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In Vitro Generation of T Cell-Reactive T Cells in Experimental Allergic Encephalomyelitis

New Schreiber Costa

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IN VITRO GENERATION OF T CELL-REACTIVE T CELLS IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is an acute neurological autoimmune disease induced in a variety of species by injection of brain or spinal cord tissue in complete Freund's adjuvant (Patterson 1971, 1977). The active encephalitogenic component in central nervous system (CNS) tissue has been identified as the basic protein of myelin (MBP) (Einstein et al., 1962). Different amino acid sequences of a given MBP molecule are recognized as the major encephalitogenic determinant in various species, with pronounced species variability (Chao and Einstein, 1970; Shapira et al., 1971; Chou et al., 1977; Kibler et al., 1977; Pettinelli et al., 1982; Fritz et al., 1983). Using different techniques, at least eight distinct antigenic determinants for delayed type hypersensitivity and three for antibody production were shown in the protein, but they do not correlate with the regions responsible for the encephalitogenicity (Driscoll et al., 1974).

EAE is characterized by a variety of clinical manifestations, mainly paralysis which culminates sometimes in death, and with histopathologic changes consisting of focal areas of intense perivascular cellular infiltration, consisting mainly of T lymphocytes (helper/inducer subset) (Pettinelli and MacFarlin, 1981; Sriram et al., 1982;
Dialynas et al., 1983; Lando and Ben-Nun, 1984; Wekerle, 1984; Trotter et al., 1985). Although controversy exists concerning the role of antibodies in the pathogenesis of EAE, there is convincing evidence for the involvement of cell-mediated immune reaction towards MBP that parallels the induction of the disease. The most important clue is the transfer of clinical disease by MBP sensitive T cells. In both rats and mice it has been shown that the T cell population is responsible for this function (Ortiz and Weigle, 1976).

In view of the immunological nature of EAE, attempts have been made in several laboratories to prevent, reverse or suppress the disease in animals challenged with MBP. This has been attempt by desensitization procedures using the specific antigens relevant to the system (Arnon and Teitelbaum, 1980); by using monoclonal antibodies to the L3T4 (helper cell) surface marker (Waldor et al., 1985) and by using immunosuppressive treatment with cyclosporine A (Ryfell et al., 1982; Bolton et al., 1982; Fredane et al., 1983; Hinrich et al., 1983; Borel et al., 1986; Shuller-Levis et al., 1986; Ellerman et al., 1988).

However, there is a lack of information regarding immunospecific therapy and immunoregulation in murine EAE dealing with suppressor cell control of the autoaggressive lymphocyte clones. Furthermore, establishment of suppressor T cell lines could prove to be a suitable therapeutic approach.
Therefore, the present study attempts to generate in vitro T cell clones or lines specific for MBP-specific T cell and to examine the ability of these T anti-T cell clones or lines to inhibit the passive transfer of disease by MBP-specific T clones or lines.
REVIEW OF THE LITERATURE

I. Historical

In 1885, Pasteur introduced his vaccine for prevention of rabies (Patterson, 1977). He stated that the vaccine consisted of brain infected with a strain of rabies virus that had been attenuated or fixed by repeated passage in rabbits. Patients bitten by dogs suspected of having rabies received repeated injections of the vaccine in efforts to induce active immunity to virulent or "street" rabies virus. Acute neurological disease developed in occasional subjects receiving a course of vaccine prophylaxis. By 1888, it was clear that these "neuroparalytic complications" represented an acute inflammatory process of the CNS, i.e., encephalitis, myelitis, or encephalomyelitis. Increasing suspicion during the 1920's that post-rabies vaccinal encephalomyelitis was an allergic reaction to nervous tissue antigens accelerated studies in experimental animals related to the immunogenic potential of mammalian tissues. By 1928, Witebsky and Steinfeld showed that the brain tissue contained specific antigenic constituents which stimulated antibodies specifically reactive with CNS tissue.
It remained for Rivers et al. (1933 and 1935) to discover that an acute disseminated encephalomyelitis could be produced in monkeys receiving repeated injection of nervous tissues extracts or emulsions over a period of many weeks or months which resembled post-rabies vaccinal encephalomyelitis. This finding was confirmed by Ferraro and Jervis (1940) who introduced rabbit brain into monkeys 29 to 103 times over a period of 112 to 405 days. The animals first showed neurological signs after 3 to 13 months. Ferraro (1944) concluded that the reaction in the nervous system was of an allergic nature—a view which found active support so that the experimental disease was called "allergic" (or isoallergic) encephalomyelitis.

