cell growth factor</td>
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INTRODUCTION

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that was first identified as a distinctive disease over a century ago by the French neurologist, Charcot (Charcot, 1877). His greatest contribution to medicine was in linking the symptoms of disease in life with the pathological findings in the nervous system after death. Charcot called this new disease 'sclérose en plaques'. The 'sclérose' of his title means hardening, referring to the scarring that is the end result of the damage in the CNS caused by MS. The initial MS lesion consists of inflammation around the veins and venules in myelin-containing regions of the CNS (Waksman, 1981). The plaque is the basic lesion of multiple sclerosis, formed by fusion of multiple smaller lesions and showing inflammation, demyelination and/or scarring (Raine, 1983).

MS is one of the most common organic neurological disorders and the most frequently encountered human demyelinating disease. In the United States the distribution of MS falls into three zones: high incidence (30 and above/100,000) above the 37th parallel; medium incidence (5 to 29/100,000) between 30° - 33°; and low incidence (below 5/100,000) in the southern states, between 12° - 19° north latitude (Kurtzke, 1988). Risk factors for MS include age, sex, race, genetics and geography. Onset characteristically is in early adult life, between ages 20 and 40, in
approximately 70% of cases (Kurtzke, 1970) and most prevalence studies report higher rates among females than males, with a ratio of about 1.5:1 (Kurtzke, 1988). Caucasians are more affected than colored races, and specific histocompatibility (HLA) antigen associations have been linked with susceptibility to disease (McDonald, 1984). Geographic distributions indicate that all high and medium MS risk areas are in Europe or its colonies like Canada, the United States, New Zealand and Australia, while risk in the tropics and in all of Asia is low (Kurtzke, 1980).

Clinically, multiple sclerosis is highly variable, and there is no specific diagnostic test. In approximately 60 percent of patients, MS occurs as a series of attacks separated by periods of partial or complete remission and frequently followed by a phase of chronic progression (Waksman and Reynolds, 1984). In the early stages of disease the remissions are usually associated with return of normal neurologic function, but with each remission there is less improvement and increased neurologic dysfunction. Some symptoms and signs last less than 24 hours. These recurrences are likely caused by metabolic or physiologic influences on a previously damaged and demyelinated area of CNS and are called pseudoexacerbations (Poser, 1980).

In addition to the remitting-relapsing form of MS, there are three other clinical courses that have been described as malignant or acute, chronic progressive and benign (McFarlin and MacFarland, 1982). In the malignant form of MS, neurologic dysfunction progresses rapidly over a few weeks or months and may or may not demonstrate periods of incomplete remission. Fewer than 5-10 percent of patients
have a malignant clinical course and this form of the disease is very rapidly fatal (Paty and Poser, 1984). In patients with a chronic progressive form of MS (30 percent of patients) neurologic dysfunction gradually worsens after onset. Remissions and relapses are not clearly defined and the rate of progression varies considerably from one person to another (McFarlin and MacFarland, 1982).

Unlike these relatively severe forms of disease, the benign form of MS seldom displays any exacerbations and those that do appear are often mild and are followed by complete recovery. At least 20 percent of MS patients have a benign course (Paty and Poser, 1984). Because demyelination is occasionally found at autopsy in asymptomatic persons, it seems likely that a subclinical form of disease can also occur (McFarlin and MacFarland, 1982).

The primary element of tissue damage in MS is destruction of the myelin sheath around nerve fibers leaving the axons intact - known as primary demyelination (Muller, 1949). The clinical features of MS are extremely varied because no myelinated system is exempt from plaque formation. Balance abnormalities and other motor symptoms are probably the single commonest cause of disability in MS, followed by sensory loss, visual dysfunction related to optic neuritis and weakness or paraparesis (Kurtzke, 1970). Loss of bladder control and impotence are very common problems. More unusual clinical features of MS are the occurrence of dementia (Peyser et al., 1980), psychotic manifestations (Matthews, 1979), epilepsy (McAlpine et al., 1972), trigeminal neuralgia or tic douloureux (Rushton and Olafson, 1965) and
Although the cause and pathogenesis of MS are unknown, it is widely believed that immune abnormalities are related to the disease. There are at least three theories regarding the etiology of MS. First, traditional epidemiologic studies strongly support the hypothesis that the MS process is triggered in genetically predisposed individuals by exogenous factors, most likely viral infection (Johnson, 1975; Arnason et al., 1974; Macchi, 1987). Second, MS may be an autoimmune disease involving loss of immune regulation to some antigen of myelin or myelin-producing cells in the CNS (Waksman, 1981). A third hypothesis holds that metabolic changes or infection have affected glial cell function in MS brains (McFarlin and McFarland, 1982). The autoimmune theory is supported by the animal model experimental autoimmune encephalomyelitis (EAE) which shares similar immunologic and clinical responses with MS, as well as similar histopathologic changes (Paterson 1977; 1978; 1979; Alvord, 1984; Raine, 1984).

I. EAE: General Features.

Experimental autoimmune encephalomyelitis (EAE), first described 55 years ago (Rivers et al., 1933), is a cell-mediated autoimmune disease of the CNS which can be induced in a variety of experimental animals such as rats, mice, guinea pigs, rabbits, monkeys and sheep (Paterson, 1966; 1976). Acute EAE is induced by
immunizing animals with whole nervous tissue, myelin, purified myelin components such as myelin basic protein (MBP), or encephalitogenic peptides of MBP, emulsified in complete Freund's adjuvant (CFA) (Paterson, 1976; 1978; Paterson and Swanborg, 1988).

The inbred Lewis rat (RT-1\sup{L}) is highly susceptible to EAE and is the prototype animal for EAE research (Vandenbark and Hinrichs, 1974). Clinical neurologic signs of EAE usually appear in rats within 10 to 21 days after sensitization with encephalitogenic antigen and CFA (Paterson, 1976). Clinical symptoms of EAE include weight loss, loss of tail tonicity, ataxic gait, paraparesis, paralysis of both hind legs, fecal impaction and urinary retention due to autonomic nervous system dysfunction from spinal cord injury (Paterson, 1966; 1976). The histopathologic changes in the CNS consist of perivascular mononuclear cell infiltration in which the predominant cell type is the T lymphocyte belonging to the "helper/inducer" subset (Wekerle, 1984). The inflammatory infiltrates consist of many small and large lymphocytes, histiocytes, some plasma cells and macrophages (Waksman, 1981; Paterson, 1976). Perivascular accumulation of fibrinogen is one of the earliest immunopathologic changes in EAE (Oldstone and Dixon, 1968). Demyelination may also be present at sites of cellular infiltration, but normally it is not prominent early in the course of EAE and in acutely fatal courses may be absent (Arnason, 1983). However, striking demyelination is often observed in the guinea pig and rat models of EAE induced by whole spinal cord in CFA (Shaw and Alvord, 1984). Rats that have recovered from a primary course of EAE are resistant to disease reinduction (Waxman et al., 1982; Willenborg, 1979). It was observed, however, that clinical disease could
be reinduced in EAE-recovered rats by a secondary challenge with MBP-CFA and a saline extract of *Bordetella pertussis* (Waxman et al., 1983). This observation could be attributed to increased vascular permeability induced by the *B. pertussis* extract (Waxman et al., 1984). The mechanism responsible for recovery and further resistance to EAE in the rat has not been determined.

Cell-mediated immune mechanisms have been shown to play a major causitive role in EAE (Weigle, 1980; Paterson, 1978). The definitive evidence for the cellular nature of the disease was shown in the passive transfer of EAE to naive recipients by lymph node cells (Paterson, 1960; Stone, 1961). T lymphocytes were shown to be responsible for transfer since anti-thy 1.1 serum plus complement prevented transfer of disease (Ortiz-Ortiz et al., 1976). Gonatas and Howard (1974) provided even more direct evidence for a major role for T cells in EAE. Lewis rats were thymectomized at 5 weeks of age, irradiated at 9 weeks, and then reconstituted with bone marrow cells from syngeneic donors depleted of T cells. Such rats were unable to develop EAE after sensitization with either whole CNS tissue or MBP in CFA. Ortiz-Ortiz and Weigle (1976) reported that both EAE and anti-MBP antibody were readily induced in thymectomized, irradiated Lewis rats reconstituted with normal thymus and bone marrow cells and challenged with MBP-CFA. If the thymus cells were first treated with MBP heavily labeled with $^{125}$I so as to eliminate (suicide) specific T cells, the recipients did not develop EAE or antibody to MBP. On the other hand, if only the B cells were eliminated by $^{125}$I-MBP treatment, EAE was not inhibited. These results showed that although both T and B cells can respond to MBP, only the T cell is responsible for induction of EAE.
Additional evidence for EAE being a T cell mediated disease involved the isolation, growth, and characterization of rat T cell lines and clones specifically reactive against GP-MBP (Ben-Nun et al., 1981; Vandenbark et al., 1985; Lemire and Weigle, 1986). These lines induced clinical and pathologic EAE in syngeneic rats 3 to 5 days after i.v. injection of $10^5$ to $10^6$ T line cells. Furthermore, attenuated line cells could be used to protect rats against active EAE induction (Ben-Nun and Cohen, 1981). In order to demonstrate in vivo transfer activity of the line cells, prior activation with MBP presented by histocompatible accessory cells was necessary (Neparstek et al., 1983).

The major problem with adoptive transfer of EAE was the very large number of T cells required to transfer disease - $10^8$ to $10^9$ cells are usually necessary (Paterson et al., 1975). A significant advance was made with the demonstration that if spleen cells from EAE donors were incubated with concanavalin A (Con A) in tissue culture prior to transfer, as few as $10^7$ cells could then adoptively transfer disease (Pannitch and McFarlin, 1977). Soon after this observation, it was shown that incubation with MBP in culture also facilitated adoptive transfer of disease (Richert et al., 1979; Driscoll et al., 1979) as did lymph node cells activated with Con A (Ortiz-Ortiz and Weigle, 1982) or with a combination of Con A and MBP (Ovadia and Paterson, 1981). In fact, MBP was found to be several times more effective than Con A (Richert et al., 1981) and could be augmented even further by the addition of interleukin 1 (Mannie et al., 1987). Lectins were also able to activate spleen cells for adoptive transfer (Takenaka et al., 1986). It became apparent that in vitro activation permitted transfer
of disease with spleen cells from animals that had fully recovered from acute EAE (Holda et al., 1980). This observation established that T effector cells or their precursors (memory cells) persist in recovered animals but are held in check.

Adoptive transfer of EAE with primed cells did not protect recovered recipients from a second passive transfer of EAE or from EAE induced by active immunization (Hinrichs et al., 1981). In addition, animals that had recovered from actively induced EAE and were refractory to further attempts at active EAE induction, were fully susceptible to passively induced EAE mediated by in vitro conditioned cells (Hinrichs et al., 1981). These findings indicate that effector T cells become relatively refractory to control mechanisms once they have been driven to a terminal state of differentiation (achieved by in vitro activation) and that control is more likely achieved at the level of a precursor cell (Lando et al., 1979b).

Recently, techniques have been developed that permit the maintenance of T cell lines or clones for prolonged periods in tissue culture (Ben-Nun et al., 1981; Ben-Nun and Cohen, 1982; Vandenbark et al., 1985; Lemire and Weigle, 1986). T cells from rats with actively induced EAE can be expanded in vitro with MBP and then maintained by alternating cycles of exposure to T cell growth factor followed by exposure to MBP in the presence of accessory cells. When exposed to MBP or to Con A (in the presence of irradiated thymocytes as a source of antigen presenting cells), these lines will proliferate and adoptively transfer EAE to naive syngeneic rats (Ben-Nun et al., 1981; Ben-Nun and Cohen, 1982). As few as $10^8$ cells prepared in this way will transfer disease.
MBP is the major constituent of CNS tissue which is responsible for encephalitogenic activity (Kies, 1965; 1973). MBP represents almost 30% of total myelin protein and can be extracted directly from myelin by dilute acids (0.1M HCl) (Gupta, 1987). It has been purified from several different animal species as well as from humans. Complete amino acid sequences for human and bovine basic protein were the first to be established (Carnegie et al., 1967; Eylar and Thompson, 1969). MBP consists of 170 amino acids, has a molecular weight of approximately 18,500 daltons (Hashim, 1978) and there is considerable homology between MBP derived from various species (Carnegie, 1971a). It is cationic and distinguishable by the absence of sulfhydryl groups. Unlike histone proteins, MBP has a high content of histidine and proline and contains one tryptophan residue at position 116 (Shapira et al., 1971). The latter is part of the major antigenic determinant in both bovine and human MBP known to induce EAE in guinea pigs (Eylar et al., 1970; Carnegie, 1971b). The encephalitogenic determinant is different for each species and depends on the species from which the protein is prepared and on the species in which EAE is induced. The most encephalitogenic MBP for the Lewis rat is that derived from guinea pig CNS (McFarlin et al., 1973) and the minimum sequence required to induce EAE is contained within amino acids 68 to 88 (Chou et al., 1976). An important structural feature of MBP is its open conformation with little secondary and tertiary structure (Braun and Brostoff, 1977) which is thought to explain its susceptibility to proteolytic degradation (Smith, 1977).
II. Genetics of EAE.

Genetics has emerged as one of the major factors determining susceptibility to autoimmune diseases in both animals and man. Genes controlling specific immune responses (Ir genes) to various exogenous antigens have been identified and are found within the major histocompatibility complex (MHC) (Benacerraf and McDevitt, 1972). Ir genes have been shown to influence susceptibility to a variety of experimental autoimmune diseases, i.e. thyroiditis (Rose et al., 1977), myasthenia gravis (Fuchs et al., 1976), collagen arthritis (Wooley et al., 1981), polyneuritis (Steinman et al., 1981) and encephalomyelitis (Bernard, 1976; Gasser et al., 1973; 1975; 1978; Gunther et al., 1978; Lando, 1979b; Lindh and Källen, 1978; Moore et al., 1980; Teitelbaum et al., 1978; Williams and Moore, 1973).

Genetic analysis of EAE has shown that both the incidence and the severity of the disease are determined by genes linked to the MHC in the rat (Gasser et al., 1973; Williams and Moore, 1973; Gunther et al., 1978), in the mouse (Levine and Sowinski, 1973; Bernard, 1976; Fritz et al., 1985; Lando et al., 1980) and in the guinea pig (Webb et al., 1973; Lisak et al., 1975).

Analysis of a group of congenic rat strains (Gunther et al., 1978) along with backcross studies (Gasser et al., 1975) have suggested that at least two categories of susceptibility genes are operative in EAE in rats. One class, as mentioned above, is associated with the MHC locus RT1, and has been designated as the Ir-EAE gene by Williams and Moore (1973). The second class involves non-MHC-linked genes, which
have greater or lesser control on susceptibility depending on the species and strains studied (Gasser et al., 1978; Lindh and Källen, 1978; Källen and Lodgberg, 1982, Happ et al., 1988). Genetic control in the rat is not identical in all strain combinations. In one case (BN x Lewis), it is closely controlled by a single autosomal gene which, although closely linked to the MHC, is distinct from it (Williams and Moore, 1973; Gasser et al., 1973). In other strain combinations, such as BN x DA, it is completely independent of the MHC (Gasser et al., 1975). Gasser and coworkers (1983) have examined the genetic unresponsiveness of the Lewis Resistant (LeR) and Black-Hooded (BH) rats and found that both of these strains were unresponsive (i.e. resistant) due to non-RT1-linked genes. The EAE resistant AO rat appears to be resistant due to a dominant autosomal gene, not linked to the MHC (Mostarica-Stojkovic et al., 1983).

Studies in mice have yielded conflicting results concerning the role of the H-2 complex in susceptibility to EAE. While Levine and Sowinski (1974) concluded that the MHC does not control susceptibility to EAE in mice, Bernard (1976) reported that susceptibility is controlled by genes linked to the H-2^a and H-2^b haplotypes. Some reports have concluded that EAE responsiveness is under the primary control of genes outside the H-2 complex (Lando et al., 1979b; Teitelbaum et al., 1978) while others suggest that the H-2 complex modifies the degree of EAE responsiveness (Montgomery and Rauch, 1982). It has been reported in some mouse strains that susceptibility is controlled by the combination of H-2 and vasoactive amine sensitization genes since Bordetella pertussis is required for optimal disease induction.
in the mouse (Linthicum and Frelinger, 1982; Knobler et al., 1985; Teucher et al., 1987).

Association between susceptibility to EAE and the MHC has also been reported in guinea pigs (Kies et al., 1975; Lisak et al., 1975). Inbred strain 13 and (strain 2 x strain 13)F1((2x13)F1) guinea pigs are highly susceptible to acute EAE compared with strain 2 animals (Ben-Nun et al., 1981). Teitelbaum et al. (1978) demonstrated that susceptibility to EAE is controlled probably by two genes, one gene is linked to the MHC of strain 13 and the other gene is located outside the MHC. Susceptibility to EAE in this species was also correlated with the immune response to the major encephalitogenic determinant of the basic protein molecule (Teitelbaum et al., 1977).

II. Resistance mechanisms in EAE.

Although it is well known that genes influence susceptibility and resistance to EAE, the mechanisms responsible for genetic control are unknown. Several EAE resistant rat strains have been studied to determine the immunologic mechanisms that mediate their resistance. The Brown Norway (BN) rat lacks the Ir-EAE gene and develops neither histologic nor clinical symptoms of EAE when injected with conventional doses of MBP-CFA (Williams and Moore, 1973). However, much higher doses of GP-MBP (i.e. 200μg) combined with CFA and B. pertussis were shown to induce EAE in the BN rat (Lennon et al., 1976). Williams et al. (1973a, 1973b) and Newlin and Gasser (1973) have demonstrated that BN lymph node cells (LNC)
respond less vigorously than Lewis LNC to the mitogens PHA and Con A. In addition, lymphocytes from MBP-sensitized BN rats are unable to proliferate to the same degree as Lewis lymphocytes in response to MBP (Sheffield et al., 1977). Moreover, BN LNC did not produce macrophage migration inhibition factor when cultured in vitro with the major encephalitogenic fragment (EF) of MBP, nor did these rats develop delayed hypersensitivity skin reactions to the EF (Lindh and Källen, 1978). BN resistance then, is thought to be due to a lack of EF-reactive T cells, thus resulting in a block in the ability to develop educated effector T cells capable of reacting with myelin (Singer et al., 1981; McFarlin et al., 1975a; 1975b). Singer, Moore and Williams (1981) concluded that BN resistance to acute EAE development was due to MHC-linked, Ir-EAE gene function which was visible at the level of macrophage presentation of antigen to the T cell. A related study by Beraud et al. (1986) found that Lewis APC induced the emergence of a T cell line that responded to the 68-88 peptide, whereas BN APC induced a line that lost its ability to respond to the 68-88 peptide, but responded to the 43-67 portion of the 43-88 MBP fragment. Thus, the genome of the APC can influence the immunodominance of MBP epitopes. More recently, another defect has been reported for the BN rat. In experiments comparing la induction on astrocytes by gamma interferon, it was found that BN astrocytes expressed significantly lower levels of la than Lewis astrocytes (Massa et al., 1987). Similar data were obtained from an analysis of EAE-susceptible and EAE-resistant mouse strains (SJL and BALB/c, respectively) (Massa et al., 1987), which suggests that this phenomenon may be more generalized and not limited to only one species. Hyperinduction of la was found to be restricted to astrocytes, since
peritoneal macrophages of susceptible and resistant strains exhibit identical profiles of \( \text{Ia} \) induction (Massa et al., 1987).

Another EAE resistant strain, the AO rat, has been reported to have a defect in its ability to produce IL-2 following Con A stimulation of spleen cells (Mostarica-Stojkovic et al., 1985). The difference in IL-2 production was determined to be the consequence of differences in T cell sub-populations in the AO versus the EAE susceptible DA strain (Lukic et al., 1987).

Suppressor cells have been implicated in the recovery phase and subsequent resistance to EAE in Lewis rats (Willenborg, 1979; Welch et al., 1980). These ideas stemmed from previous findings in which antigen-specific suppressor T cells were found to regulate EAE in Lewis rats rendered specifically unresponsive to EAE by pretreatment with MBP in non-encephalitogenic form (i.e., administered intravenously in soluble form or intradermally, emulsified in incomplete Freund's adjuvant) (Swierkosz and Swanborg, 1975; Welch and Swanborg, 1976; Welch et al., 1980). Other reports showed that unresponsiveness to EAE can be transferred by adoptive transfer of suppressor cells from tolerized donors (Swierkosz and Swanborg, 1977; Adda et al., 1977; Beraud et al., 1982; Lando et al., 1979a).

It has been demonstrated that loss of effective suppressor T cell function can lead to autoimmune diseases (Waldman and Broder, 1977). This suggests that resistance to EAE may be due to the presence of suppressor cells in resistant strains. Several researchers have addressed this possibility by pretreatment of resistant and
low susceptible strains with the potent immunosuppressive agent cyclophosphamide (CY) which can inhibit or delay induction of suppressor cells. Low doses of CY (20mg/kg) have been shown to be more effective than higher doses (>100mg/kg) in eliminating suppressor cell function (Lando et al., 1979a). Källen et al. (1986) showed that low dose CY treatment (40mg/kg) two days before immunization increased susceptibility for EAE in Fisher and PVG rats but not in BN rats or (BN x F)F1 hybrids. These authors concluded that only Fisher and PVG rats have an EAE suppressive mechanism dependent on CY-sensitive suppressor lymphocytes. Using the same treatment schedule, Källen and Lodgberg (1982) were unable to demonstrate CY-sensitive suppressor cells in the resistant Lew/Mol substrain. Pretreatment with low dose CY abrogates the resistance of AO rats to EAE, while also enhancing IL-2 production and increasing CD4:CD8 ratios (Mostarica-Stojkovic et al., 1982). Teitelbaum et al. (1978) treated several resistant and susceptible mouse strains with a low dose of cyclophosphamide (20mg/kg) two days before EAE induction. Their results showed: (1) susceptible strains were unaffected; (2) the resistant strains Balb/c, Balb/B10 and NZB developed full blown EAE of the same severity and incidence as the susceptible SJL/J mice; and (3) certain resistant strains with different H-2 haplotypes were not affected by CY treatment. These results were interpreted as indicating the presence of two types of resistance: one sensitive to CY because CY could eliminate suppressive cells and the other due to a lack of immune response genes necessary for eliciting a response to MBP. Further support for the second type of resistant strain was obtained when poor or no delayed hypersensitivity response to the small mouse basic encephalitogen (SMBE) was obtained in A/J mice which lack
the Ir gene to EAE, whereas Balb/c mice with Ir-EAE developed a good cell mediated immune response to the SMBE (Teitelbaum et al., 1978).

Antibody may exert a protective or regulatory function in EAE. In inbred mouse strains, though not in rat strains, those refractory to EAE make even more anti-MBP antibody than susceptible strains (Barnett and McFarlin, 1981; McFarlin et al., 1975b).

Strain 2 guinea pigs have been shown to be relatively resistant to EAE induction. Lisak et al. (1969) demonstrated minimal delayed skin reactivity to homologous MBP in the strain 2 group. Lymphocytes from strain 2 guinea pigs showed diminished proliferative responses to GP-MBP compared to strain 13 GP. These responses were shown not to be due to a generalized decrease in responsiveness, as shown by mitogen and irrelevant antigen stimulation (Lisak et al., 1975). Geczy et al. (1984) reported that activation of macrophage procoagulant activity is important in the manifestation of EAE in susceptible strain 13 GP and that control of the autoimmune response to brain antigens in the resistant strain 2 GP is coincident with specific production of anticoagulant activity by mononuclear cells. Studies have examined whether guinea pigs with EAE show differential expression of la antigens in the CNS. Sobel and Colvin (1985) used monoclonal antibodies to polymorphic epitopes on strain 2 and strain 13 la molecules to analyze mononuclear and endothelial cells in CNS tissues from parental strains and F₁ hybrids. They found that F₁ GP with EAE had markedly increased strain 13 la, but not strain 2 la, on CNS parenchymal vessels and mononuclear inflammatory cells. These results suggest for
the first time that specific MHC products are selectively expressed on cells in situ in the CNS of immunologically heterogeneous individuals.

IV. EAE as a model for MS.

EAE is a promising disease model system for analyzing the mechanisms underlying neuroimmunologic disorders of animals and humans. The disease is considered an appropriate model system for studying multiple sclerosis (Paterson, 1977; 1978; 1980; Arnason, 1983; Alvord, 1984). The best model for MS is the chronic relapsing EAE (CR-EAE) model which can be induced in strain 13 guinea pigs and SJL mice (Raine and Traugott, 1984a) with the injection of whole spinal cord homogenate and adjuvant (Stone and Lerner, 1965). The low mortality, relapsing and remitting clinical course, immunologic patterns, genetic predisposition, large demyelinating lesions and profound sparing of axons are key points of similarity and have been the basis for this model being the most heavily applied to therapeutic investigations relevant to MS (Raine, 1983; Raine and Traugott, 1984b).

From the histopathological standpoint, this model of EAE resembles MS in many respects. CNS disease is most evident in the white matter where very large foci of demyelination are observed and the optic nerves are also affected (Raine, 1983). In many animal species, peripheral nerve demyelinating lesions may be seen in addition to CNS ones (Pender and Sears, 1982) and although rare, peripheral nerve damage has been observed in MS (Arnason, 1983). In MS as well as in EAE, the initial lesions are perivascular mononuclear cell infiltrates and the inflammatory events
include edema, formation of perivascular cuffs and infiltration of the parenchyma by T and B lymphocytes and macrophages (Arnason, 1983). CNS remyelination and oligodendrocyte survival have been observed in both CR-EAE (Raine and Traugott, 1984b; Raine, 1983) and in MS (Tourtellotte and Ma, 1978).

MBP has been investigated as a possible antigen in the autoimmune response to myelin observed in MS. MBP is present in the cerebrospinal fluid (CSF) of patients with MS and levels appear to correlate with the extent of recent demyelination (Whitaker, 1977; Alvord et al., 1984; Cohen et al., 1976). MBP has been found to be an antigenic component of circulating immune complexes from some MS patients (Dasgupta et al., 1984). Antibodies to MBP are found in the serum and CSF of persons with MS (Panitch et al., 1980; Paterson et al., 1981) and are well documented in animals with EAE (Paterson, 1976; Lassman et al., 1984).

Researchers find that between 50% and 90% of patients with MS have increased levels of CNS Ig synthesized locally as well as oligoclonal bands in their CSF (Kabat et al., 1942; Oger et al., 1983; Livrea et al., 1988). The CSF shows elevated levels of IgM, IgA, and IgG. The IgG isolated is mainly of the IgG₁ subclass. In addition to a restricted electrophoretic heterogeneity, there is an altered kappa/lambda light chain ratio (Ebers, 1984). Elevated oligoclonal antiviral (especially anti-measles) and anti-neuroantigen (anti-MBP) antibodies can be found (Davison, 1980). CSF complement-fixing antibody reacting with crude MS brain has been detected (Ryberg, 1978). Oligoclonal bands have been observed in EAE in rabbits
(Whitacre et al., 1981; 1982), in guinea pigs (Mehta et al., 1984), in monkeys (Chu et al., 1984) and in rats (Whittaker and Whitacre, 1984).

*In vitro* studies showed that sera from animals with EAE were found to demyelinate CNS organotypic cultures prepared from neonatal rat cerebellum (Bornstein and Appel, 1961) and to inhibit myelin formation when applied to unmyelinated CNS explants (Bornstein and Raine, 1970). This demyelinating factor seems to be immunoglobulin directed against galactocerebroside, a component of myelin (Lisak, 1984). Recent studies in EAE have shown that alternate and classical complement pathways can be activated in normal serum by contact with live myelinated CNS cultures and extracted myelin, respectively (Silberberg et al., 1982; Vanguri et al., 1982). Sera from some MS patients also specifically demyelinated rodent CNS tissue cultures (Bornstein, 1963; Ulrich and Lardi, 1978). Serum and CSF of patients in acute exacerbation were more likely to have such activity and the activity was said to be heat labile, complement dependent and found in the immunoglobulin (IgG and IgM) fraction of serum (Kim et al., 1970).

In examining demyelinating factors *in vivo*, serum and CSF of MS patients were shown to cause demyelination *in vivo* in the tadpole optic nerve (Tabira et al., 1976) and MS serum demonstrated *in vivo* demyelination after intraneural injection into guinea pig optic nerve (Sergott et al., 1983). In guinea pigs with CR-EAE, serum was obtained and tested for *in vivo* demyelinating activity by infusion into the subarachnoid space of normal rats. The anti-myelin/oligodendrocyte glycoprotein antibody titers showed good correlation with the *in vivo* demyelinating activity of the
sera \( r = 0.91 \), suggesting that these antibodies may be involved in the demyelination observed in CR-EAE (Linnington and Lassmann, 1987).

Development of EAE is closely associated with delayed hypersensitivity (DH) to CNS tissue or MBP, as demonstrated by skin tests or \textit{in vitro} correlates of cellular immunity such as macrophage migration inhibition (Alvord et al., 1975). Although studies of MS patients yield no evidence of DH cutaneous reactivity (Lisak et al., 1968), there is some evidence, although controversial, for macrophage migration inhibition factor production by PBL of MS patients after \textit{in vitro} stimulation with MBP (Sheremata et al., 1974; 1976).

As mentioned before, susceptibility to EAE induction is genetically controlled and this control resembles the genetic mechanisms involved in spontaneously occurring human disease. The relationship between occurrence of MS and HLA haplotype was first established in the early 1970's when weak associations with HLA-A3 and B7 were reported in caucasians of European descent (Naito et al., 1972; Jershild et al., 1972). More recent studies (reviewed in McDonald, 1984) have found stronger associations with products of the D and DR loci (e.g. HLA-DR2) for northern European and North American caucasians (Batchelor et al., 1978; Ryder and Svejgaard, 1981). In Germany, France and the United Kingdom, significant associations have been found with HLA-DR3 as well as DR2 (McDonald, 1984). Several specific ethnic groups have MS susceptibility patterns that can be linked to HLA types, while in other races, MS is rare or absent (e.g. Bantu, Inuit, American Indian, Yakut and Huterite) (McDonald, 1984; Waksman, 1981). Interestingly, MS is a
rare disease in the Hungarian Gypsies and in the Japanese, yet the incidence of HLA-DR2 in these groups is quite high (Palffy, 1982; Naito et al., 1982). This observation suggests that the presence of HLA-DR2 alone is not sufficient for the development of the disease, and that more than one genetic factor may be involved in the etiology of MS. These results agree with the genetic susceptibility associations found in EAE. Although susceptibility in both diseases has been linked to the MHC locus, there exist other factors such as non-MHC genes, geography, environmental stresses and many others that may play an important role in pathogenesis.

These studies indicate a remarkable degree of concordance between EAE and MS in regard to clinical course, pathology of the CNS lesion, genetic associations with susceptibility, immunoglobulin abnormalities in the CNS, circulating antibodies that mediate demyelination, and immunologic reactivity to MBP.

V. EAE: Analysis of the resistance mechanisms in the LeR rat.

This study was designed to analyze the immune mechanisms involved in the genetic resistance of the Lewis Resistant or LeR rat. Studying the EAE-susceptible Lewis rat and the syngeneic EAE-resistant LeR rat offers a unique opportunity to investigate how genes influence resistance to autoimmune neural tissue destruction.

Waxman et al. (1981) observed resistance to EAE clinical disease induction in an apparent mutant rat strain derived from a large inbred colony of Lewis rats. The colony was originally derived from Simonsen Laboratories (Gilroy, California) and was
initially susceptible to EAE induction (Vandenbark and Hinrichs, 1974). The colony had been maintained as a closed colony for approximately five years allowing random breeding of rats. When members of that colony were sensitized with MBP-CFA, only 23% of these randomly bred rats developed paralysis (n=180), compared with 92% incidence of paralysis in Lewis rats from commercial sources (n=161). Histopathologic evaluation corresponded with clinical assessments. From the original resistant F₀ rats, breeding pairs were selected for total resistance at each generation and by the F₃ generation, an entirely resistant lineage was obtained (Waxman et al., 1981). These rats were then designated Lewis Resistant (LeR). These authors found that LeR resistance remained stable through the F₉ generation of brother-sister mating.

To determine if EAE resistance in the LeR rat was a genetic trait, 100% resistant F₃ LeR rats were crossed with Lewis (Le) rats and the EAE susceptibility of (Le x LeR)F₁ progeny was examined. Ninety-five percent of the F₁ progeny of Le males x LeR females and of Le females x LeR males were resistant to EAE induction, and both male and female F₁ progeny were resistant (Waxman et al., 1981). Thus, the resistance appeared to be inherited as a dominant, autosomal trait. The LeR strain has its roots in the Le colony kept at Simonsen Laboratories, which coincides with the origin of another EAE resistant strain, the Lew/Mol rat (Källen and Lodgberg, 1982). LeR and Lew/Mol rats differ with respect to the EAE susceptibility of their F₁-hybrids with EAE susceptible rats. In contrast to about 50% of the (Lew/Mol x Lew/Mol)F₁-hybrids, only 5% of the (LeR x Le)F₁-hybrids developed neurological signs of EAE (Waxman et al., 1981; Källen and Lodgberg, 1982). In other words, when
these two resistant strains were crossed with the susceptible strain from which they were derived, the LeR progeny were more resistant to EAE induction than the Lew/Mol progeny.

(LeR x Le)F₁ rats were then backcrossed to Le rats, and progeny were tested for their susceptibility to EAE induction (Waxman et al., 1981). The progeny rats’ susceptibility segregated in a ratio of 25% entirely resistant to 75% susceptible. The susceptible group consisted of 46% that developed paralytic EAE and the remaining 29% developed EAE with mitigated neurologic symptoms. Waxman et al. (1981) found that the simplest explanation of these results was that resistance is mediated by a single gene. Variability in the expression of this gene caused by an unknown factor might account for the mitigated symptoms seen in some backcross animals. These findings are also compatible with the hypothesis that complete EAE resistance is dependent upon the inheritance of two unlinked genes. Possibly, one of these genes is a strong resistance gene and the other one is a relatively weak resistance gene. Assuming that the genes involved segregate in a Mendelian pattern, one-fourth of the backcross progeny should inherit both genes thus conferring complete EAE resistance. Another one-fourth of the backcross rats would be expected to inherit only the strong resistance gene, which theoretically might only mitigate clinical signs of EAE without the weak resistance gene. The remaining one-half of the backcross rats, which inherited either the weak resistance gene alone or neither resistance gene would make up the group of entirely susceptible rats which developed paralytic EAE (Waxman et al., 1981).
Waxman et al. (1981) also reported that LeR lymphoid cells, derived from rats sensitized with MBP and subjected to an in vitro activation step, were unable to passively transfer EAE to naive Lewis recipients. Lewis lymphoid cells readily transferred disease into naive LeR recipients, indicating there is no physiologic barrier to EAE induction in LeR rats. These same authors examined whether LeR rats manifest EAE resistance due to an impairment in their cellular immune reactivity. LeR rats developed significant ovalbumin specific cellular reactivity suggesting that EAE resistance is not the result of a nonspecific T cell deficit. In addition, it was found that unlike the EAE resistant BN rat, LeR rats developed both MBP and EF-specific cellular reactivity at levels comparable to the Lewis rat. From their passive transfer data, Waxman et al. (1981) concluded that EAE resistance in the LeR rat may be the result of an immunologic deficit or it may be due to suppressor cell activity expressed during the differentiation of antigen-reactive cells into EAE-inducing effector cells.

As discussed previously, there is evidence that EAE susceptibility is dependent upon the inheritance of an Ir gene (Ir-EAE) closely linked to the rat MHC locus, RT1. It seems likely that LeR possess Ir-EAE for EAE susceptibility because they develop EF-specific reactivity and they share at least some common serologically detectable MHC antigens with Lewis rats (Waxman et al., 1981). Gasser et al. (1983) have shown that the gene for nonsusceptibility (i.e. resistance) in the LeR rat is not linked to the RT1.

Studies have shown that EAE can be induced in strongly EAE resistant rat strains using augmented sensitization or activation methods. In the BN rat, EAE can
be induced if rats are sensitized with whole rat spinal cord combined with *Bordetella pertussis* or carbonyl iron adjuvants (Levine and Sowinski, 1975). Lennon et al. (1976) were also able to induce EAE in BN rats by using a 200μg dose of GP-MBP combined with CFA and *B. pertussis*, i.e. 20 times the MBP dose needed to induce EAE in Lewis rats. Driscoll et al. (1985) reported that addition of bacterial lipopolysaccharide (LPS) plus MBP to LNC or spleen cell cultures from MBP sensitized LeR rats caused them to successfully transfer EAE. Con A activated spleen cell cultures, however, could not be made to transfer EAE with the addition of LPS.

The Lewis/LeR model offers a unique opportunity to investigate how genes influence susceptibility to autoimmune disease. In other combinations of susceptible and resistant strains, cellular resistance mechanisms are difficult to study due to multigenic differences between strains and histocompatibility barriers which restrict the investigation of cell interactions. In contrast, backcross data suggest that resistance in LeR rats is mediated by only one or two genes (Waxman et al., 1981). In addition, there do not appear to be histocompatibility barriers between Le and LeR rats (Waxman et al., 1981). This facilitates the construction of LeR → Le and Le → LeR radiation chimeras, which are a powerful tool for analyzing cell interactions *in vivo* which might mediate resistance.

The first objective of this project was to determine if the resistance observed in the LeR rat was immunologically mediated. This goal was approached through establishment of radiation-induced bone marrow chimeras and mixed cell chimeras between Lewis and LeR rats. Construction of radiation chimeras allows the
manipulation of hematopoietic-lymphoid components of the donor in the context of the host target tissue, thus allowing the separation of immune-mediated regulation of disease from other host factors. The second objective was to determine if the mechanism of resistance was similar to that reported for other EAE-resistant animals. This goal was approached through investigation of the macrophages and suppressor cells of the LeR rat, lymphokine secretion, Ia expression, antigen presentation, and general MBP-specific cellular immune responses of this rat strain.
MATERIALS AND METHODS

Animals.

Male Lewis (Le, RT-1\(^{\text{a}}\)) and ACI (RT-1\(^{\text{av}}\)) rats, 6 to 12 weeks old, were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). LeR rat breeding pairs were obtained from Dr. William Hickey (University of Pennsylvania, Philadelphia, PA.) where they had been maintained as a closed breeding colony since 1983. The LeR colony was maintained by brother-sister mating at The Ohio State University and resistance to EAE induction was checked at every generation. All rats were maintained on laboratory chow and tap water unless otherwise stated.

Preparation of myelin basic protein.

Human myelin basic protein (HuMBP) was prepared from human cortex (obtained through the efforts of Dr. Allan Yates, Department of Pathology) by the methods of Deibler et al. (1972) and Swanborg et al. (1974). Briefly, 200g of frozen human brains were homogenized in chloroform/methanol (2:1, v/v) and the residue then washed and acid extracted using 0.01N HCl. The acid extract was precipitated using 50% saturated ammonium sulfate, resuspended in 0.01N HCl, dialyzed against deionized water and lyophilized. MBP prepared in this manner is generally at least 95% homogeneous, as characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Deibler et al., 1972).
Induction and Clinical Evaluation of EAE.