Another advance was gained by the addition to brain tissue of what was known as the "Freund adjuvant" for the purpose of bringing about experimental disease more rapidly and regularly. Freund and McDermott (1942) demonstrated that a mixture of lanolin-like substances, paraffin oil, and killed tubercle bacilli, induced a prompt and high antibody response when added to a wide variety of antigens. Thus, in 1947 Freund et al.; Morgan; Kabat et al. and Morrison simultaneously reported that a single injection of nervous tissue emulsified in complete Freund's adjuvant (CFA), (containing mycobacteria), regularly induced an accelerated and extremely severe form of disseminated encephalomyelitis in guinea pigs, monkeys, and rabbits within a matter of 2 or 3 weeks.
The experimental production of neurological signs and lesions in different animals, however, was unpredictable. Later, Olitsky and Yager (1949) showed however that mice, when immunized with CNS tissue incorporated with a modified adjuvant, were ill sooner with fewer exposures than the other species of animals.

II. The Role of Myelin Basic Protein Reactive T Cells

There was ample evidence that induction of EAE by the injections of CNS tissue results in the generation of both antibodies and cell-mediated immunity. However, controversy existed concerning the role of antibodies in the pathogenesis of EAE. Thomas et al. (1950); Lumsden et al. (1950) found a poor correlation with titers of circulating brain antibodies. Patterson (1977) was not able to transfer the disease to normal animals with immune serum but it was found to be possible to transfer the disease by one inoculum of sensitized lymphoid cells.

Arnason et al. (1962) showed that neonatally thymectomized rats had a markedly reduced capacity to develop EAE. This finding was difficult to interpret because such animals also were compromised with respect to production of antibody. A single report by Blaw et al. (1967) that EAE occurred in chickens rendered hypo or aggamaglobulinemic by prior extirpation of the bursa of Fabricius was difficult to interpret because of the small number of birds that were truly aggamaglobulinemic.
Gonatas and Howard (1974) were the first to secure definitive evidence that T cells were a prerequisite for development of EAE in rats. Adult Lewis rats were thymectomized, subjected to total body irradiation, and reconstituted with bone marrow cells from thymectomized, thoracic duct-drained donors, thereby assuring that no T cells were represented in the bone marrow suspension. Such rats were totally lacking in their capacity to develop EAE following sensitization to guinea pig spinal cord or MBP-CFA. Absence of MBP-binding antibody indicated that the rats were deficient in T cells collaborating with antibody-producing B cells. The absence of helper T cells provided indirect evidence that the rats were truly depleted of all T cell subpopulations but left the question of whether antibody might have a role in the disease.

The studies of Ortiz-Ortiz and Weigle (1976) and Ortiz 'et al. (1976) have provided compelling evidence that a specific T cell sub-population causes EAE. They found that EAE could not be induced in thymectomized rats, but that grafting of thymus cells restored susceptibility to EAE following priming with MBP. However, they were unable to transfer clinical EAE after depleting T cells with anti-T cell antiserum, although they were able to induce anti-MBP antibodies. EAE could be transferred to T cell deficient nude rats by injection with immune cells from syngeneic EAE donor rats (Hinrichs and Humphries, 1983).
Finally, EAE has been induced by the transfer of encephalitogenic T cell lines and clones (Ben-Nun et al., 1981; Ben-Nun and Lando, 1983; Trotter et al., 1985).