Acute EAE was induced by a single injection of 100μg human MBP (HuMBP) in 0.1 ml complete Freund's adjuvant (CFA) administered intradermally in the hind footpads. The 100μg HuMBP dose was determined to be the optimal EAE-inducing dose for the Lewis rat, while the LeR rat maintained its resistance. The CFA contained 4 mg/ml killed Mycobacterium tuberculosis (Jamaica strain) suspended in the emulsifier, Arlacel A, and mineral oil (Marcol 52). Animals were evaluated for signs of neurologic impairment beginning on day 10 after sensitization and concluding at the time of complete clinical recovery (days 18-20). Rats were assigned a daily clinical grade as follows: 0 = no symptoms, 0.5 = distal limp tail, 1.0 = complete loss of tail tonicity, 2.0 = ataxia, 3.0 = hindquarter weakness with partial paralysis, 4.0 = hindquarter paralysis usually accompanied by fecal impaction and incontinence. This protocol for the clinical assessment of EAE has been utilized extensively in previous publications (Bitar and Whitacre, 1988; Källen and Lodgberg, 1982; Lando et al., 1979a; Moore et al., 1980; Waxman et al., 1981).

Histopathologic Evaluation of EAE.

All rats were sacrificed after recovery from EAE or 21 days after sensitization if no clinical signs appeared. Brains and spinal cords were fixed in 10% formalin, dehydrated and embedded in paraffin. Transverse sections (7μm) of the thalamus, mesencephalon and cerebellum-pons as well as longitudinal sections of the entire spinal cord were stained with hematoxylin and eosin. Duplicate histologic slides from
each animal were assessed for the presence of perivascular mononuclear cell infiltrates and scored as follows: no lesions = 0, 1-10 lesions = 1+, 11-30 lesions = 2+, and >30 lesions = 3+ (Bitar and Whitacre, 1988).

**Lymphocyte Proliferation Assay.**

At various times after HuMBP sensitization, rats were sacrificed by exsanguination under ether anesthesia and spleens or lymph nodes draining the sites of antigen injection (inguinal, popliteal, and periaortic) were removed. Lymphoid organs were trimmed and expressed through 120 mesh stainless steel screens to obtain single cell suspensions. Cells were suspended in Hanks' Balanced Salt Solution (HBSS) (M.A. Bioproducts) washed two times by centrifugation (140 x g) and viability was determined by trypan blue dye exclusion. Unless stated otherwise, cells were suspended in "basic culture medium" which contains the following ingredients: RPMI 1640 (M. A. Bioproducts), 2 mM L-glutamine (M. A. Bioproducts), 5 x 10^-6 M 2-mercaptoethanol (Sigma), 25 mM HEPES buffer (Gibco), 50 units/ml penicillin and 50 μg/ml streptomycin (M. A. Bioproducts) and 2% fresh autologous rat serum. Lymph node cells (LNC) and spleen cells were cultured in 0.2 ml volumes at 37°C and 5% CO₂ in flat-bottomed 96 well tissue culture plates (Costar) for 3-7 days. Cultures contained 2 or 4 x 10^5 cells/well incubated in the presence or absence of the following stimulants: HuMBP (0-500μg/well), OVA (0-500μg/well), purified protein derivative (PPD) (0-20μg/well) (Parke-Davis) or Con A (0-25μg/well) (Miles). During the final 18 hours of incubation, cultures were pulsed with ^3H-thymidine (1μCi/well) (Amersham). Cells were harvested using a semiautomatic cell harvester (Skatron) and incorporation of radiolabel was measured in a liquid scintillation counter (Beckman). Results are
expressed as counts per minute (cpm) or stimulation index (S.I.) = cpm with antigen/cpm without antigen.

**Popliteal Lymph Node Assay (PLNA).**

Using the procedure of Källen and Logdberg (1982), spleen cell suspensions were prepared from naive donor animals (Le, LeR and ACI) at a concentration of $6 \times 10^7$ spleen cells/0.1 ml. Naive recipient Le, LeR and ACI rats were injected subcutaneously in the left hind footpad with 0.1 ml homologous cells and in the right hind footpad with heterologous spleen cells. Four days later, recipient rats were sacrificed and both popliteal lymph nodes (PLN) were removed, trimmed and weighed immediately. Results are reported as "relative PLN weight increase" determined as follows: right (heterologous) PLN weight/ left (homologous) PLN weight.

**Mixed Lymphocyte Culture.**

Lymph node cell suspensions were obtained from naive Lewis, LeR and ACI rats. LNC were washed three times in HBSS and then resuspended in basic culture medium supplemented with 0.25mg/L Fungizone (Squibb) and 5% heat inactivated horse serum. Cell concentrations were adjusted to $6 \times 10^6$/ml for stimulator cells and $4 \times 10^6$/ml for responder cells. Stimulator LNC were irradiated with 1500 rads using a Gammacell 40 $^{137}$Cs source (Atomic Energy of Canada, Ltd.). Responder cells (100 µl) and stimulator LNC (100 µl) were then plated in round-bottomed 96 well tissue culture plates (Linbro) and incubated at 37°C and 5% CO$_2$ for five days. During the last 18 hours of culture, wells were pulsed with $^3$H-thymidine (0.8µCi/well), harvested using a semi-automatic cell harvester and incorporation of radiolabel was measured.
by liquid scintillation counting. The data are expressed as the mean cpm of triplicate cultures ± SEM of the responding LNC.

Delayed Type Hypersensitivity (DTH).

*In vivo* cell mediated immune responses, measured 20 days after footpad immunization with 100μg HuMBP-CFA, were assessed by eliciting a delayed type hypersensitivity (DTH) reaction in the pinna of the left ear of Le and LeR rats. The right ear was used for tagging (identification purposes). Unsensitized rats served as controls. Rats were injected intradermally in the ear with 10μg HuMBP dissolved in 0.05 ml saline. Ear thickness was measured before injection and at 24 hr and 48 hr post injection with skinfold thickness calipers (Mitutoyo). All rats were coded and measured independently by two different persons to minimize bias. The data are expressed as the mean increase in ear thickness (inches) ± SEM.

T cell Line Generation and Adoptive Transfer of EAE.

MBP-specific T cell lines were generated from Lewis and LeR rats using the procedure of Vandenbark et al. (1985) and Ben-Nun et al. (1981). Lymph nodes draining the site of footpad injection were obtained from Lewis or LeR rats 9 days after MBP-CFA sensitization and prepared as a single cell suspension. The LNC (7 x 10⁶/ml) were cultured in 10 x 60mm tissue culture petri dishes (Falcon) in basic culture medium containing 2% fresh autologous rat serum and 100μg/ml MBP for three days. Lymphoblasts were then isolated by Ficoll-Hypaque density gradient centrifugation (240 x g, 25 min. at RT) using Lymphocyte Separating Medium (LSM) (Litton Bionetics). The cells were washed and cultured in 20 x 100mm tissue culture
petri dishes (Falcon) in medium containing 10% fetal bovine serum and 10% (v/v) rat T cell growth factor (48 hour supernatant from Con A-stimulated rat spleen cells). After 4-8 days in culture, the cells were washed and restimulated with MBP (10-60 µg/ml) for three days in the presence of syngeneic gamma irradiated (3300 R) thymocytes (10^7/ml) as a source of antigen presenting cells. The LNC were then alternately expanded in rat T cell growth factor or restimulated with antigen and accessory cells. *In vitro* lymphocyte proliferative assays were used to test for the antigen specificity of T cell lines by incorporation of ^3^H-thymidine in the presence of MBP and PPD. Proliferative assays involved placing 50µl of LNC (10^4/well), 50µl irradiated (3300R) thymocytes as feeder cells (10^6/well), 100µl antigen (1mg/ml) and media to a total volume of 300µl/well in flat-bottomed 96 well tissue culture plates (Costar). Plates were incubated for 72hr, pulsed with ^3^H-thymidine (1µCi/well), and 18 hours later plates were harvested and proliferation measured by uptake of radiolabel.

HuMBP-specific T cell lines from Lewis and LeR rats were evaluated for their ability to induce clinical EAE. T cells (10^7) collected after a 3-day culture with MBP and irradiated (3300R) thymocytes, were injected intravenously into naive Lewis recipients (Vandenbark et al., 1985). Clinical signs were monitored from 5 to 10 days after injection.

*Cyclophosphamide (CY) treatment.*

Solutions of cyclophosphamide (Neosar, Adria Laboratories, Inc.) were freshly reconstituted in sterile water. Recipient Lewis and LeR rats received intraperitoneal injections of 20, 40 or 60mg/kg CY. Control animals were injected with the same
volume of PBS. Two days later, recipient rats were sensitized with 100μg HuMBP-CFA and observed daily for clinical signs of EAE.

**IL-2 Generation and CTLL 20 IL-2 Bioassay.**

For generation of IL-2, donor rats (normal Le and LeR) were sacrificed and their spleens were removed into cold HBSS. Spleens were trimmed and expressed through a stainless steel mesh screen to generate single cell suspensions. The cells were washed 3 times in HBSS, resuspended in basic culture medium containing 5% fetal bovine serum and incubated in 75cm² tissue culture flasks. Spleen cells (5 x 10⁶/ml) were stimulated with 2.5μg/ml Con A for 48 hours. At the end of the incubation period, the contents of the flasks were centrifuged, supernatants aspirated, filtered through 0.2μm filters and stored at -20°C. For assay of IL-2 activity, 100μl of culture supernatant was placed in the well of a 96-well plate. Serial two-fold dilutions of the supernatants were made across the plate and then 10⁴ washed CTLL-20 cells were added to each well. These cultures were then incubated for a total of 24 hours including a final 6 hour pulse with ³H-thymidine (0.5 μCi/well).

CTLL-20, an IL-2-dependent murine cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and 10% supernatant from Con A-stimulated rat spleen cells as a source of IL-2. The amount of IL-2 contained in test supernatants was expressed in terms of units of IL-2 activity/ml and was determined by comparison to a known IL-2 standard (315 units/ml).
Limiting Dilution Analysis (LDA) for TCGF-Secreting T cells.

This procedure was modified in our laboratory for the rat based on methods published for LDA in the human (Orosz et al., 1987). LNC and spleen cell suspensions from rats sensitized with MBP-CFA 11 or 14 days previously were obtained as described for the lymphocyte proliferation assay. After washing 3 times in HBSS, responder LNC and spleen cell concentrations were adjusted to $1.6 \times 10^6$/ml in basic culture medium containing 2% autologous rat serum. Thymocytes from naive, syngeneic donor rats were used as a source of antigen presenting cells as described previously for T cell lines. Thymocytes were washed once and resuspended in HBSS, irradiated with 7500 rads. These cells were then washed an additional 3 times in HBSS and the thymocyte concentration was adjusted to $40 \times 10^6$/ml in basic culture medium with 2% autologous rat serum.

Dilutions of responder LNC or spleen cells (25μl/well) were added to Linbro V-bottomed microtiter plates, usually in replicate sets of 24 microwells for each serial two-fold dilution, beginning with 40,000 cells/well and ending with 312 cells/well. To these wells were added $1 \times 10^6$ syngeneic irradiated thymocytes (25μl/well) and 25μl of HuMBP (100μg/ml). Microcultures containing responder cells, irradiated thymocytes and antigen were incubated overnight (18 hr) at 37°C and 5% CO₂. After incubation, the plates were irradiated with 2500R and $1 \times 10^3$ CTLL-20 cells (a TCGF-dependent cell line, described previously) were added in 25μl volumes. The microcultures were incubated an additional 24 hr including a terminal 18hr pulse with 0.5μCi $^3$H-thymidine. Microcultures were harvested onto glass fiber filters and radioactivity was measured by scintillation counting.
Minimal estimates of the helper T cell frequency were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the limiting dilution microwells and the percentage of replicate wells that failed to produce detectable TCGF (Taswell, 1981). Limiting dilution microcultures were considered positive for MBP-specific TCGF production if thymidine incorporation by CTLL-20 cells was greater than the thymidine incorporation (mean plus 3 SD) observed in microwells containing thymocytes, MBP and CTLL-20 cells, but no responder cells. Frequency calculations were determined by Chi square minimization analysis as described by Taswell (1981). This analysis yields minimal frequency estimates (1/f) and ranges of frequencies, the 95% confidence limits of the frequency estimate and a Chi square estimate of probability (p), where significance is indicated by p ≥ 0.05.

Passive Transfer of EAE.

The EAE passive transfer procedure of Panitch and McFarlin (1977) and Richert et al. (1979) was utilized. Briefly, spleen cells or LNC were obtained 12 days after HuMBP-CFA sensitization and were cultured for 3 days at 37°C in 5% CO₂ in basic culture medium supplemented with 5% fetal calf serum. In vitro activation of cells involved two different protocols with: (1) cell concentration at 2 x 10⁶/ml in medium containing either 1μg/ml Con A or 20μg/ml HuMBP, or (2) cell concentration at 5 x 10⁶/ml in medium containing 50μg/ml HuMBP. Following a 3 day culture period, the cells were harvested, washed, and the number of viable cells determined by trypan blue dye exclusion. In vitro activated LNC or spleen cells were then infused
intravenously via the lateral tail vein into normal recipients, which were monitored for clinical signs of EAE.

**Direct Spleen Cell Transfer.**

Normal Lewis and LeR rats were used as spleen cell donors. Donor rats were anesthetized and sacrificed by exsanguination. Spleens were removed, trimmed and expressed through screens and the resulting single cell suspension was washed 3 times in HBSS. Normal Lewis and LeR recipient rats were injected intravenously with either $250 \times 10^6$ or $500 \times 10^6$ spleen cells. Three to five days later, recipient rats were sensitized with HuMBP and observed for clinical signs of EAE.

**OX8$^+$ Spleen Cell Panning.**

Lewis and LeR spleen cell suspensions were obtained as described in the previous section. Labelling with OX8 monoclonal antibody consisted of incubating $35 \times 10^6$ spleen cells together with a 1:30 dilution of OX8 (Serotec mouse monoclonal anti-rat-CD8 ascites) in 1 ml basic culture medium containing 10% FBS for 1 hour at 4°C. Plates (Lab-Tek, 100 x 25 mm) were sensitized for 1 hour at RT with 10 ml rabbit anti-mouse IgG (heavy and light chain specific, Zymed) (10µg/ml in 0.05M Tris, pH 9.5). Plates were rinsed 3 times with PBS containing 5% FBS and stored at 4°C with 10ml PBS with 5% FBS until used. Spleen cell tubes were spun down and resuspended in 3ml PBS with 5% FBS. The contents of one tube (3ml) was added dropwise to each sensitized plate and incubated 1 hour at 4°C. To obtain non-adherent cells, plates were gently swirled, contents decanted, and a similar process repeated twice. Non-adherent cells were pooled and washed 3 times in HBSS.
Adherent cells were obtained for flow cytometric analysis by vigorously pipetting plates with PBS with 5% FBS for 15 min./plate. Adherent cells were pooled and washed as above. Unfractionated, non-adherent and adherent spleen cells were stained for flow cytometric analysis as described below. Non-adherent cell recovery after OX8 panning was 74% for Lewis spleen cells and 58% for LeR spleen cells. Flow cytometric analysis of unfractionated versus non-adherent cells showed a decrease in OX8+ cells from 20% to 2% for Lewis splenocytes and from 23% to 4% for LeR splenocytes.

Unfractionated and non-adherent cells were injected (100 x 10⁶) intravenously into recipient Lewis rats. Three days later, rats were sensitized with HuMBP and observed for clinical signs of EAE.

Determination of Cell Phenotypes.

Lymph node cells (2 x 10⁶) in 100μl of PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide were incubated with an equal volume of mouse anti-rat monoclonal antibodies (ascites, diluted 1:20). Antibodies (Accurate Chemicals, Westbury, N.Y.) were specific for total T cells and macrophages (W3/13), T helper cells (W3/25), T suppressor/cytotoxic cells (OX8), Lewis-specific polymorphic determinant on the RT1.B-encoded la molecule (OX3) and the monomorphic determinant on the RT1.B-encoded la molecule (OX6). After incubation for 30 min. on ice, the cells were washed twice and a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ goat anti-rat IgG (Tago Inc., Burlingame, CA.) was added. After another 30 min. incubation on ice, the cells were washed 2 times in PBS without
BSA and then analyzed using an EPICS-C flow cytometer (Coulter Instruments). Control cells were stained only with the secondary antibody. For each test, data was collected from 2500 cells, and measurements of forward and 90° light scatter as well as log green fluorescence were obtained.

**Ia Induction by Gamma Interferon.**

Thymocyte cell suspensions were prepared from normal Lewis and LeR rats, washed 3 times in HBSS, and aliquots were immediately stained with monoclonal antibodies for flow cytometric analysis as described above. The remaining thymocytes were placed in basic culture medium containing 10% fetal calf serum in the presence of 10, 50 or 100 U/ml recombinant rat gamma interferon (Amgen Biologicals). Control thymocytes received no treatment. Three days after culture initiation, thymocytes were washed and stained with monoclonal antibodies as described above.

**Construction of Radiation Chimeras.**

Recipient Lewis and LeR rats were anesthetized and exposed to 1000 rads total body irradiation in a Gammatron 40 irradiator (Atomic Energy of Canada, Ltd.) using a $^{137}$Cs source. This dose of radiation was uniformly lethal within 7-16 days for non-reconstituted rats which were included in each experiment. Bone marrow cell (BMC) suspensions were prepared from the femur, tibia and humerus of normal Lewis and LeR rats, washed 3 times in HBSS and $100 \times 10^6$ BMC were injected intravenously into irradiated recipients via the lateral tail vein. At 12-14 weeks post-reconstitution, these BMC chimeras were challenged with HuMBP-CFA to test for EAE.
susceptibility. Supportive measures which enhance the chimera survival rate were used, including utilization of sex-matched donors and recipients as well as administration of medicated water containing Bactrim, neomycin sulfate and tetracycline for 1 week prior to and 3 weeks following irradiation.

To produce chimeras with cells from various lymphoid organs ("mixed cell chimeras"), the procedure described by Singer et al. (1981) was used. BMC were obtained as described above. Spleens and thymuses were collected from Lewis and LeR donor rats, and expressed through stainless steel screens resulting in single cell suspensions. All cell suspensions were prepared in HBSS, washed three times and injected i.v. into recipient Lewis rats in the following concentrations: $1 \times 10^8$ BMC + $2 \times 10^8$ spleen cells + $4 \times 10^8$ thymocytes. At 5-6 weeks post-reconstitution, these mixed cell chimeras were challenged with 100μg HuMBP-CFA and observed for clinical signs of EAE. Supportive measures were identical to those used for BMC chimeras.

**Depletion of Splenic Adherent Cells in Radiation Chimeras.**

Spleen cells were obtained from normal Lewis or LeR donor rats and divided into two populations: unfractionated (control) and non-adherent (depleted). Adherent cells were removed from the spleen cell preparations by first incubating $1 \times 10^8$ cells suspended in 10ml HBSS containing 10% FBS in a 60 x 100mm plastic tissue culture Petri dish (Falcon) for one hour at 37°C and then removing the supernatant containing the non-adherent cells. Cell recovery after plastic adherence was 58% for LeR spleen cells and 80% for Lewis spleen cells. This step was followed by a second adherent
cell depletion step which involved passing the plastic non-adherent cells ($1 \times 10^9$ in 4ml) over a 25ml Sephadex G10 (Pharmacia) column in HBSS containing 5% FBS (Raff and Hinrichs, 1977; Jerrells et al., 1980; Iris and Mishell, 1974). Sephadex G10 columns containing spleen cells were incubated 30 min. at $37^\circ$C in 5% CO$_2$. After incubation, 75 ml of HBSS with 5% FBS was applied to the top of each column and gravity was used to elute the non-adherent cells. Lewis and LeR recovery in the G10 column effluent was 55% and 61%, respectively. Non-specific esterase staining (Koski et al., 1976) of each population showed that the unfractionated cells contained approximately 9% esterase-positive cells, the cells recovered from the plastic adherence step contained approximately 3.7% positive cells, and the cells eluted from the G10 column contained approximately 0.4% positive cells. Thus, the final non-adherent spleen cell preparation contained less than 1% esterase-positive cells. Spleen cells (either unfractionated or non-adherent) were then infused along with BMC and thymocytes into irradiated recipients as described above for construction of radiation chimeras. After 5-6 weeks reconstitution time, chimeras were sensitized with MBP-CFA and observed for clinical signs of EAE.

**Autologous Bone Marrow Reconstitution.**

Le and LeR rats were anesthetized using ketamine with acepromazine (44 mg/kg). Each rat was fitted with a lead ring to shield the right hind leg. The shield protected the BM in a majority of the femur, tibia and fibula. Rats were then irradiated (1000 rads total body irradiation) using a $^{137}$Cs source. All rats were placed on medicated water (neomycin sulfate, Bactrim and tetracycline) 4 days prior to irradiation and at least 3 weeks following irradiation. After 12-14 weeks reconstitution,
rats were sensitized with HuMBP-CFA and observed for clinical signs of EAE. Twenty days post sensitization, all rats were sacrificed and brains and spinal cords were removed for histologic examination.

Statistical Methods.

The Student's t-test was used to compare lymph node weights in the popliteal lymph node assay, delayed type hypersensitivity reactions, and units of IL-2 activity in the CTLL-20 assay. Fisher's Exact test (one tailed) was used to compare differences in the incidence of EAE between animal treatment groups (Hollander and Wolfe, 1973). The Wilcoxon rank sum test was used to compare individual EAE clinical scores between experimental and control groups (Hollander and Wolfe, 1973). Mixed cell chimera results were also examined using a logit analysis model which determines the individual factors (bone marrow cells, spleen cells or thymus cells) and interactions between factors that contribute to the incidence of EAE (Fienberg, 1977). Comparisons within the logit analysis were performed at alpha ≤ 0.01.
RESULTS

Our laboratory received the LeR rat colony, then in the F14 generation, from Dr. Frank Waxman in 1984. Sensitization of these rats with a standard 50μg dose of guinea pig MBP (GPMBP) revealed clinical EAE in the LeR rat with severity approaching that of the Lewis controls. This result prompted an immediate scrutiny of the MBP preparation, dosage of MBP and adjuvant preparation used for sensitization of LeR rats. None of these proved to be a significant contributing factor to the increased incidence of EAE in the LeR rats.

It was determined that the approach of breeding relatively resistant rats from the existing LeR colony did not yield a reproducibly resistant population of progeny rats. Thus, Dr. Whitacre's laboratory obtained 5 LeR breeding pairs of known resistance from Dr. William F. Hickey, University of Pennsylvania. Upon their arrival in December 1984, three of the males were injected with 50μg GPMBP-CFA and were found to be resistant to EAE (Table 1). Breeding pairs were set up and at least two rats from each successive litter were sensitized to monitor their resistance. During the F1 generation, it was apparent that these animals were becoming susceptible to EAE induced by sensitization with GPMBP. Both the incidence of EAE and the severity of the disease increased in successive generations. In light of these results, we contacted Dr. Hickey and learned that his definition of resistance was based upon animals remaining clinically well following
Table 1

Susceptibility of LeR rats to EAE by generation

<table>
<thead>
<tr>
<th>Generation</th>
<th>Sensitization</th>
<th>Incidence of EAE</th>
<th>Clinical score ± SEM\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>F\textsubscript{0}</td>
<td>50 ug GPMBP</td>
<td>0/3 (0%)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>F\textsubscript{1}</td>
<td>50 ug GPMBP</td>
<td>17/24 (71%)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>F\textsubscript{2}</td>
<td>50 ug GPMBP</td>
<td>5/5 (100%)</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>Lewis controls</td>
<td>50 ug GPMBP</td>
<td>11/11 (100%)</td>
<td>4.0 ± 0.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Average maximum clinical score based on the following: distal limp tail = 0.5, limp tail = 1.0, ataxia = 2.0, partial paralysis = 3.0, paralysis = 4.0.

Table 2

Comparison of EAE clinical signs induced in response to human or guinea pig MBP-CFA in LeR and Lewis rats

<table>
<thead>
<tr>
<th>MBP preparation and dose</th>
<th>EAE incidence (avg. clinical score)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LeR</td>
</tr>
<tr>
<td>Human MBP</td>
<td></td>
</tr>
<tr>
<td>50 ug</td>
<td>0/14</td>
</tr>
<tr>
<td>75 ug</td>
<td>NT\textsuperscript{a}</td>
</tr>
<tr>
<td>100 ug</td>
<td>0/4</td>
</tr>
<tr>
<td>200 ug</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>0/21</td>
</tr>
<tr>
<td>Guinea pig MBP</td>
<td></td>
</tr>
<tr>
<td>50 ug</td>
<td>22/29 (1.55)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} NT = not tested.
injection of human MBP-CFA which is less encephalitogenic than GPMBP. Table 2 shows the results of EAE induced by human MBP (HuMBP) versus GPMBP in LeR and Le rats.

It can be seen that LeR rats were resistant to EAE induced by 50-200μg HuMBP but not GPMBP in contrast to Le rats which were susceptible to EAE induced by both preparations. These results led us to alter our routine screening of LeR litters to include sensitization with HuMBP. Since our rat colony had been completely re-derived and our definition of EAE resistance was now modified, it was necessary to document that the LeR strain indeed remained syngeneic with the Lewis strain.

Previous work has suggested that the LeR strain is histocompatible with the Lewis (Le) strain (Waxman et al., 1981; Gasser et al., 1983; Driscoll et al., 1985). To further explore the suggested histocompatibility of these rat strains, we tested both in vitro and in vivo parameters of MHC compatibility. Using the ACI rat as a known MHC incompatible strain, we found that Le and LeR LNC were mutually unreactive in one-way mixed lymphocyte reactions (Figure 1). However, vigorous proliferative responses were observed with ACI/Lewis and ACI/LeR LNC combinations. Similar results were observed in the popliteal lymph node assay which serves as an in vivo test of histocompatibility (Figure 2). LeR spleen cells introduced into Lewis rats and the opposite combination of Lewis spleen cells introduced into LeR rats both yielded lymph node weight increases well below 2, as did the autologous strain combinations. In contrast, spleen cells from either Lewis, LeR, or ACI caused profound lymph node weight increases when introduced into a known MHC incompatible partner. Thus, results from both the in vitro and in vivo
Figure 1. One-way mixed lymphocyte culture. Cell concentrations were 6 x 10^6/ml for stimulator lymphocytes and 4 x 10^6/ml for responder lymphocytes. Stimulator LNC were irradiated with 1500 rads. The data are expressed as the mean cpm of triplicate cultures ± SEM of the responding rat strain LNC.
Figure 2. Popliteal lymph node assay. Categories on the abscissa are donor strain of spleen cells→recipient rat strain. Values on the ordinate are "relative PLN weight increase" = right (heterologous) PLN weight/ left (homologous) PLN weight. Lewis→LeR and LeR→Lewis categories are not significantly different from syngeneic control groups, whereas MHC barrier categories were significantly different from syngeneic control groups at p < .005 by the Student's t-test.
assays confirm the previously suggested histocompatibility between the Lewis and LeR rats.

A. Is LeR resistance immunologically mediated?

The resistance to EAE manifested by LeR rats could be mediated by immunologic or non-immunologic host factors. The fact that LeR rats are susceptible to passive EAE induced by sensitized Lewis cells (Waxman et al., 1981) indicated that there is no apparent physiologic barrier to EAE induction in the LeR rat. However, this observation did not provide evidence that LeR resistance was mediated by an immunologic mechanism. Radiation chimeras provide a means for distinguishing between immunologic and non-immunologic resistance mechanisms. To test the hypothesis that LeR resistance is mediated by cells of the hematopoietic/immune system, LeR → Le BMC chimeras were constructed and compared to control Le → Le chimeras, and Le → LeR BMC chimeras were constructed and compared to control LeR → LeR chimeras (Table 3). Le rats reconstituted with LeR bone marrow showed 29% incidence of EAE compared to susceptible Le → Le chimeras which showed 94% incidence of disease. LeR rats reconstituted with Le BM showed 100% incidence of disease compared to resistant LeR → LeR chimeras which demonstrated no disease. The patterns of clinical and histologic severity were similar to those observed for EAE incidence. These results clearly showed that the source of the donor BMC played an important role in the determination of EAE susceptibility/ resistance patterns.
Table 3
Bone marrow chimeras of Lewis and LeR rats\(^a\)

<table>
<thead>
<tr>
<th>BM donor</th>
<th>Recipient</th>
<th>Incidence of EAE (%)</th>
<th>Average maximum clinical symptom(^b)</th>
<th>Histologic score(^d)</th>
<th>P value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis</td>
<td>LeR</td>
<td>7/7 (100%)</td>
<td>1.4 ± 0.28</td>
<td>1.85 ± 0.34</td>
<td>0.0001</td>
</tr>
<tr>
<td>LeR</td>
<td>LeR</td>
<td>0/6 (0%)</td>
<td>0 ± 0</td>
<td>0.5 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>LeR</td>
<td>Lewis</td>
<td>5/17 (29%)</td>
<td>0.38 ± 0.17</td>
<td>1.06 ± 0.21</td>
<td>0.0006</td>
</tr>
<tr>
<td>Lewis</td>
<td>Lewis</td>
<td>17/18 (94%)</td>
<td>2.2 ± 0.34</td>
<td>1.92 ± 0.37</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Bone marrow cell suspensions were prepared from the femur, tibia and humerus of donor rats, washed 3 times in HBSS and 100 x 10^6 BMC were injected intravenously into irradiated recipients. At 12-14 weeks post-reconstitution, these BMC chimeras were sensitized with MBP-CFA to test for EAE induction.

\(^b\) Average maximum clinical symptoms and average histologic score ± SEM.

\(^c\) Probability values calculated by Fisher's Exact test (one-tailed) comparing incidence of clinical EAE between Lewis->LeR versus LeR->LeR and between LeR->Lewis versus Lewis->Lewis.
Since LeR BMC transferred resistance while Lewis BMC transferred susceptibility, resistance appeared to be an immunologically mediated event. However, the possibility exists that the radiation used to induce the chimeric state could alter the EAE susceptibility patterns observed in these two rat strains. Therefore, autologous bone marrow reconstitutions were carried out, in which Lewis and LeR rats were lethally irradiated while one hind leg was encased in a lead shield. Immunologic reconstitution of the animals took place from marrow within the protected leg. Figure 3 shows that the susceptibility/resistance patterns were not significantly changed by irradiation, i.e., Lewis rats remained susceptible while LeR rats were resistant to MBP challenge. It should be noted that irradiated animals had increased disease severity as well as disease incidence when compared with non-irradiated rats.

LeR rats could be resistant due to lack of a leukocyte population critical to disease induction. Alternatively, LeR rats could be resistant due to the presence of a suppressive cell population interfering with the generation of disease-inducing effector cells. To investigate these two possibilities, Lewis BMC chimeras were constructed using mixtures of Lewis and LeR BMC in various ratios (Table 4). It can be seen that infusion of $100 \times 10^6$ LeR versus $100 \times 10^6$ Lewis BMC (groups B and A, respectively) into irradiated Lewis recipients produced a significant decrease ($p = 0.001$) in the EAE clinical score in the chimeric recipient. Recipients of a mixture containing $50 \times 10^6$ Lewis BMC and $50 \times 10^6$ LeR BMC (group C) showed an intermediate level of disease (42%) compared to groups A and B. Group D recipients reconstituted with a 75/25 mixture of Lewis and LeR BMC showed susceptibility patterns only slightly reduced (80% EAE) from those animals receiving Lewis cells alone (90% EAE). On the other hand, group E
Figure 3. Effect of irradiation and autologous BM reconstitution on EAE induction. a) Rats were sensitized with 100μg HuMBP and evaluated for incidence of EAE. b) Lewis and LeR rats were anesthetized and the right hind leg of each rat was encased in a lead leg shield. Each rat was then exposed to 1000R total body irradiation. Following 12-14 weeks bone marrow reconstitution, rats were sensitized with 100μg HuMBP and evaluated for clinical signs of EAE. Comparison of EAE incidence between non-irradiated and irradiated rats by Fisher's Exact test showed no significant differences (Lewis: p = 0.124, LeR: p = 0.544).
Table 4

Effect of mixing Lewis and LeR bone marrow cells on induction of EAE in Lewis chimeras

<table>
<thead>
<tr>
<th>Group</th>
<th># BM cells (millions)</th>
<th>Incidence of EAE (%)</th>
<th>Average max. clinical score</th>
<th>Day of onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 Lewis - 9/10 (90%)</td>
<td>2.65 ± 0.47</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>- 100 LeR 3/9 (33%)</td>
<td>0.5 ± 0.29</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50 Lewis 50 LeR 5/12 (42%)</td>
<td>0.54 ± 0.29</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>75 Lewis 25 LeR 8/10 (80%)</td>
<td>0.95 ± 0.35</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>25 Lewis 75 LeR 4/12 (33%)</td>
<td>0.58 ± 0.33</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>25 Lewis - 3/3 (100%)</td>
<td>1.3 ± 0.8</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>- 25 LeR 0/4 (0%)</td>
<td>0 ± 0</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Lewis rats were irradiated with 1000R and then injected i.v. with the indicated cell mixtures. After 12 weeks reconstitution time, recipient rats were sensitized with 100μg HuMBP and observed for clinical signs of EAE. Average maximum clinical scores are reported ± SEM. This is based on the following scale: 0.5 = distal limp tail, 1.0 = limp tail, 2.0 = ataxia, 3.0 = partial paralysis, 4.0 = total paralysis.

a Represents mean day of EAE onset.

b Significantly different at p = 0.001 by Wilcoxon rank sum test, compared to individual maximum clinical scores in group A.

c Not significantly different from group A.
animals reconstituted with a 25/75 Le/LeR BMC mixture showed resistance to EAE (33%)
that was not significantly different from those in group B treated with LeR BMC alone.
Interestingly, as few as $25 \times 10^6$ LeR BMC (group G) were able to completely inhibit EAE
induction in Lewis recipients whereas $25 \times 10^6$ Le BMC (group F) allowed complete
susceptibility to EAE. These results are suggestive of a suppressive element derived
from the LeR BMC population which exerts a dampening effect upon the Lewis BMC.

In order to define which leukocyte populations may mediate EAE resistance, a
group of mixed cell type chimeras was constructed according to the methods of Singer
et al. (1981). This approach facilitates the in vivo analysis of cell interactions which may
lead to disease susceptibility or resistance. Also, in this method there is a shorter
waiting period between reconstitution and MBP challenge. Lethally irradiated Le rats
were reconstituted with various mixtures of Le or LeR BMC, spleen cells and thymocytes.
At 5-6 weeks post-irradiation, rats were challenged with HuMBP-CFA and susceptibility
to EAE was determined. The results from four experiments are reported in Table 5. Le
recipients reconstituted entirely with LeR lymphoid cells (group B) are resistant to EAE
induction as assessed by clinical signs (group A: positive control Le $\rightarrow$ Le chimeras were
susceptible). Based on the BMC chimera results in Table 3, we would expect groups C-
E to be resistant to EAE induction, since they were reconstituted with LeR BMC.
Interestingly, groups C and E differ only in the source of spleen cells, with group E
animals demonstrating EAE resistance (similar to group B negative controls) and group
C animals displaying EAE susceptibility (not significantly different from group A positive
controls). These results indicate an important role for LeR spleen cells in mediating
resistance to EAE induction. When LeR spleen cells are replaced by Le spleen cells
Table 5

Induction of EAE in Lewis radiation chimeras reconstituted with mixtures of Lewis/LeR lymphoid cells

<table>
<thead>
<tr>
<th>Group</th>
<th>SOURCE OF CELLS:</th>
<th>Incidence of EAE (%)</th>
<th>Average maximum clinical symptom</th>
<th>Average histologic score</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM</td>
<td>Spleen</td>
<td>Thymus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Le</td>
<td>Le</td>
<td>Le</td>
<td>12/14 (86%)</td>
<td>2.29 ± 0.46</td>
</tr>
<tr>
<td>B</td>
<td>LeR</td>
<td>LeR</td>
<td>LeR</td>
<td>0/18 (0%)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>C</td>
<td>LeR</td>
<td>Le</td>
<td>Le</td>
<td>11/13 (85%)</td>
<td>1.89 ± 0.41</td>
</tr>
<tr>
<td>D</td>
<td>LeR</td>
<td>Le</td>
<td>LeR</td>
<td>6/16 (38%)</td>
<td>0.91 ± 0.3</td>
</tr>
<tr>
<td>E</td>
<td>LeR</td>
<td>LeR</td>
<td>Le</td>
<td>1/13 (8%)</td>
<td>0.23 ± 0.23</td>
</tr>
<tr>
<td>F</td>
<td>Le</td>
<td>LeR</td>
<td>LeR</td>
<td>6/14 (43%)</td>
<td>0.75 ± 0.33</td>
</tr>
<tr>
<td>G</td>
<td>Le</td>
<td>Le</td>
<td>LeR</td>
<td>5/6 (83%)</td>
<td>3.0 ± 0.68</td>
</tr>
<tr>
<td>H</td>
<td>Le</td>
<td>LeR</td>
<td>Le</td>
<td>2/10 (20%)</td>
<td>0.8 ± 0.53</td>
</tr>
</tbody>
</table>

a Lewis (Le) rat recipients were lethally irradiated and injected i.v. with the indicated cell mixtures. 5-6 weeks later, rats were sensitized with 100μg MBP-CFA and observed for clinical signs of EAE.

b A logit analysis showed that spleen cells (highly significant), bone marrow cells (significant) and the interaction between bone marrow and thymus cells (significant) are important factors in EAE resistance.

c Average maximum clinical symptoms and average histologic score ± SEM.

d Probability values calculated using the Wilcoxon rank sum test comparing individual clinical scores between the experimental groups B-H and group A.

e Not significant.
(groups C and D), there is greater disease susceptibility. Similarly, we would expect
groups F-H to be susceptible to EAE induction, since they were reconstituted with Le
BMC. However, groups F and H demonstrate a low incidence (43% and 20%,
respectively) and a significantly lower level of clinical disease severity (p < .0066
compared to severity in group A). Again, the presence of LeR spleen cells in groups F
and H appears to play a significant role in overriding susceptibility. Group G recipients,
on the other hand, lack LeR spleen cells and are highly susceptible to clinical EAE
induction, suggesting LeR thymocytes alone are not capable of overriding EAE
susceptibility. In general, histologic scores correlated with clinical scores, showing
increased numbers of lesions in highly susceptible groups.

If LeR spleen cells play an important role in mediating resistance, then conversely,
do Le spleen cells override resistance and/or mediate susceptibility? This appears to be
the case in groups C and G, where the presence of Le spleen cells in the context of LeR
or Le BMC gives 85% and 83% incidence of EAE, respectively. Group D also contains
Le spleen cells, but in the context of LeR BM and LeR thymocytes. Perhaps interactions
are occurring between particular cell types and thereby affecting the incidence of EAE.
To analyze this possibility further and to test whether individual cell populations or
interactions between cell types were affecting the incidence of EAE, a special statistical
test, a logit analysis, was performed. This analysis showed that the statistically significant
factors in the determination of EAE resistance are the source of spleen cells (highly
significant), the source of bone marrow cells (significant) and the interaction between
bone marrow and thymus cells (significant). Thus, the logit analysis confirmed a very
important role for spleen cells in mediating resistance/susceptibility to EAE. In addition,
an interaction between LeR BMC and LeR thymus cells serves to explain why the animals in group D showed significantly less severe EAE, when the presence of Le spleen suggests that they should be susceptible.