Results of several studies using immunohistologic analyses of the infiltrating cells in acute EAE, had established that the predominant cell of the early acute EAE lesion was the T lymphocyte. Furthermore, the predominant T cell population belonged to the helper/inducer subset which was characterized by the W3/25 marker in the rat (Wekerle, 1984), and by Lyt-1$^+$2$^-$ and L3T4 determinants in the mouse (Pettinelli and MacFarlin, 1981; Sriram et al., 1982; Dialynas et al., 1983; Lando and Ben-Nun, 1984; Trotter et al., 1985). However, cytotoxic/suppressor (or precursor) Lyt-2$^+$ T cells as well as B cells were present at lower frequencies (10% and 12% respectively) (Sriram et al., 1982). B cells and macrophages appeared at later stage, 10 days post-inoculation with bovine white matter and CFA, and were restricted to the meninges and perivascular spaces. On the other hand, T cells were demonstrable as early as 5 days post-inoculation, before the onset of clinical signs and were predominantly found within the CNS parenchyma of guinea-pigs (Traugott et al., 1982).

A neuropathological study of EAE (MacKay et al., 1973) emphasized the many similarities between the histopathological hallmarks of the experimental animal disease and those characterizing multiple sclerosis (MS)
in man. This was especially true in those forms of the human disease that were characterized by an acute or subacute course leading to death in a comparatively short period of time. However, chronic and relapsing forms of EAE also had similarities to MS (Wisniewski et al., 1977, Raine et al., 1974). Mocktarian et al. (1984) reported that a single transfer of MBP-sensitized lymph node cells or T cells, in the absence of a peripheral antigen depot, led to both acute EAE with significant primary demyelination, and chronic relapsing disease with the lesions typical of demyelination over a long period. Zamvil et al. (1985) generated MBP-specific T-cell clones restricted to class II (Ia) antigens of the major histocompatibility complex (MHC) from PL/J and (PL/SJL)F₁ mice following sensitization to rat MBP. Two such IaU-restricted T-cell clones that proliferated in response to the encephalitogenic N-terminal MBP peptide and recognized a shared determinant with mouse (self) MBP caused paralysis in 100% of (PL/SJL)F₁ mice tested. Relapsing paralysis followed in two-thirds of the recipients after recovery from acute paralysis, whereas one third developed chronic persistent paralysis. These findings had major implications for the immunological mechanisms involved in experimental and human demyelinating diseases, reinforcing the view that EAE was a meaningful laboratory model for MS.
In summary, the autoimmune nature of EAE can be established by several criteria: 1) the tissue damage which followed the injection of the CNS material was restricted to this tissue; and 2) sensitized lymphocytes were capable of transferring the disease to normal recipients.

III. Induction of EAE

EAE can be induced in a two different ways: by active induction and by passive induction.

a) Active induction

This is accomplished in a variety of species by injection of CNS antigens, mainly MBP (Levine and Wenk, 1961; Patterson and Bell, 1962; and Laatsch et al., 1962). The induction leads to a severe inflammation of the CNS white matter within 10 to 15 days (Levine and Wenk, 1961; Patterson and Bell, 1962).

b) Passive induction

This is accomplished by transfer of encephalitogenic MBP-reactive T-cell lines or clones into naive mice or rats (Ben-Nun et al., 1981 and 1983; Naparstek et al., 1983; and Pettinelli and McFarlin, 1981). Here, the inflammation of the CNS white matter usually occurs within 5 to 8 days.
IV. Studies of Myelin Basic Protein

The discovery that MBP was responsible for a major portion of the encephalitogenic activity of CNS tissue (Einstein et al., 1962) opened the doors for a large amount of research on this antigenic constituent.