To determine if macrophages in the spleen cell populations played a role in susceptibility or resistance to EAE induction in mixed cell chimeras, groups C and E (from Table 5) were re-tested using spleen cell suspensions depleted of adherent cells. Depletion consisted of a plastic adherence step followed by passage of the non-adherent cells over a Sephadex G10 column. This procedure reduced the percentage of esterase positive cells from 9% to less than 0.5%. When the adherent cell-depleted populations were infused into mixed cell chimeras, no detectable differences were observed compared to chimeras given control unfractionated spleen cell preparations (Table 6). Thus, the spleen cell mediated resistance does not appear to be caused by splenic adherent cells.

Cell mixing experiments were also employed in the mixed cell chimera studies in order to examine the question of a suppressive element in the LeR spleen. In these experiments, LeR and Lewis spleen cells were mixed in various ratios prior to infusion into irradiated Lewis recipients. The results shown in Table 7 reveal that control groups A and B receiving entirely Lewis or LeR cells were totally susceptible and resistant, respectively. Furthermore, control groups C and D receiving LeR or Lewis spleen cells in the context of LeR BMC and Lewis thymus were likewise resistant and susceptible, respectively. Mixing equal numbers of Lewis and LeR spleen cells prior to infusion resulted in total resistance (group E) in the recipient rats, as did a 75/25 LeR/Lewis
Table 6

Effect of adherent spleen cell depletion on induction of EAE in radiation chimeras

<table>
<thead>
<tr>
<th>Group</th>
<th>SOURCE OF CELLS:</th>
<th>Incidence of EAE(^b) (%)</th>
<th>Average maximum clinical symptom(^c) incidence</th>
<th>Histology average score(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LeR Le (unfractionated) Le</td>
<td>3/4 (75%)</td>
<td>0.38 ± 0.13</td>
<td>4/4</td>
</tr>
<tr>
<td>2</td>
<td>LeR Le (nonadherent) Le or LeR</td>
<td>6/10 (60%)</td>
<td>0.65 ± 0.25</td>
<td>7/10</td>
</tr>
<tr>
<td>3</td>
<td>LeR LeR (unfractionated) Le</td>
<td>1/4 (25%)</td>
<td>0.13 ± 0.13</td>
<td>1/4</td>
</tr>
<tr>
<td>4</td>
<td>LeR LeR (nonadherent) Le or LeR</td>
<td>0/8 (0%)</td>
<td>0.00 ± 0.0</td>
<td>3/8</td>
</tr>
</tbody>
</table>

\(^a\) Lewis rat recipients were lethally irradiated and injected i.v. with the indicated cell mixtures. 5-6 weeks later, all rats were sensitized with 100 \(\mu\)g human MBP-CFA and observed for clinical signs of EAE. Control spleen cells were not depleted of adherent cells. Depletion of adherent cells involved plastic adherence followed by passage over Sephadex G10.

\(^b\) Using a logit analysis, no significant effects on the incidence of EAE were obtained by depleting adherent spleen cells. However, this same analysis confirmed that the presence of LeR spleen cells conferred significant resistance on the resulting chimera.

\(^c\) Average maximum clinical symptoms and average histologic score ± SEM.
Table 7

Effect of mixing Lewis and LeR spleen cells on induction of EAE in Lewis chimeras

<table>
<thead>
<tr>
<th>Group</th>
<th>Source of cells:</th>
<th>Incidence of EAE</th>
<th>Average maximum clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM   Spleen Thymus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Le   Le   Le</td>
<td>3/3 (100%)</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>B</td>
<td>LeR  LeR  LeR</td>
<td>0/3 (0%)</td>
<td>0.0 (S)</td>
</tr>
<tr>
<td>C</td>
<td>LeR  LeR  Le</td>
<td>1/6 (17%)</td>
<td>0.3 ± 0.3 (S)</td>
</tr>
<tr>
<td>D</td>
<td>LeR  Le   Le</td>
<td>2/3 (66%)</td>
<td>0.8 ± 0.6 (NS)</td>
</tr>
<tr>
<td>E</td>
<td>LeR  1/2 LeR 1/2 Le</td>
<td>0/6 (0%)</td>
<td>0.0 (S)</td>
</tr>
<tr>
<td>F</td>
<td>LeR  3/4 LeR 1/4 Le</td>
<td>0/7 (0%)</td>
<td>0.0 (S)</td>
</tr>
<tr>
<td>G</td>
<td>LeR  1/4 LeR 3/4 Le</td>
<td>2/4 (50%)</td>
<td>1.8 ± 1.0 (NS)</td>
</tr>
</tbody>
</table>

a Lewis rats were exposed to 1000R total body irradiation and then injected intravenously with the indicated cell mixtures. After 5-6 weeks reconstitution time, recipients were sensitized with MBP-CFA and evaluated for clinical signs of EAE.

b Groups A and B are controls and groups C and D confirm the role of LeR spleen cells in mediating EAE resistance. Groups E, F and G show the effect of mixing different proportions of Lewis and LeR spleen cells in the context of LeR BM and Le spleen.

c Average maximum clinical score ± SEM. Letters in parentheses represent significantly different (S) or not significantly different (NS) compared to individual maximum clinical scores in group A (p ≤ 0.01) by the Wilcoxon rank sum test.
mixture (group F). However, a 25/75 LeR/Lewis mixture (group G) resulted in an intermediate level of EAE with a relatively severe clinical score. In this experiment, it would appear that the LeR spleen cells are indeed exerting a suppressive influence upon the Lewis spleen cell component. It also appears that relatively small numbers of LeR cells in the context of Lewis cells are not able to mediate suppression.

From the studies outlined in this section, four main conclusions can be drawn. First, LeR resistance is indeed immunologically mediated since Lewis rats reconstituted with LeR lymphoid cells are resistant to EAE. Second, the LeR spleen appears to be the important site for localization of resistance since the presence of LeR spleen cells in mixed cell chimeras correlates with resistance. Third, the resistance observed in the LeR rat is not directly mediated by macrophages, since removal of splenic adherent cells does not change EAE susceptibility/resistance patterns. And fourth, LeR cells appeared to exert a suppressive influence upon the Lewis cell component in BMC and spleen cell mixing experiments.

B. Is the mechanism of resistance observed in the LeR rat similar to that for other EAE resistant animals?

The resistance mechanism(s) operative in the LeR rat were investigated in light of resistance mechanisms reported for other EAE resistant animals. First, we questioned whether the MBP-specific immune response of LeR rats differs from Le rats. Waxman et al. (1981) used the technique of macrophage migration inhibition to examine the cellular reactivity of LeR rats to GPMBP and reported that the LeR rat showed no defect
in cellular reactivity to whole MBP or to the encephalitogenic 68-88 peptide when tested at day 21 post-challenge. Thus, it appeared that the mechanism of LeR resistance was different from that of the EAE resistant BN rat which cannot generate reactivity to GPMBP or its EF (McFarlin et al., 1975b). We chose to re-examine the LeR antigen-specific response for several reasons: 1) the colony of LeR rats had been completely re-derived, 2) the EAE-inducing antigen was now human rather than guinea pig MBP, 3) the MBP-specific response may differ depending upon the length of time after sensitization at which the response is tested.

Our initial experiments examined the proliferative responses of Le and LeR LNC and spleen cells. Both Le and LeR rats were sensitized with 100μg HuMBP-CFA and 18 days later their LNC and spleen cells were cultured with several concentrations of HuMBP, purified protein derivative (PPD—a component of the adjuvant) and Con A. LNC and spleen cell proliferative responses are shown in Figures 4a and 4b, respectively. No differences were observed between Lewis and LeR proliferative responses to HuMBP, PPD or to Con A (not shown) suggesting that the LeR rat shows no antigen-specific or antigen non-specific deficits in its lymphocyte proliferative responses. To further characterize the T cell reactivity in LeR rats, we then tested LNC obtained at different time points after sensitization (i.e. days 9, 12, 15, 18, 21) and also different lengths of culture (i.e. 72 hr, 96 hr, and 120 hr) to determine if the kinetics of the proliferative response vary between Le and LeR rats. Figure 5 shows the proliferative response of LeR versus Le LNC at representative timepoints following MBP challenge. LeR proliferative responses at day 9 postchallenge were significantly decreased compared to Le responses, but the later timepoints (12, 15, 18, and 21 days) showed no differences. Optimal culture length
Figure 4. Proliferative responses of Le and LeR LNC and spleen cells. Le and LeR rats were sensitized with 100μg HuMBP-CFA and 18 days later their LNC (a) and spleen cells (b) were cultured with HuMBP, purified protein derivative (PPD) and Con A (not shown). The data represent mean CPM of quadruplicate cultures of LNC or spleen cells from 3 Lewis rats and 5 LeR rats.
Figure 5. Kinetics of the MBP-specific proliferative response by Lewis and LeR LNC. At representative timepoints (days 9, 12, 18) after sensitization with MBP, LNC were obtained from 5 Lewis and 5 LeR rats and cells were cultured with various concentrations of HuMBP for 72 hr after which proliferation was measured by $^3$H-thymidine uptake. The data represent the mean CPM of quadruplicate cultures ± one SD.
was determined to be 72hrs. This suggested that early in the MBP response, during antigen presentation to T cells and education of precursor lymphocytes, the LeR T cells were not recognizing and responding to the MBP antigen to the same degree as the Le T cells. Alternatively, the number of responding LeR T cells may have been reduced due to decreased expression of class II major histocompatibility complex molecules (Ia molecules) on the surface of LeR antigen presenting cells (APC). These APC are required to complex with and present antigen to the T lymphocytes.

As an in vivo correlate to the lymphocyte proliferative responses, we also examined the MBP-specific delayed hypersensitivity responses demonstrated by Lewis and LeR rats. Twenty days following footpad sensitization with 100μg HuMBP, rats were injected intradermally in the pinna of the ear with a solution of HuMBP in saline, and ear swelling was measured at 24 and 48 hours with skinfold thickness calipers. In Figure 6, it can be seen that the ear swelling observed in LeR rats was not significantly different from that observed in Lewis rats. Thus, with the exception of a decrease in the very early LeR proliferative response to MBP, we found no differences between Lewis and LeR rats in MBP specific cellular reactivity, as measured in vitro or in vivo.

The overall cellular composition of Lewis and LeR lymph nodes and spleens was examined for the distribution of rat lymphocyte subsets. This approach would allow recognition of large shifts in lymphocyte subpopulations. Lewis and LeR LNC and spleen cells, removed 11 days after sensitization with HuMBP-CFA, were reacted with mouse monoclonal antibodies specific for total T cells and macrophages (W3/13), T helper cells (W3/25), T suppressor/cytotoxic cells (OX8), and Ia antigens (OX3 and OX6) present on
Figure 6. MBP-specific delayed type hypersensitivity (DTH) response in Lewis and LeR rats. Twenty days after sensitization with 100μg HuMBP-CFA, DTH responses were measured by injecting 10μg HuMBP into the pinna of the ear and measuring ear swelling at 24 and 48 hours post injection. Mean increase in ear thickness (inches) ± SEM at 48 hours is reported for 14 Lewis, 18 LeR and unsensitized control rats (2 Lewis and 2 LeR). Lewis and LeR DTH responses were not significantly different as measured by the Student's T test (0.1 < p ≤ 0.4).
macrophages, B cells and activated T cells. Labelled cells were then reacted with FITC-conjugated F(ab')₂ goat anti-mouse IgG and examined by flow cytometry. As shown in Table 8, there were no differences detected between Lewis and LeR rats in distribution of any of the cell population tested. Therefore, the resistance observed in the LeR rat is not due to an absolute loss of any of these phenotypically identifiable cell populations.

It has been reported by Mostarica-Stojkovic et al. (1982) that AO rats, but not DA rats, are resistant to EAE induction. These investigators have found that Con A stimulated spleen cell cultures derived from AO rats consistently produce lower amounts of interleukin 2 (IL-2) than similar cultures from DA rats (Mostarica-Stojkovic et al., 1985). It is hypothesized that IL-2 is under genetic control and that this genetic control is expressed at the level of the lymphokine-secreting T cell (Lukic et al., 1987). This approach was investigated in the LeR rat by examining the amount of IL-2 secreted by Con A-stimulated LeR spleen cells in comparison with similarly treated Lewis cells. In Table 9, the results of 2 separate experiments, testing 4 Lewis and 4 LeR rats, are shown. Statistical analysis of the IL-2 levels obtained in the culture supernates derived from Lewis versus LeR rats showed no significant differences.

We elected to utilize the approach of limiting dilution analysis in order to obtain the actual frequency of HuMBP-reactive IL-2 secreting lymphocytes (i.e. T helper cells) in Lewis and LeR lymph nodes and spleens (Table 10). Lymphocytes were assayed directly after their removal from animals. This assay takes advantage of the addition of IL-2 dependent cells (CTLL-20) directly to irradiated LNC cultures, thereby increasing the sensitivity of detection of IL-2 (Orosz et al., 1987). Representative limiting dilution
### Table 8

Monoclonal antibody profile of Lewis and LeR spleen and LNC at 11 days post MBP-sensitization

<table>
<thead>
<tr>
<th></th>
<th>Controlb</th>
<th>OX3</th>
<th>W3/13</th>
<th>OX6</th>
<th>OX8</th>
<th>W3/25</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeR spleen</td>
<td>3.5</td>
<td>11.8</td>
<td>32.5</td>
<td>11.5</td>
<td>15.9</td>
<td>5.2</td>
</tr>
<tr>
<td>Lewis spleen</td>
<td>3.4</td>
<td>15.0</td>
<td>33.0</td>
<td>12.0</td>
<td>11.8</td>
<td>5.8</td>
</tr>
<tr>
<td>LeR LNC</td>
<td>4.5</td>
<td>8.5</td>
<td>93.6</td>
<td>5.7</td>
<td>25.8</td>
<td>60.5</td>
</tr>
<tr>
<td>Lewis LNC</td>
<td>6.7</td>
<td>10.4</td>
<td>91.6</td>
<td>7.7</td>
<td>23.9</td>
<td>64.7</td>
</tr>
</tbody>
</table>

* Values represent the percent positive cells stained with monoclonal antibodies. Monoclonal antibody designations: OX3 = Lewis-specific polymorphic determinant on RT1.B-encoded la molecule; W3/13 = total T cells; OX6 = monomorphic determinant on the RT1.B-encoded la molecule; OX8 = T suppressor/cytotoxic cells; W3/25 = T helper cells.

* Secondary antibody only.
Table 9

IL-2 levels in 48 hour Con A supernatants from Lewis and LeR rat spleen cells

<table>
<thead>
<tr>
<th>ANIMAL #</th>
<th>UNITS OF IL-2/ml b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>Lewis 1</td>
<td>489</td>
</tr>
<tr>
<td>Lewis 2</td>
<td>158</td>
</tr>
<tr>
<td>Lewis 3</td>
<td>403</td>
</tr>
<tr>
<td>Lewis 4</td>
<td>489</td>
</tr>
</tbody>
</table>

Mean IL-2 levels ± SD : 384 ± 156 262 ± 68 323 ± 129

| LeR 1    | 229    | 181    | 205      |
| LeR 2    | 158    | 286    | 222      |
| LeR 3    | 153    | 382    | 268      |
| LeR 4    | 153    | 535    | 344      |

Mean IL-2 levels ± SD : 173 ± 37 346 ± 150 259 ± 137

a Lewis and LeR individual IL-2 levels not significantly different by the Student's t test.
b Calculated by using an IL-2 standard = 315 units/ml.
**Table 10**

Limiting dilution analysis for frequency of Lewis and LeR IL-2 secreting cells^a^ (Frequency per 100,000 cells)

<table>
<thead>
<tr>
<th>LER-8-88: Day 11-Direct LDA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ANIMAL #</td>
<td>LNC</td>
<td></td>
</tr>
<tr>
<td>Lewis 126</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>Lewis 125</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td>LeR 128</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>LeR 127</td>
<td>18.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LER-12-88: Day 11-Direct LDA</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ANIMAL #</td>
<td>LNC</td>
<td>SPLEEN CELLS</td>
<td></td>
</tr>
<tr>
<td>Lewis 272</td>
<td>8.9</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>Lewis 273</td>
<td>8.6</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>LeR 278</td>
<td>6.1</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>LeR 277</td>
<td>6.5</td>
<td>NC ^b^</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LER-12-88: Day 14-Direct LDA</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ANIMAL #</td>
<td>LNC</td>
<td>SPLEEN CELLS</td>
<td></td>
</tr>
<tr>
<td>Lewis 276</td>
<td>10.3</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>Lewis 281</td>
<td>7.8</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>LeR 279</td>
<td>5.8</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>LeR 282</td>
<td>7.6</td>
<td>6.8</td>
<td></td>
</tr>
</tbody>
</table>

^a^ Summary of data by Chi Square Minimization analysis.

^b^ Not calculable.

^c^ Lewis and LeR frequencies not significantly different by an approximate normal test for proportions (Devore, 1987).
analysis scattergrams demonstrating the MBP reactivity of Lewis and LeR responder LNC are shown in Figure 7. The number of microcultures that give CPM below the background line (mean CPM $+ 3\text{SD}$ of 48 wells containing APC, antigen, and CTLL-20 cells without responder cells) is used to calculate the frequency of antigen reactive cells.

Eleven days after MBP sensitization, the average frequency of antigen reactive cells was $13.9$ per $10^5$ LNC and $6.3$ per $10^5$ spleen cells in the Lewis rat, whereas LeR LNC and spleen cell frequencies were $10.7$ and $11.9$ per $10^5$ cells, respectively. In addition the average frequencies did not change significantly when tested at 14 days after MBP sensitization. Statistical analysis showed that the frequency of antigen reactive T cells was not significantly different between Lewis and LeR rats. Thus, the resistance mechanism operative in the LeR rat does not appear to be a decreased ability to secrete IL-2, as is proposed for other rat strains resistant to EAE. Moreover, the absolute number of MBP-reactive lymphocytes is not decreased in the LeR rat.

C. Are suppressor T cells involved in LeR resistance?

It has been demonstrated that suppressor cells can exert a regulatory influence on the development of EAE (Swierkosz and Swanborg, 1977; Adda et al., 1977; Beraud et al., 1982; Lando et al., 1979a). Therefore, LeR resistance could be due to the presence of a hyperactive suppressor cell population. This possibility was tested in four ways: 1) by cyclophosphamide treatment of Lewis and LeR rats in order to deplete rats of suppressor cells, 2) by direct spleen cell transfers between Lewis and LeR rats followed by EAE induction, 3) by depletion of phenotypic suppressor T cells (OX8$^+$) from spleen cell suspensions prior to direct cell transfer, and 4) by transfer of EAE with in vitro
Figure 7. Limiting dilution analysis: MBP-specific response of Lewis and LeR LNC. Serial dilutions of responder LNC were cultured in the presence of syngeneic irradiated thymocytes and 100μg/ml HuMBP for 18 hr after which the microtiter plates were irradiated and 1 x 10^3 CTLL-20 cells were added. After a 24 hr culture including a terminal 18 hr pulse with ^3^H-thymidine, microcultures were harvested and CTLL-20 proliferation was measured by uptake of radiolabel. Scattergram represents 24 replicate microwells for each responder cell dilution.
activation of Lewis and LeR LNC.

Low dose cyclophosphamide treatment, which can inhibit or delay induction of suppressor cells, has been shown to increase susceptibility for EAE in Fisher and PVG rats (Källen et al., 1986), in AO rats (Mostarica-Stojkovic et al., 1982) and in the mouse strains Balb/c, Balb/B10 and NZB (Teitelbaum et al., 1978). Cyclophosphamide treatment (20, 40, and 60 mg/kg) of Lewis and LeR rats was carried out 2 days prior to sensitization with HuMBP-CFA. As shown in Table 11, cyclophosphamide treatment of LeR rats produced no change in the resistance of these animals to EAE. Similar treatment of Lewis rats also resulted in no change in disease susceptibility.

If potent suppressor cells play an important role in LeR resistance, then it might be possible to transfer these cells to normal non-chimeric animals and demonstrate inhibition of EAE induction. To investigate this possibility further, direct spleen cell transfers between LeR and Lewis rats were carried out. In these studies, 250 x 10^6 spleen cells from normal Lewis or LeR rats were injected intravenously into naive, recipient Lewis or LeR rats which were then sensitized with HuMBP-CFA 3 or 5 days later. As can be seen in Table 12, control LeR rats receiving LeR spleen cells remained resistant to EAE induction, while control Lewis rats receiving Lewis spleen cells remained susceptible. However, LeR rats injected with Lewis spleen cells remained largely refractory to EAE, indicating suppression of the introduced Lewis cells. It is possible that the introduced cells did not have sufficient room in the recipient spleen to populate that organ. This possibility is less credible when considering the group of Lewis recipients injected with LeR spleen cells. Here the effect of resistant LeR cells introduced into a
Table 11

Effect of cyclophosphamide treatment on Induction of EAE in Lewis and LeR rats

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Incidence of EAE (%)</th>
<th>Average maximum clinical score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20mg/kg CY:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR</td>
<td>PBS</td>
<td>1/8 (12.5%)</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>LeR</td>
<td>CY</td>
<td>0/10 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Lewis</td>
<td>PBS</td>
<td>5/8 (63%)</td>
<td>0.88 ± 0.34</td>
</tr>
<tr>
<td>Lewis</td>
<td>CY</td>
<td>4/10 (40%)</td>
<td>0.4 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>40mg/kg CY:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR</td>
<td>PBS</td>
<td>0/7 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>LeR</td>
<td>CY</td>
<td>0/10 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Lewis</td>
<td>PBS</td>
<td>2/5 (40%)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Lewis</td>
<td>CY</td>
<td>6/10 (60%)</td>
<td>0.35 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg CY:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR</td>
<td>CY</td>
<td>0/2 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Lewis</td>
<td>CY</td>
<td>2/2 (100%)</td>
<td>0.5 ± 0.0</td>
</tr>
</tbody>
</table>

a Solutions of cyclophosphamide (CY) were freshly prepared and recipient rats were injected i.p. 2 days prior to MBP challenge at dosages of 20mg/kg, 40mg/kg or 60mg/kg. Control animals were injected in the same manner with Phosphate Buffered Saline (PBS).

b NS = probability values were not significantly different based on Fisher's Exact test comparing CY-treated animals to their PBS-treated controls within treatment groups.
Table 12

Direct transfer of spleen cells: effect on EAE induction in Lewis and LeR rats\textsuperscript{a}

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Incidence of EAE (%)</th>
<th>Mean clinical score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeR</td>
<td>LeR</td>
<td>0/5 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>Lewis</td>
<td>LeR</td>
<td>3/10 (30%)</td>
<td>0.2 ± 0.11\textsuperscript{b}</td>
</tr>
<tr>
<td>Lewis</td>
<td>Lewis</td>
<td>3/4 (75%)</td>
<td>0.75 ± 0.43</td>
</tr>
<tr>
<td>LeR</td>
<td>Lewis</td>
<td>2/9 (22%)</td>
<td>0.11 ± 0.07\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Spleen cell suspensions were obtained from naive donor rats and 250 x 10\textsuperscript{6} cells were injected intravenously into naive recipients as indicated. Three to five days after cell injections, rats were sensitized with HuMBP-CFA and observed for clinical signs of EAE.

\textsuperscript{b} Lewis -> LeR scores are not significantly different from LeR -> LeR scores.

\textsuperscript{c} LeR -> Lewis scores are significantly different from Lewis -> Lewis scores, \( p = 0.03 \).
susceptible Lewis rat results in resistance. Perhaps the introduced LeR cells are acting to suppress the development of encephalitogenic Lewis cells.

To determine whether the observed suppression in the spleen cell direct transfer experiment was due to T suppressor cells, OX8 \(^+\) cells were depleted from both Lewis and LeR splenocyte preparations. In these panning studies, spleen cells were incubated with OX8 antibody (mouse monoclonal anti-rat-CD8 ascites), then OX8 \(^+\) cells were depleted by incubation of spleen cells on plates previously sensitized with rabbit anti-mouse IgG. Either unfractionated or OX8 depleted cells (100 x 10\(^6\)) were injected intravenously into normal recipients three days prior to MBP sensitization. The results, shown in Table 13, demonstrate that depletion of OX8 \(^+\) cells from Lewis splenocytes had no effect on EAE induction in either Lewis or LeR recipients. Depletion of OX8 \(^+\) cells from LeR splenocytes, however, increased the EAE incidence from 33\% to 71\% between unfractionated splenocytes versus OX8 depleted LeR spleen cells, respectively. The average maximum clinical score also increased from 0.16 to 0.71 between these same groups. These results suggest that OX8 \(^+\) T suppressor cells in LeR spleens mediate suppression in LeR \(\rightarrow\) Le direct spleen cell transfers since their removal causes an increase in EAE incidence and severity.

The final approach in investigation of suppressor T cell involvement in LeR resistance focused on EAE transfer. LNC and spleen cells from Lewis and LeR rats previously sensitized with HuMBP-CFA, were cultured \textit{in vitro} with HuMBP 3 days prior to transfer. Preliminary experiments carried out using Lewis donors and recipients showed: 1) that \textit{in vitro} activated LNC optimally transferred EAE whereas spleen cells did
Table 13

Effect of depletion of OX8\(^+\) spleen cells on EAE induction\(^a\)

<table>
<thead>
<tr>
<th>Donor + Treatment</th>
<th>Recipient</th>
<th>Incidence of EAE (%)</th>
<th>Average maximum clinical score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis spleen</td>
<td>Lewis</td>
<td>5/6 (83%)</td>
<td>0.66 ± 0.16</td>
</tr>
<tr>
<td>unfractionated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR spleen</td>
<td>Lewis</td>
<td>2/6 (33%)</td>
<td>0.16 ± 0.11</td>
</tr>
<tr>
<td>unfractionated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis spleen</td>
<td>Lewis</td>
<td>5/6 (83%)</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td>OX8 depleted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR spleen</td>
<td>Lewis</td>
<td>5/7 (71%)</td>
<td>0.71 ± 0.26</td>
</tr>
<tr>
<td>OX8 depleted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis or LeR spleen</td>
<td>LeR</td>
<td>0/4 (0%)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>unfractionated or</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lewis OX8 depleted</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Spleen cells were incubated with OX8 antibody (mouse monoclonal anti-rat-CD8 ascites), then OX8\(^+\) cells were depleted by incubation of spleen cells on plates previously sensitized with rabbit anti-mouse IgG. Unfractionated or OX8 depleted cells (100 x 10\(^6\)) were injected intravenously into normal recipients three days prior to MBP sensitization.
not, 2) the cell concentration used for *in vitro* culture should be increased from $2 \times 10^6$/ml to $5 \times 10^6$/ml, and 3) the dose of cells injected should be increased from $50 \times 10^6$ to $100 \times 10^6$ LNC (Table 14). Using the above conditions to potentiate EAE transfer, reciprocal cell transfers from Le → LeR and LeR → Le were performed. As shown in Table 15, Lewis recipients receiving Lewis LNC all showed clinical signs of disease. In contrast, Lewis rats receiving LeR LNC showed no signs of EAE. Interestingly, the majority of LeR recipients developed EAE, whether they received LeR LNC or Lewis LNC.

In summarizing those experiments examining suppressor T cell involvement in LeR resistance, it appears that transfer of OX8⁻ T suppressor cells and both transfer approaches, i.e., the direct transfer of normal spleen cells and the transfer of EAE using sensitized and activated LNC do argue in favor of a role for suppressor cells. Specifically, the fact that normal LeR spleen cells introduced into an otherwise immunocompetent susceptible Lewis rat renders the Lewis rat resistant suggests the presence of a rather potent suppressive element in the LeR spleen cell population. In addition, depletion of phenotypic suppressor T cells from LeR splenocyte preparations prior to direct cell transfer caused increased EAE in Lewis recipients. However, cyclophosphamide treatment failed to demonstrate abrogation of LeR resistance.

**D. Are T cell recognition/antigen presentation defects involved in LeR resistance?**

It has been shown that LeR spleen cells failed to transfer disease to naive Le rats, however, LeR recipients were susceptible to passive disease induction mediated by sensitized and *in vitro* conditioned Le spleen cells (Waxman et al., 1981). These
**Table 14**

**Preliminary in vitro activation and EAE transfer with Lewis rats**

<table>
<thead>
<tr>
<th>Group</th>
<th># and Type of Cells</th>
<th>Incidence of EAE (%)</th>
<th>Score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50 x 10^6 Spleen</td>
<td>0/5 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>B</td>
<td>100 x 10^6 Spleen</td>
<td>0/5 (0%)</td>
<td>0.0</td>
</tr>
<tr>
<td>C</td>
<td>50 x 10^6 LNC</td>
<td>4/5 (80%)</td>
<td>0.4 ± .22</td>
</tr>
<tr>
<td>D</td>
<td>100 x 10^6 LNC</td>
<td>2/2 (100%)</td>
<td>0.75 ± .35</td>
</tr>
</tbody>
</table>

LNC and spleen cells from Lewis donors were cultured for three days at 5 x 10^6 cells/ml in the presence of 50μg/ml HuMBP. Following culture the cells were washed and injected intravenously into Lewis recipients. Rats were then observed for clinical signs of EAE.

**Table 15**

**Passive transfer of EAE by in vitro cultured lymph node cells**

<table>
<thead>
<tr>
<th>LNC Donor --&gt; Recipient</th>
<th>#cells transferred</th>
<th>Incidence of EAE (%)</th>
<th>Average maximum clinical score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis --&gt; Lewis</td>
<td>100 x 10^6</td>
<td>5/5 (100%)</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>LeR --&gt; Lewis</td>
<td>100 x 10^6</td>
<td>0/8 (0%)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>LeR --&gt; LeR</td>
<td>100 x 10^6</td>
<td>4/5 (80%)</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Lewis --&gt; LeR</td>
<td>100 x 10^6</td>
<td>4/7 (57%)</td>
<td>0.36 ± 0.14</td>
</tr>
</tbody>
</table>

Culture conditions: 5 x 10^6 cells/ml, 50 μg HuMBP/ml, 72h culture in 75cm² flasks lying down.

b Significantly different (p = 0.0008) from Lewis-->Lewis EAE incidence by Fisher's Exact test.

c Not significantly different from LeR-->LeR EAE incidence by Fisher's Exact test.
observations suggested that resistance is expressed during the development of diseaseinducing effector cells from specific antigen reactive cells, a step which involves antigen presentation and recognition. In order to compare the MBP-specific immune responses in more detail, HuMBP-specific T cell lines from Lewis and LeR rats were derived. Utilizing the selection procedures described by Ben-Nun et al. (1981b) and Vandenbark et al. (1985), lymph nodes draining the site of footpad MBP-CFA injection were removed and LNC cultured in the presence of MBP. Subsequently, the activated cells were harvested and placed in culture in the presence of IL-2. LNC were then restimulated in the presence of MBP and syngeneic irradiated thymocytes as a source of APC. Following the first re-stimulation, the LNC were observed to proliferate only in response to the selecting antigen. To perform presentation experiments, Lewis and LeR T cell lines were derived and selected using homologous APC. Proliferative responses were then tested using both the homologous and heterologous APC together with MBP. It can be seen in Figure 8a that the Lewis T cell line proliferated vigorously in the presence of MBP and Le thymocytes and less vigorously in the presence of MBP and LeR thymocytes. Surprisingly, the LeR T cell line also proliferated more vigorously in the presence of Lewis APC than in the presence of LeR APC. Both Figures 8a and 8b demonstrate that the overall proliferative response to MBP demonstrated by the Lewis T line cells was far better than that demonstrated by the LeR T line cells. This relative level of activity was also obvious in the ability of each T cell line to transfer EAE. Lewis HuMBP-specific T cell lines following stimulation with MBP and homologous APC, transferred more severe EAE than did similarly treated LeR T line cells (Figure 9). These results suggest that the LeR APC are contributing significantly to the decreased LeR proliferative responses.
Figure 8. Antigen presentation by thymocytes to Lewis and LeR T cell lines. (a) Lewis T cell lines (b) LeR T cell lines. T line cells \((10^4)\) and irradiated thymocytes \((10^6)\) were placed in culture wells with HuMBP antigen and incubated 72 hr. Plates were pulsed with \(^3\)H-thymidine \((1 \mu\text{Ci/well})\) for 18 hr and harvested. Proliferation was measured by uptake of radiolabel. Error bars represent ± one standard deviation.
Figure 9. Transfer of EAE with Lewis and LeR T cell lines. T line cells ($10^7$) collected after a 3-day culture with irradiated thymocytes and HuMBP, were injected i.v. into naive Lewis recipients. Clinical signs were monitored from 5 to 10 days after injection. Error bars represent one SEM.
We also examined the ability of LeR and Lewis thymocytes to present antigen to LeR or Lewis T cells using limiting dilution analysis (LDA). In these experiments, LNC were derived from HuMBP sensitized Lewis or LeR rats and exposed to MBP with homologous or heterologous irradiated thymocytes. The amount of IL-2 released by varying numbers of LNC was measured. Table 16 shows that in every case, use of LeR thymocytes resulted in detection of a greater number of antigen-reactive LNC than stimulation of the identical LNC with Lewis thymocytes. This enhanced antigen presenting cell capability was demonstrable with both Lewis and LeR LNC. Perhaps the LeR thymocyte population is in a higher state of activation and therefore may be secreting mediators, such as gamma interferon or IL-2, that activate LNC cultures regardless of their origin. These results appear to disagree with APC experiments performed with T cell lines. However, it could be argued that LDA cultures are more representative of the in vivo situation than T cell line studies since T cell lines are very highly selected for their antigen specificity and may have lost some of their natural reactivities by virtue of long-term culture.

Since BN rat astrocytes were shown to express significantly decreased levels of la after gamma interferon treatment (Massa et al., 1987), it was of interest to examine the relative capacity of Lewis versus LeR APC populations to express MHC class II antigens both before and after exposure to gamma interferon. We chose to examine thymocytes, since they are the most efficient APC in the generation of MBP-specific T lymphocyte lines, and also in extension of our findings previously obtained with the T cell lines. Monoclonal antibody staining of thymocytes was performed similarly to staining of LNC. Fresh thymocytes removed directly from Lewis and LeR rats (not shown) gave
Table 16

Limiting dilution analysis comparing MBP presentation to Lewis and LeR LNC by homologous and heterologous thymocytes

<table>
<thead>
<tr>
<th>LNC source</th>
<th>Thymocyte source</th>
<th>Frequency per 100,000 cells</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewis 486</td>
<td>Lewis</td>
<td>8.1</td>
<td>6.5 - 9.7</td>
</tr>
<tr>
<td>Lewis 486</td>
<td>LeR</td>
<td>33.1</td>
<td>27.4 - 38.8</td>
</tr>
<tr>
<td>Lewis 490</td>
<td>Lewis</td>
<td>24.1</td>
<td>18.9 - 29.3</td>
</tr>
<tr>
<td>Lewis 490</td>
<td>LeR</td>
<td>75.4</td>
<td>61.8 - 89.1</td>
</tr>
<tr>
<td>Lewis 487</td>
<td>Lewis</td>
<td>13.3</td>
<td>10.4 - 16.2</td>
</tr>
<tr>
<td>Lewis 487</td>
<td>LeR</td>
<td>52.6</td>
<td>43.7 - 61.5</td>
</tr>
<tr>
<td>LeR 491</td>
<td>LeR</td>
<td>11.8</td>
<td>9.2 - 14.4</td>
</tr>
<tr>
<td>LeR 491</td>
<td>Lewis</td>
<td>2.1</td>
<td>1.5 - 2.6</td>
</tr>
<tr>
<td>LeR 489</td>
<td>LeR</td>
<td>11.2</td>
<td>8.7 - 13.7</td>
</tr>
<tr>
<td>LeR 489</td>
<td>Lewis</td>
<td>2.6</td>
<td>1.9 - 3.2</td>
</tr>
<tr>
<td>LeR 488</td>
<td>LeR</td>
<td>23.0</td>
<td>19.0 - 27.0</td>
</tr>
<tr>
<td>LeR 488</td>
<td>Lewis</td>
<td>4.7</td>
<td>3.6 - 5.7</td>
</tr>
</tbody>
</table>

a Summary of data by chi square minimization.
b Frequency of antigen reactive cells as calculated by chi square minimization.
comparable antibody profiles to thymocytes cultured for three days without stimulants (Table 17). After culturing thymocytes in basic culture medium containing 10% fetal bovine serum and 10, 50 and 100 U/ml gamma interferon for three days, it was observed that the percent of cells labelled by OX6 was reduced in LeR thymocyte preparations cultured in the absence of interferon (Table 17). Moreover, there was a decrease in OX3+ thymocytes in LeR (60.1%) versus Lewis (82.6%) cell preparations exposed to 100U/ml interferon (Figure 10). It should be noted that no increases were observed in OX3 or OX6 staining of thymocytes after gamma interferon culture. Perhaps this particular population of thymocytes is not inducible by treatment with gamma interferon. Overall, comparing the extent of MHC class II expression on Lewis versus LeR thymocytes, we did not observe the extent of differences previously reported for BN versus Lewis astrocytes.