MBP represents approximately one-third of the total protein of myelin and about 1% of the weight of the whole nervous tissue. It is extracted from delipidated mammalian brain or spinal cord at acid pH ranges of 1.0-2.5, and can be purified by various combinations of resin-gel chromatography (Rauch and Einstein, 1974). Even the most "pure" MBP preparations, however, reveal varying degrees of microheterogeneity (Chou et al., 1976). This microheterogeneity may well account for differences in specific activity of MBP preparations reported by different laboratories. MBP is a heat-stable, acid-resistant, relatively flat molecule with a molecular weight in the range of 18,000-22,000 daltons (Rauch and Einstein, 1974). Amino acid sequencing has revealed about 170 residues for all MBP preparations derived from nervous tissue of all mammalian species, except in rats. Rat MBP has in addition to a 170-residue molecule, a smaller molecular weight species MBP lacking a 40-amino acid sequence comprising the carboxyl terminal position of the parent molecule (Matersen et al., 1972).

The myelin basic protein molecule has been characterized by using limited pepsin digestion. This cleaves
the MBP into 3 fragments, consisting of amino acid 1 to 37, 38 to 87 and 88 to 170 (Chou et al., 1977). It has been found that the ability of the fragments to induce EAE differs among species and strains within a species. For example, in the rat, the encephalitogenic determinant is located within the peptide encompassed by residues 68-88 (Chou et al., 1977 and Kibler et al., 1977). Pettinelli et al. (1982) has shown both by direct immunization and by passive transfer experiments that a major encephalitogenic determinant for the SJL/J mouse is located in the carboxy terminal end at 89-170 of guinea pig myelin basic protein (GPMBP). However, in subsequent studies (Fritz et al., 1983) it was found that in the PL/J mouse strain, the encephalitogenic determinant is situated within the amino terminal end (residues 1-37) of GPMBP. When intact GPMBP was used, both PL/J and SJL/J mice developed EAE. (SJL/PL)F₁ mice developed EAE with intact GPBP.

V. Genetic Studies of EAE

It has been established that immune responses to many antigens may be regulated by distinct immune response (Ir) genes. Many such Ir genes are associated with the major histocompatibility complex (MHC) of the particular species and map within the I region. In addition, Ir genes not linked to the MHC were also demonstrated as influencing the immune response to a variety of antigens (Gasser and Silvers, 1974; Benacerraf
and Katz, 1975). Thus, the genetics of the immune response is complicated and may involve several genes and cooperation between genes (Dorf et al. 1974), and may also vary between species (Gunther et al., 1976). Moreover, the immune response to different determinants on the same molecule may be under different genetic control (Maron et al., 1973). Genetic differences in immune response may also play an important role in susceptibility to a variety of diseases, including autoimmune diseases, in both animals and man. This was demonstrated also for EAE in several species.

Genetic studies of EAE in rats demonstrated that the genetic control was not identical in all strain combinations. In one case (BN X Lewis), it was controlled by a single autosomal gene which, although closely linked to the MHC, was rather distinct from it (Williams and Moore, 1973, Gasser et al., 1973). In the other strain combinations such as (BN X DA), it was completely independent of the MHC (Gasser et al., 1975). Williams and Moore (1973) concluded that an autosomal dominant gene linked to the histocompatibility locus determined susceptibility to EAE by acting as a immune response (Ir) gene. Studies in mice yielded controversial results concerning the role of the H-2 complex in susceptibility to EAE. In the mouse some authors have shown linkage to the H-2$^S$ and H-2$^D$ haplotype (Bernard, 1976 and Raine et al., 1980), whereas others have been unable to confirm
these observations (Levine and Sowinski, 1974; Montgomery and Rauch, 1982; Lando et al., 1979).

Arnon, 1981, studied the susceptibility to EAE in various mouse strains and in their F₁ hybrids with the reportedly susceptible strain SJL/J (Lando et al., 1979). The results showed in the SJL/J strain 48% developed clinical incidence of the disease and all mice showed histological changes in the brain, but the F₁ hybrid of SJL/J with the resistant strain BALB/c was found to be much more sensitive to EAE than the parent strain SJL/J. Crossing SJL/J with the congenic strains of BALB/c (BALB/C₃H and BALB.B10), which were of the same genetic background but differ in their H-2 complex, resulted in F₁ hybrids with high sensitivity to EAE. Similarly, the F₁ hybrid of SJL/J X NZB was very sensitive as manifested both in severity and incidence of clinical and histological disease. On the other hand, crossing SJL/J with DBA/2 which is H-2d as is BALB/c and NZB, or with C57BL/6J which is H-2b as BALB.B10, resulted in hybrids which were less sensitive to EAE than the parental strain SJL/J.