E. Summary

In summarizing these results, the following conclusions can be drawn: 1) LeR resistance appears to be immunologic and can be localized to the spleen where a non-macrophage suppressive cell population appears to reside. 2) EAE resistance mechanisms in the LeR rat do not appear to resemble mechanisms in other EAE resistant strains, such as decreased MBP-reactivity, lack of a delayed hypersensitivity response to MBP, deficient IL-2 secretion, or loss of any cell subpopulations. 3) Direct spleen cell transfer and passive EAE transfer studies indicate possible suppressor T cell involvement in LeR resistance. However, resistance is not abrogated by treatment with cyclophosphamide. 4) LeR MBP-specific T lymphocyte lines are less effective at antigen-
### Table 17

Monoclonal antibody profile of normal Lewis and LeR thymocytes with and without gamma interferon treatment

<table>
<thead>
<tr>
<th></th>
<th>Control b</th>
<th>OX3</th>
<th>W3/13</th>
<th>OX6</th>
<th>OX8</th>
<th>W3/25</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No treatment:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR thym</td>
<td>0.92</td>
<td>73.8</td>
<td>99.5</td>
<td>8.01</td>
<td>86.4</td>
<td>95.1</td>
</tr>
<tr>
<td>Lewis thym</td>
<td>1.13</td>
<td>68.2</td>
<td>99.6</td>
<td>16.1</td>
<td>90.3</td>
<td>94.1</td>
</tr>
<tr>
<td><strong>10 Units/ml gamma interferon:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR thym</td>
<td>1.4</td>
<td>74.1</td>
<td>99.9</td>
<td>10.1</td>
<td>89.9</td>
<td>95.0</td>
</tr>
<tr>
<td>Lewis thym</td>
<td>3.1</td>
<td>66.5</td>
<td>97.2</td>
<td>12.6</td>
<td>92.3</td>
<td>95.9</td>
</tr>
<tr>
<td><strong>50 Units/ml gamma interferon:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR thym</td>
<td>0.8</td>
<td>88.2</td>
<td>99.8</td>
<td>4.8</td>
<td>83.8</td>
<td>90.2</td>
</tr>
<tr>
<td>Lewis thym</td>
<td>0.8</td>
<td>82.8</td>
<td>99.8</td>
<td>8.2</td>
<td>88.9</td>
<td>89.1</td>
</tr>
<tr>
<td><strong>100 Units/ml gamma interferon:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LeR thym</td>
<td>0.9</td>
<td>60.1</td>
<td>99.8</td>
<td>6.3</td>
<td>84.9</td>
<td>91.2</td>
</tr>
<tr>
<td>Lewis thym</td>
<td>1.6</td>
<td>82.6</td>
<td>99.8</td>
<td>6.6</td>
<td>86.7</td>
<td>92.2</td>
</tr>
</tbody>
</table>

a  Values represent the percent positive cells stained with monoclonal antibodies. Monoclonal antibody designations: OX3 = Lewis-specific polymorphic determinant on RT1.B-encoded la molecule; W3/13 = total T cells; OX6 = monomorphic determinant on the RT1.B-encoded la molecule; OX8 = T suppressor/cytotoxic cells; W3/25 = T helper cells.

b  Secondary antibody only.
Figure 10. Log green fluorescence histograms of control and OX3 stained thymocytes in LeR versus Lewis cell preparations exposed to 100U/ml gamma interferon. Both control and OX3 stained thymocytes were cultured for 3 days in the presence of 100U/ml recombinant rat gamma interferon. a) LeR control thymocytes stained with secondary antibody alone. b) LeR OX3-stained thymocytes. c) Lewis control thymocytes stained with secondary antibody alone. d) Lewis OX3-stained thymocytes. The vertical axis represents cell count from a sample of 2500 cells. The horizontal axis represents log fluorescence intensity based on 255 channels. Laser power was 500mW.
specific proliferative responses and EAE transfer than are similarly prepared Lewis lines. LeR thymocytes appear to have altered antigen presenting functions compared to Lewis thymocytes, although, LeR thymocytes do not appear to have marked deficiencies in expression or induction of MHC class II antigens.
DISCUSSION

The principal findings of this study can be summarized in four parts. First, investigation of whether EAE resistance in the LeR rat is immunologically mediated was carried out taking advantage of the observation that differential disease susceptibility between Le and LeR rats could be demonstrated using human MBP as the sensitizing antigen. Both in vitro and in vivo assays confirmed the previously suggested histocompatibility at the MHC between Le and LeR rats. Resistance to EAE induction in the LeR rat appeared to be an immunologically mediated event since LeR BMC transferred resistance while Le BMC transferred susceptibility. In addition, these results suggested little or no contribution by radiation resistant host tissue elements present in recipient rats. In fact, in autologous bone marrow transplants, radiation did not change the inherent susceptibility or resistance patterns in chimeric animals since Lewis rats remained susceptible to EAE while LeR rats maintained their resistance. In BMC mixing experiments, the LeR BMC population appeared to contain a suppressive element that exerted a dampening effect on the Lewis BMC. This suppressive element appeared to be localized to the spleen since both spleen cell mixing and mixed cell chimera experiments demonstrated that the donor origin of the spleen cell component correlated with EAE susceptibility. The source of BMC and the interaction between BMC and thymus cells also appeared to play an important role in the determination of EAE resistance in mixed cell chimeras. The resistance observed
in the LeR rat is not directly mediated by macrophages, since removal of splenic adherent cells did not change resistance patterns.

The second group of findings in this study involves the question of whether the LeR resistance mechanism(s) resembles mechanisms for other EAE resistant strains. Kinetic studies of MBP-specific proliferative responses showed that LeR responses at day 9 postchallenge were significantly decreased compared to Le responses, however later timepoints showed no differences. This suggested that LeR LNC are delayed in recognizing and responding to the MBP antigen. MBP-specific in vivo reactivity, as measured by delayed hypersensitivity, also showed no defect in cellular reactivity in the LeR rat. LeR T cell reactivity to mitogen stimulation was comparable to Lewis reactivity, indicating that a non-specific deficit in T lymphocyte function was not demonstrable. The resistance observed in the LeR rat is not due to an absolute loss of any phenotypically identifiable cell population such as those defined by monoclonal antibodies specific for total T cells and macrophages, T helper cells, T suppressor/cytotoxic cells, and Ia antigens present on macrophages, B cells and activated T cells. No significant difference in the ability to secrete IL-2 was observed between Le and LeR rats, when supernates from spleen cells non-specifically activated with Con A, and LNC and spleen cells activated with HuMBP were tested.

Third, in summarizing those experiments examining suppressor T cell involvement in LeR resistance, it appears that both transfer approaches, i.e. the direct transfer of normal spleen cells and the transfer of EAE utilizing sensitized and activated LNC, argue in favor of a role for suppressor cells. In addition, depletion of
OX8+ T suppressor cells from LeR spleen cell preparations caused an increase in EAE incidence and severity in Lewis recipients of direct spleen cell transfers. However, CY-sensitive suppressor cells do not appear to mediate resistance in the LeR rat. These results are similar to those reported by Källen and Lodgberg (1982) for the Lew/Mol and Lew/Mai rat strains, which are also resistant and susceptible to EAE induction, respectively. These authors were unable to abrogate EAE resistance in the Lew/Mol rat by pretreatment with CY. Perhaps the resistance observed in the LeR rat is also mediated by a cyclophosphamide-insensitive suppressor cell.

The fourth and last group of findings addresses whether T cell recognition and/or antigen presentation defects are involved in LeR resistance. In investigation of this question, MBP-specific T cell lines were derived from Lewis and LeR LNC. Lewis T cell lines proliferated to HuMBP at approximately twice the level of the LeR T cell lines. This relative level of activity was also observed in the ability of each T cell line to transfer EAE. Lewis HuMBP-specific T cell lines transferred more severe EAE than did LeR T cell lines. Perhaps Lewis T cell lines contain more encephalitogenic clones than LeR T cell lines, which may be due to recognition of-and activation by-different portions of the MBP molecule. Lewis thymocytes as APC appeared to be better stimulators of proliferation than LeR APC which suggests that LeR APC are contributing to the decreased LeR proliferative responses. APC defects, however, were not apparent in the LDA experiments examining HuMBP presentation to freshly isolated LNC by homologous and heterologous thymocytes. Here, it appears that LeR thymocytes were more efficient than Lewis thymocytes at antigen presentation to both Lewis and LeR LNC. LeR thymocytes showed only minor differences compared
to Lewis thymocytes in the expression of MHC class II antigens, both prior to and following gamma interferon treatment. Thus, differences observed in LeR antigen presentation function do not appear to correlate with the level of MHC class II antigen expression.

Genetic analysis of EAE has shown that both the incidence and the severity of the disease are determined by genes linked to the MHC in the mouse (Levine and Sowinski, 1973; Bernard, 1976; Fritz et al., 1985; Lando et al., 1980) and in the rat (Gasser et al., 1973; Gunther et al., 1978; Williams and Moore, 1973). Another class of genes operative in EAE are those not linked to the MHC (Gasser et al., 1978; Lando et al., 1979b; Lindh and Källen, 1978; Happ et al., 1988; Källen and Logdberg, 1982; Levine and Sowinski, 1974). Currently, it is not known how many non-H-2 genes are involved in the murine autoimmune response to MBP. A two gene model for genetic control of murine EAE has been proposed by Linthicum and Frelinger (1982) suggesting that induction of clinical EAE requires the appropriate genes in the MHC as well as a gene responsible for *Bordetella pertussis*-induced histamine sensitivity. Williams and Moore (1973) suggest that the Ir-EAE gene is more likely to determine reactivity at the level of antigen specificity, while the quantitative expression of this reaction may be influenced by other genes including some which may control thymus-derived cell function. Gasser et al. (1983) have shown that the gene for nonsusceptibility (i.e. resistance) in the LeR rat is not linked to the RT1 locus. Thus, the RT1^L^ haplotype of the Lewis and LeR strains appears to promote susceptibility (Gasser et al., 1983). This argues in favor of a simple mutational event that occurred in the LeR line giving rise to an EAE resistant phenotype. It is not known how this
resistant phenotype manifests itself in vivo. Resistance could be immunologic, that is, derived from hematopoietic cells, or it could be non-immunologic (i.e., non-bone marrow derived).

Radiation-induced bone marrow and mixed cell chimera procedures allow the manipulation of components of the reticuloendothelial-lymphocyte compartment, with the apparent exception of radiation-resistant cells in the thymus (Singer et al., 1981; Doherty et al., 1981), while leaving the host tissue, including the CNS, intact. Thus, by lethally irradiating adult rats with little or no susceptibility to EAE induction and replacing their bone marrow-derived compartment with cells from an MHC-matched highly susceptible strain, or vice versa, one can attribute genetically determined resistance to either the BM-derived or target CNS compartments.

Several groups of investigators have examined reciprocal bone marrow transplantation producing chimeric animals with the immune system of the donor and the target tissue of the host allowing the separation of immune-mediated regulation of disease from other host factors. Singer et al. (1981) concluded that BN resistance is expressed at the level of the lymphoid system (i.e. bone marrow derived) and not at the level of the target organ, the CNS. BN resistance appeared to be a non-transferrable radiation-resistant block in the ability to generate sensitized autoreactive T cells. These authors also reported that suppressor mechanisms did not appear to play a major role in BN resistance. Other investigators have reported what appeared to be a regulatory role for the non-bone marrow-derived compartment (i.e., the CNS) in controlling clinical expression of EAE. Korngold et al. (1986) and Lublin et al.
(1986) observed a low incidence of EAE in B10.S → B10.S and SJL → B10.S chimeras versus high incidence of EAE in SJL → SJL and B10.S → SJL chimeras. These results demonstrated that the immune system from the resistant B10.S mouse is capable of mediating EAE only when present in a susceptible SJL host, while the SJL immune system was restricted in its ability to induce disease when present in a resistant B10.S host. These authors concluded that in the B10.S/SJL chimera system, restriction to the development of EAE may reside outside the immune system, perhaps involving antigen recognition or presentation in the CNS itself.

In examining bone marrow derived versus non-bone marrow derived effects, it was concluded that LeR resistance does appear to be bone marrow derived and localized to the splenic compartment. Hinrichs et al. (1987) predicted that chimeric recipients of the (Le x BN) F, → BN construct would possess non-bone marrow derived cells allogeneic to the transferred Le spleen cells used for passive EAE transfer. Their results showed that these chimeric recipients developed and recovered from clinical disease with the same kinetics as syngeneic recipients and therefore any MHC-restricted antigen presenting function by these non-bone marrow derived cells is not required for the development of adoptively transferred EAE. Although we were not using adoptively transferred EAE, these results are somewhat analogous to our BM chimera results (Table 3) in which LeR → Le chimeras were resistant and Le → LeR chimeras were susceptible. If non-bone marrow derived cells, such as astrocytes or CNS endothelial cells, are important in mediating LeR resistance to EAE, then we would expect Le → LeR chimeras to be resistant. Since they were not, this argues in favor of bone marrow derived donor elements in mediating resistance to EAE and
suggests little or no contribution by radiation-resistant host tissue elements in LeR resistance. Radiation may have other effects, however, and cannot be dismissed as unimportant.

There have been many reports about the effects of irradiation on EAE. Paterson and Beisaw (1963) and Vitale et al. (1966) found that rats exposed to total body x-irradiation well before CNS tissue-adjuvant sensitization showed a consistent decrease in their ability to develop EAE. Interestingly, total body irradiation of rats when given after CNS-adjuvant sensitization caused animals to develop more severe clinical neurologic signs compared to non-irradiated control rats (Allegretti and Matosić, 1961). In support of these findings, Paterson et al. (1975) reported that whole body irradiation of recipient Lewis rats exerted a considerable augmenting influence on the passive transfer of EAE in this rat strain. Increasing doses of total body irradiation prior to injection of sensitized donor cells resulted in an increased incidence of paralysis and an increased number of lesions in the brain and spinal cord. Optimum lymphoid cell transfer occurred with irradiation doses of 850 to 1000 rads, and shielding experiments suggested that the irradiation was required to extend over the length of the spinal cord in order to augment transfer of EAE (Paterson et al., 1975). These authors interpreted their observations to indicate that irradiation directly influences the CNS target and favors development of lesions, perhaps by increasing the permeability of the blood brain barrier and by augmenting binding and interaction of sensitized cells or antibody with CNS antigen(s).
In our hands, total body irradiation of chimeras with 1000 rads augmented clinical signs of EAE to a small degree, but, this enhancement was not statistically significant when compared to non-irradiated rats. The important observations in this experiment were first, that irradiation did not abrogate resistance to EAE in LeR rats and second, irradiation did not reduce Lewis rat susceptibility.

To isolate and clarify the mechanism of LeR resistance, it was necessary to examine mechanisms from other EAE resistant strains. There is evidence that EAE resistant BN rats fail to develop cellular reactivity \textit{in vitro} and delayed hypersensitivity responses, \textit{in vivo}, for the critical disease inducing antigenic determinant located within amino acids 68-88 of the MBP molecule (McFarlin et al., 1975a; 1975b). In addition, BN rat astrocytes but not peritoneal macrophages have been found to express significantly decreased levels of la molecules compared to comparable Lewis cell populations (Massa et al., 1987b). In contrast to BN rats, Lewis rats develop cellular reactivity for MBP and peptide 68-88, and this reactivity was detectable after primary and secondary MBP-CFA challenge (McFarlin et al., 1975a; 1975b; Waxman et al., 1981). Using a lymphocyte proliferation assay, we examined the LeR response to HuMBP, PPD and the mitogen Con A. No overall differences were observed between Lewis and LeR proliferative responses suggesting that the LeR rat, unlike the BN rat, shows neither antigen-specific nor antigen non-specific defects in its \textit{in vitro} lymphocyte proliferation. The LDA assay did not detect differences between Le and LeR rats in frequency of MBP-reactive lymphocytes in lymph nodes or spleens (Table 10). Analysis of the kinetics of the HuMBP-specific proliferation revealed a delay in the response of LeR rats, suggesting that LeR T cells were not recognizing and
responding to the MBP antigen to the same degree as Le T cells during the early recognition of antigen. This could relate to decreased expression of class II major histocompatibility complex molecules on the surface of LeR APC. Alternatively, LeR rats may display some defect in processing or presentation of antigen to lymphocytes. LeR rats may show differences in the composition of lymphocyte subpopulations or in IL-2 secretion, as has been suggested for the EAE-resistant AO rat (Lukic et al., 1987). Another possibility is that LeR rats possess suppressor cells that inhibit antigen-specific responses.

First, in examining lymphocyte subpopulations and MHC class II expression, it was observed that monoclonal antibody profiles of Lewis and LeR LNC, spleen cells and thymocytes showed no significant differences in phenotypically identifiable lymphocyte subpopulations between the two strains. Staining with markers that detect class II molecules also did not demonstrate differences. Both of these observations suggest that the delay in the LeR proliferative response to HuMBP is not a function of LeR lymphocyte composition nor of decreased class II MHC expression in the cell populations examined.

Antigen presenting cells, necessary for processing and presentation of antigen to lymphocytes, have been implicated in several strains as a critical component of their immune reactivity. Ia+ cells of non-bone marrow origin located in the brain (e.g. endothelial cells, astrocytes) have been proposed to play a significant and perhaps determining role in the expression of a cell-mediated response to neuroantigens (Hickey and Kimura, 1988; Fontana et al., 1984; McCarron et al., 1986; Fierz et al.,
Endothelial cells isolated from the cerebral vasculature express Ia when isolated from SJL mice with clinical EAE, but Ia+ endothelial cells are not found in similar cell preparations from normal syngeneic mice (McCarron et al., 1986). In guinea pigs immunized with MBP, endothelial cells become Ia+ just before disease onset (Sobel et al., 1984). However, in (Strain 2 x Strain 13) F₁ hybrids, only the high-responder strain 13 haplotype is expressed on endothelial cells of MBP-sensitized guinea pigs (Sobel and Colvin, 1985). Large numbers of Ia+ cells are also found in multiple sclerosis brain lesions, especially in the peripheral areas of the expanding plaque (Traugott et al., 1985). These observations have led some investigators to suggest that endothelial cells may have a relevant role in antigen presentation in vivo (Sobel et al., 1984; Sakai et al., 1986) both in the expression of antigen-specific delayed-type hypersensitivity responses (Burger and Vetto, 1982) and in the development of the autoimmune response to antigens of the central and peripheral nervous system. In experiments comparing gamma interferon induced expression of Ia, it was found that astrocytes from EAE susceptible strains (Lewis rat and SJL mouse) expressed much higher levels of Ia than did astrocytes from EAE resistant strains (BN rat and BALB/c mouse) (Massa et al., 1987b). These authors concluded that astrocyte hyperinducibility may be a major strain and tissue specific factor that contributes to Ia restricted DTH reactions in the CNS.

Both astrocytes and macrophages have been considered to play a crucial role in BN rat resistance (Massa et al., 1987b; Singer et al., 1981; Beraud et al., 1976). In guinea pigs, recognition of the disease inducing MBP components has been linked to
the interaction of lymphocytes and macrophages (Ben-Nun et al., 1981a). In the Balb/c mouse (Massa et al., 1987b) and the B10.S mouse (Lublin et al., 1986; Korngold et al., 1986), non-bone marrow derived cells such as astrocytes, have been implicated in EAE resistance. Thymocytes are frequently used as APC with rat in vitro assays because they are the most efficient APC in the generation of MBP-specific T lymphocyte lines. In examining antigen presentation by LeR thymocytes, two apparently conflicting results were obtained. On the one hand, LeR APC appear to be defective in presentation of MBP to MBP-specific T lymphocyte lines, and on the other hand LeR APC demonstrated heightened activation of- and presentation to- LNC in the LDA assay. It could be argued that T lymphocyte lines represent a population of cells that have been very highly selected for and thus, may not have similar reactivities compared to uncultured lymphocytes obtained directly from animals. Therefore, antigen presentation experiments with these highly selected T cell lines may not reflect in vivo APC capabilities. The LDA assay, however, utilizes responder lymphocytes obtained directly from animals. This population of cells is much more heterogeneous and serves as a better model for in vivo cell reactivities. Therefore, the capacity of LeR thymocytes to present antigen, as observed in the LDA assay, demonstrates no deficiencies. In addition, expression of MHC class II antigens on LeR thymocytes was not observed to be significantly different from expression on Lewis thymocytes either prior to or after gamma interferon treatment (Table 16). Only the highest dose of gamma interferon (100U/ml) produced small differences between Lewis and LeR thymocytes, suggesting that these thymocyte populations may not be appropriately inducible with gamma interferon. Since LeR thymocytes have been observed to exhibit differing antigen presenting capabilities depending on the assay, it is still
possible that alterations in APC function may play a role in EAE resistance. To clarify this, it would be of interest to examine the gamma interferon induction of Ia on other APC in the LeR rat, such as astrocytes and macrophages.

Macrophages are thought to play a critical role in presentation of MBP in an immunogenic form to initiator lymphocytes of guinea pig origin (Ben-Nun et al., 1981a). If macrophages (or macrophage-derived factors) must interact directly with antigen-stimulated T cells in order to provide the second signal necessary for effector cell activation, the reaction becomes dependent on the number of macrophages available. Since the rat spleen is known to contain a suppressive macrophage population (Welch et al., 1978), the possibility existed that the LeR rat simply has a greater proportion of such macrophages than does the Lewis rat. Depletion of splenic adherent cells did not affect LeR spleen cell-mediated resistance suggesting that LeR macrophages did not play an important role in resistance to EAE. It is nevertheless conceivable that macrophage-derived factors and/or the process of irradiation could activate resident host-derived macrophages which are radiation resistant. These cells could present MBP to T cells and the eventual induction of EAE would be dependent on the host splenic macrophage or dendritic cell element. Our data are inconsistent with this hypothesis since, first, susceptibility to EAE induction before and after adherent cell removal still correlates with the origin of the spleen cell component and not the recipient strain and second, LeR rats can be made susceptible after transplanting them with Le BM.
The possibility exists that in the LeR rat, there are in vivo influences which affect the outcome of an MBP-stimulated immune response which are not detectable in vitro. In examination of this question, an in vivo correlate of lymphocyte reactivity was examined: the MBP-specific DTH response. LeR delayed hypersensitivity responses were not significantly different from Lewis DTH responses, implying no LeR defect in vivo in antigen recognition, processing or lymphocyte trafficking into the ear.

Further examination of LeR resistance had to include the possibility that a defect in lymphokine secretion, specifically IL-2, might be mediating resistance. It has been reported by Mostarica-Stojkovic et al. (1982) that AO rats, but not DA rats, are resistant to EAE induction. The AO rat strain has been reported to have a defect in its ability to produce IL-2 following Con A stimulation of spleen cells (Mostarica-Stojkovic et al., 1985). The difference in IL-2 production was determined to be the consequence of differences in T cell subpopulations in the AO versus the susceptible DA rat strain (Lukic et al., 1987). This defect was investigated in the LeR rat by first, examining the amount of IL-2 secreted by Con A stimulated LeR spleen cells in comparison to Lewis spleen cells and second, by determining the frequency of antigen-reactive cells (i.e. lymphokine secreting cells) through limiting dilution analysis. Neither of these assays demonstrated significant differences in IL-2 secretion between Lewis and LeR lymphocytes. Thus, the resistance mechanism operative in the LeR rat does not appear to be a decreased ability to secrete IL-2, as is proposed for the AO rat strain.
In examining suppression in EAE, it has been proposed that resistance or low
susceptibility to EAE in some animals is caused by the induction of clones of
suppressor T cells after challenge with MBP. Ben-Nun and Cohen (1982b) proposed
that the secondary resistance which develops after recovery from acute EAE in
susceptible rats is correlated with the appearance of specific suppressor lymphocytes
in the spleen and thymus. Much of the evidence in the present study points to a
suppressor cell mechanism in mediating LeR resistance to EAE. BMC and spleen cell
mixing experiments in radiation chimeras suggested that a suppressive cell population
in LeR rats was overriding Lewis susceptibility. Probing this further, direct spleen cell
transfers clearly demonstrated a transferrable component in the LeR spleen that
suppressed induction of EAE in Lewis recipients. In addition, depletion of phenotypic
suppressor cells from LeR splenocytes caused an increase in EAE in Lewis recipients,
strongly implicating OX8+ suppressor cells in EAE resistance. Lewis splenocytes
transferred to LeR recipients did not result in a fully susceptible animal, which
suggests that host-derived suppressor cells were active in these naive non-irradiated
recipients. Previous results, specifically the susceptibility to EAE observed in Lewis →
LeR BMC chimeras, suggests that these host-derived suppressor cells are radiation
sensitive.

The induction of suppressor clones may be inhibited or delayed with low
doses of cyclophosphamide (CY) (L'age et al., 1978), a phosphoric acid ester diamide
of nitrogen mustard (Paterson, 1976). Källen et al. (1986) demonstrated that the
timing of CY treatment is also important since CY treatment 2 days before
immunization - but not 4 days before, nor 4 or 8 days after immunization - appeared
to increase susceptibility for EAE in Fisher (F) and PVG rats. No effects of CY treatment on the EAE susceptibility of BN rats or on (BN x F)F1 hybrids were found (Källen et al., 1986). EAE in the resistant Lew/Mol substrain also could not be potentiated with a similar dose (40 mg/kg) of CY (Källen and Logdberg, 1982). These results are consistent with those published for mice (Teitelbaum et al., 1978) with some strains becoming susceptible after CY treatment while others remain unaffected. OX8+ spleen suppressor cells in rats have been reported to produce a T cell derived factor inhibitory for DNA synthesis (Chiba et al., 1985). In vivo pretreatment with 50 mg/kg CY has been observed to abolish the production of this factor, supporting the interpretation of Källen et al. (1986) that CY may regulate functionally important OX8+ spleen suppressor cells in the rat.

One possible explanation for the lack of a CY effect on BN and (BN x F)F1 hybrids is that strains lacking immune response genes for EAE do not react to CY because they are unable to mount an immune response to MBP (Teitelbaum et al., 1978). It is also possible that these rats demonstrate a CY insensitive suppressor mechanism. That could explain why the (BN x F)F1 hybrids, which show some sensitivity to EAE, cannot be made further susceptible with CY pretreatment, in spite of the presence of immune response genes and CY-sensitive suppressor mechanisms from the F strain. Studies in man and mice (Klajman et al., 1984; Diamantstein et al., 1981) have shown the existence of both CY sensitive and CY insensitive T suppressor cells. Thus, our observations that low dose CY treatment in the LeR rat does not abrogate EAE resistance is not a finding totally without precedent. Perhaps the
suppressor cells mediating LeR resistance have different characteristics than the conventional CY-sensitive cells.

Suppressor cells of several types have been described previously. In developing hematopoietic tissue in mice, the presence of veto cells has been described (Miller, 1980; Muraoka and Miller, 1980). Such cells inhibit the activity of T cells reacting to antigen on their surface, effectively "vetoing" such activity. Thus, veto cells are potential candidate suppressor cells in LeR resistance to EAE.

Nonspecific suppression mediated by cells termed "natural suppressor" cells has been observed by Sykes et al. (1988) in mixed allogeneic chimeras in mice. They showed that cells mediating this suppression were not classical T lymphocytes, do not express surface la antigen, cannot be removed by treatment with anti-asialo GM₁ serum and C (therefore, are not LAK or NK cells), nor by adherence to G10 columns. These cells were, however, eliminated by irradiation. Similar suppressor cells have been described in other systems. Holda et al. (1985) and Maier et al. (1985) have developed a murine model for chronic graft versus host (GVH) reaction between donor (B10.D2) and recipient (Balb/c) mice that are identical at their H-2 and mls loci and are non-reactive in a mixed leukocyte reaction, but they differ at minor histocompatibility antigens. These authors found that mice undergoing chronic GVH disease across minor histocompatibility barriers have suppressor cells within their spleens. These natural suppressor (NS) cells inhibit the proliferation of lymphocytes, as tested by their ability to suppress the response of normal spleen cells to mitogen stimulation (Maier et al., 1985). However, these suppressor cells require T cell signals.
for their activation and for their ability to suppress. Thus, the gradual depletion of cells from the spleens of GVH mice may be associated with the activity of NS cells on the resident population of the spleen. As cells respond and react to various antigens, the NS might inhibit their proliferation. Therefore, over time fewer and fewer cells would survive.

Cells with the functional and phenotypic characteristics of natural suppressors have been found in a number of locations and after a variety of procedures. These cells bear the "null" phenotype, are not MHC-restricted in their suppressive activity and resemble large granular lymphocytes. In general they are not cytolytic and do not kill conventional NK targets (Holda et al., 1985). NS cells are found in normal hematopoietic tissue such as adult bone marrow (Dorschkind and Rosse, 1982) or in fetal or neonatal hematopoietic tissue (Weigensberg et al., 1984). NS cells are particularly prominent during hematopoietic tissue regeneration such as is seen after total lymphoid irradiation (Strober, 1984) or following injury by cyclophosphamide (Sege et al., 1985).

Natural suppressor cells appear to act by an antiproliferative mechanism, and although they do not show MHC restriction, a certain degree of specificity can be superimposed on this system (Holda et al., 1985). For example, if NS activity requires T cell signals one could see how T cells specifically responding to an antigen might generate enough T cell signals to stimulate neighboring NS cells to down-regulate this T cell response. Such a mechanism might be artificially manipulated to induce antigen-specific tolerance. It is possible that NS cells have a physiologic role in
elimination of autoreactive clones. Autoreactive T cell clones, when activated by their appropriate autoantigen in areas rich in NS cells (hematopoietic tissue) would be prevented from proliferating by those NS cells. According to this theory such autoreactive T cells may either be functionally eliminated or suppressed and unable to undergo clonal expansion. Thus, from our observations of suppression in the LeR system, "natural suppressor"-like cells are potential candidate suppressor cells in LeR resistance to EAE.

The adoptive transfer of paralytic EAE into LeR rats by sensitized Le leukocytes (Waxman et al., 1981, and Table 15) is striking in view of previously established genetic restrictions which preclude the transfer of EAE from Le into allogeneic or even MHC-compatible rat strains (Hinrichs et al., 1981). In the reciprocal transfer, Le rats did not develop EAE after adoptive transfer of LeR cells. One could speculate that this represents a one-way allogeneic recognition of LeR cells by Le rats. However, our results suggest that Le and LeR leukocytes are mutually non-reactive in one-way mixed lymphocyte cultures (Figure 1) and by the PLNA (Figure 2). These results suggested that Le and LeR rats are syngeneic at the MHC, however, minor histocompatibility antigen differences are not demonstrable with the MLC (Maier et al., 1985). Thus, one explanation for lack of any observed EAE in LeR → Lewis animals may be due to a one way recognition of LeR minor histocompatibility antigens by Lewis cells and subsequent clearance of LeR effector cells. Effects of these minor histocompatibility antigens may be observable in the LDA assay shown in the top half of Table 16, where higher frequencies of antigen-reactive cells were observed in every Lewis LNC/LeR APC combination compared to Lewis/Lewis groups. This suggests
Lewis LNC recognize not only antigen but also minor histocompatibility antigens on LeR APC, thus giving higher frequencies of reactive cells. The reciprocal recognition of Lewis minor histocompatibility antigens by LeR LNC does not appear to be present in the bottom half of Table 16. Perhaps here, LeR LNC recognize different portions of the MBP molecule on LeR APC, but Lewis APC do not bind optimally to these different MBP epitopes. Thus, the resistance to EAE induction seen in LeR rats may be a result of a deficit in differentiation of antigen-reactive cells to effector precursor cells in vivo and/or the education of such effector precursor cells during the in vitro culture period. Our finding of a lack of EAE transfer in LeR → Le combinations is in agreement with the earlier observations of Waxman et al. (1981). They reported that LeR spleen cells, activated in vitro with Con A, were unable to transfer EAE to Lewis rats, but, LeR rats were perfectly capable of developing EAE when given preformed Lewis effector cells. It should be noted that a different culture stimulant and source of lymphoid cells were used in their studies.

In vitro activation of LNC and passive transfer of EAE demonstrated similar results to those of Waxman et al. (1981) in which LeR in vitro activated cells were unable to passively transfer EAE to Lewis recipients. The unexpected finding of EAE in the LeR → LeR transfer perhaps could be related to the observations of Ben-Nun, Eisenstein and Cohen (1982). In this report, MBP-specific T cell lines were developed from EAE susceptible rats, and from EAE resistant PVG and BN rats. The BN line failed to recognize the EF of MBP and did not mediate EAE, however, the PVG line was able to induce EAE in PVG recipients. Thus, the EAE resistant Ir phenotype of PVG rats cannot be attributed to a failure of their APC to present the disease-inducing
portion of MBP (Ben-Nun et al., 1981a) or to a failure of their T cells to recognize the EF of MBP, but may be explained by reversible inhibitory or suppressive mechanisms (Cohen and Wekerle, 1973). In support of this idea, Ben-Nun and Cohen (1982b) reported that suppressed EAE effector T lymphocytes can be rescued as cell lines from Lewis rats that have recovered from active EAE and have acquired resistance to reinduction of active disease. An alternative explanation for LeR → LeR passive EAE transfer might relate to the findings of Driscoll et al. (1985). These authors observed that the inability of LeR lymphoid cells to transfer EAE after culture with MBP could be overcome by incorporating lipopolysaccharide (LPS) in the MBP-culture medium or by simultaneous transfer of LPS-activated antigen non-specific spleen cells with the MBP sensitized cells. LPS is known to stimulate a wide range of immunologic activities (Morrison and Ryan, 1979), including stimulation of protein kinase C (Wightman and Raetz, 1984), enhancement of arachidonic acid metabolism in various cells such as astrocytes (Fontana et al., 1982), and induction of la molecules on Lewis rat astrocytes (Massa and ter Meulen, 1987a). In addition, no specific LPS-responding cells have been identified. A bacterial infection in the LeR recipients would have affected only transfer into LeR recipients and not Lewis recipients. If LeR rats display a defect in la expression similar to BN rats, then LPS treatment would cause enhancement of such antigen expression. On the other hand, our data indicates that LeR rats have a potent suppressor cell population which inhibits MBP-specific cells from initiating lesions in the CNS. LPS might act upon these suppressor cells, rendering them inefficient blockers of CNS T-cell autoreactivity.
The finding that LeR resistance appears to be mediated by suppressor cells is very interesting, since decreased T suppressor cell numbers and suppressor function have been implicated as determining factors in immune dysregulation in MS (Antel et al., 1978; 1979; Gonzalez et al., 1978). Antel et al. (1979) found that suppressor cell activity fell to low and sometimes undetectable levels during exacerbations of MS. As an exacerbation ended, suppressor activity rebounded to high or normal levels. Subnormal T suppressor function is most apparent in those patients with active (i.e. progressive or relapsing) disease (Antel et al., 1979; Huddlestone and Oldstone, 1979). Using monoclonal antibodies which recognize mononuclear cell surface proteins, the most consistently demonstrated changes have been found in the number of T suppressor cells (T8+, T5+, Leu 2a+) (Reinherz et al., 1980; Hauser et al., 1983). In addition, some reports indicate that the density of T8 antigen is reduced in MS patients with active disease (Reder et al., 1983; 1984a; 1984b).

There are three mechanisms that have been proposed to explain the decrease of T8 cells in the peripheral blood of MS patients. The first mechanism proposes that lymphocytotoxic antibodies directed against isolated T8 cells cause their destruction. Although some lymphocytotoxic antibodies do exist in MS patients (Schocket et al., 1977), Hauser et al. (1983) were unable to demonstrate antibody-mediated destruction of T cells in MS patients. A second possible mechanism relates to modulation of surface markers in vivo (Oger et al., 1982). Weiner et al. (1984) carried out several investigations looking for re-expression of the T8 marker following culture of T cells in vitro from patients with decreased T8 cells in the peripheral blood. This group was unable to demonstrate consistent re-expression of the T8 marker. The last
mechanism involves sequestration of cells. Specifically, changes in T4:T8 ratios in peripheral blood may represent the migration of T8 cells from the peripheral blood into the CNS and perhaps into the lymphoid tissue also. This conclusion is based on the finding of increased or equal numbers of T8 cells compared to T4 cells in the brains of MS patients despite decreases of T8 cells in the peripheral blood (Weiner et al., 1984; Traugott et al., 1983; Booss et al., 1983). In a related study involving the murine model of EAE, a decrease in the Lyt 1 T cell subset was found in the blood of animals prior to the appearance of these cells in the brain (Hauser et al., 1984). It is known that Lyt 1 cells are required for adoptive transfer of murine EAE (Petinelli and McFarlin, 1981) and that Lyt 1 cells preferentially accumulate in the brain (Sriram et al., 1982). In the rat model of EAE, cells expressing the Lyt 1 analogue also preferentially accumulate in the spinal cord (Hickey et al., 1983).

Several questions remain to be answered concerning T cell changes in the peripheral blood of MS patients. If T8 cells migrate to the CNS, this does not imply that these cells mediate CNS damage or that enough cells migrate to the brain to account for the decrease in the blood. Clearly there may be more than one mechanism for T cell changes in the blood of MS patients. This may also hold true in EAE, where several possibilities exist for altered reactivity associated with suppressor T cells.

Here we propose four hypotheses to explain how suppression in LeR rats might prevent EAE. First, although most research is consistent with a cell-mediated immune mechanism in EAE, the role of soluble factors (i.e. lymphokines) has been
difficult to fully assess. In the LeR rat, we have shown that no apparent defect exists in IL-2 secretion. However, it should be noted that the IL-2 dependent cell line, CTLL-20, is also responsive to IL-4. Perhaps the observed suppression by LeR spleen cells relates to soluble factors. One possibility involves over-secretion of a lymphokine (perhaps gamma interferon or IL-4). These mediators might cause the LeR immune system to become non-specifically activated and thus, refractory to antigen-specific responses. Alternatively, hypersecretion of immune mediators might suppress the immune system by over-stimulation and subsequent down-regulation of surface molecules and/or antigen receptors. The heightened stimulation observed in LDA assays in the presence of LeR APC may in fact represent this proposed hypersecretion of cellular mediators.

Second, it has been proposed that immunoregulatory products of activated suppressor T cells could reverse or prevent disease, because they diminish immune responses to MBP by other T cell subpopulations, with or without the participation of antibody-producing B cells. Perhaps LeR suppressor cells are secreting a potent suppressor factor that acts on precursor cells in vivo and prevents the education of such effector cells into disease-inducing cells.

Third, an alternative explanation for LeR suppression might involve large numbers of suppressor cells that are not demonstrable in the lymph nodes or spleen at day 11 post-sensitization, since our monoclonal antibody profiles at that timepoint did not show any differences between Le and LeR spleen cells or LNC. Perhaps these suppressor cells migrate to the spinal cord during this crucial period, as
suggested by Hickey et al. (1983), or to other locations in the CNS, where they can suppress EAE.

A fourth explanation for LeR suppression of EAE may relate to recognition of different portions of the MBP molecule. It is possible that LeR lymphocytes recognize and respond to a non-encephalitogenic sequence of amino acids within the MBP molecule, as has been suggested for the BN rat. This would explain the observations of normal in vitro reactivity but poor disease causing ability by LeR lymphocytes.

In summary, it appears that LeR resistance is indeed bone marrow derived and therefore immunologic. The cell mediating resistance is localized to the spleen, however, it did not appear to be a macrophage. Several experiments indicated that this splenic cell component mediating resistance was a suppressor cell that was not sensitive to CY treatment. This suggested the presence of a different type of cell that might be classified as a "natural suppressor" cell or a "veto" cell. Resistance mechanisms operative in other EAE resistant strains, such as defective in vitro MBP-specific lymphocyte proliferative responses, lack of in vivo MBP-specific delayed hypersensitivity, defective IL-2 secretion, altered T cell subpopulations, and decreased Ia expression on APC were eliminated as possible mechanisms in LeR resistance. Thus, this study was designed to analyze the immune mechanisms of LeR resistance so as to provide useful information on the cell interactions required for the induction of autoimmunity. In relating these findings to human CNS autoimmune diseases, it might be hypothesized that LeR rats, with their ability to suppress EAE effector cells, are analogous to individuals that are not susceptible to CNS autoimmune disease due
to intact, functioning mechanisms that prevent or eliminate CNS autoreactive T lymphocytes. Lewis rats, on the other hand, are not able to destroy or prevent autoreactive T cell clones, and might be analogous to an MS patient, since both are genetically and functionally susceptible to disease.

Further studies into the mechanism involved in LeR resistance might include examining the relative capacity of Lewis versus LeR astrocytes and peritoneal exudate cells to express MHC class II antigens after gamma interferon induction. It would be interesting to examine secretion of other cellular mediators by LeR cells, such as gamma interferon or IL-4. Interleukin 4 effects could be differentiated from IL-2 effects in IL-2 bioassays by blocking IL-2 receptors on CTLL-20 cells (with anti-IL-2 receptor). Thus, any remaining significant proliferation of CTLL-20 cells would be due to IL-4. Further studies might examine LeR anti-MBP antibody levels in serum and saliva and comparison of these to antibody levels in Lewis rats to determine whether antibody plays a role in LeR resistance. Additional experiments could examine the effects of splenectomy or thymectomy on induction of EAE in LeR rats. Alternatively, spleen and thymus irradiation shielding experiments could be performed to examine radio-resistant versus susceptible cell populations in these organs, and their role in EAE resistance. It would be interesting to examine LeR reactivity to MBP fragments (both encephalitogenic and non-encephalitogenic) and to determine whether LeR lymphocytes develop specificity for a particular amino acid sequence.

Our data indicate an important role for suppressor cells in LeR resistance. Therapeutic approaches applicable to MS could include isolating and using specific
suppressor cell-derived factors or determining ways to differentially activate suppressor cells that are deficient in MS patients. It seems plausible that understanding the mechanisms involved in resistance to EAE in the LeR rat may help increase our knowledge of MS and open new avenues of research into the disease mechanisms occurring in MS.
REFERENCES