These results demonstrated that there was no pure dominant Mendelian inheritance of susceptibility to EAE mice.

To investigate the possible linkage of EAE susceptibility to the H-2S haplotype, Arnon (1981) tried to induce the disease in two strains that possessed the H-2S
haplotype (ASW and B10S, as well as in their F1 hybrids with BALB/c). In all these strain combinations he failed to induce the disease. These results were compatible with those obtained by Levine and Sowinsky (1974) but differed, however, from the results of Bernard (1976). Arnon's data definitely indicated that the susceptibility to EAE was not dependent solely on the H-2S.

Therefore, it can be summarized that there are at least two categories of susceptibility/resistance genes playing a role in EAE (Gunther et al., 1978, and Gasser et al., 1975). One is related to the MHC and the other genes is unrelated to the MHC (Kallen and Logdberg, 1982; Levine and Sowinski, 1974). Linthicum and Frelinger (1982) identified one of these non-MHC genes as being a histamine sensitization gene in the mouse.

VI. Immunomodulation of EAE

Resistance to EAE not only appears to be a genetically determinant trait, but also can be acquired in genetically EAE-susceptible rats. Susceptible Lewis rats, which had spontaneously recovered from acute EAE, become resistant to EAE induction by a later second injection of MBP in complete Freund's adjuvant (CFA). Injection of myelin basic protein in incomplete Freund's adjuvant (IFA), which does not induce EAE, renders the recipient resistant to subsequent encephalitogenic treatments, and even pretreatment with CFA without MBP has a protective
effect (Kies and Alvord, 1958; Svet-Moldavsky et al., 1959). Adda et al. (1977) found that resistance to EAE could be transferred to naive recipients by lymphoid cells from recovered or resistant rats. Arnon (1981) and Welch et al. (1980) suggested that suppressor T cells, at least in rats, were the key cells in self limitation of and subsequent resistance to EAE.

There is significantly less known about the mechanisms of resistance in the mouse than in the rat. Lando et al. (1980) showed that hybridization between some EAE-resistant mouse strains such as DBA/2 or CB57BL/6J and the sensitive SJL/J resulted in crosses which exhibited much less sensitivity than the parental strain. This was indicative of a suppressive effect of the resistant parent. To support that statement, Lando et al. (1979), tested the effect of cyclophosphamide (CY) 2 days before induction of disease in the susceptible mice SJL/J and (SJL/J X BALB/c)F₁, and no differences were observed in the incidence or severity of EAE. On the other hand, CY had a marked effect in a proportion of resistant strains (Lando et al., 1980). The most dramatic effect was in BALB/c mice, which after treatment with CY succumbed to the disease to the same extent and with the same severity as the susceptible SJL/J mice. However, in other resistant strain such as DBA/2, CY had no effect on disease induction. The dose of CY used was reported to
eliminate specifically a population of T suppressor cells in other systems (Chiorazzi et al., 1977).

An effect comparable to that exerted by the cyclophosphamide could be obtained by other immunosuppressive treatments, such as low dose irradiation (Chiorazzi et al., 1976). Arnon (1981) observed that sublethal irradiation with 350R, 2 days before the encephalitogenic challenge, brought about development of EAE in 50% of the BALB/c mice, which were otherwise resistant to the disease. It can be concluded that CY or low dose irradiation can convert one type of resistant strain into a susceptible strain, while these treatments have no effect on another type of resistant strain. It was suggested that in strains of the first type a gene that is responsible for susceptibility to EAE did exist but the natural high level of suppressor cells in these strains prevented the manifestation of disease. Following treatment with CY these suppressor cells were eliminated and disease was overt. Indeed, it was demonstrated earlier by Lage-Stehr and Diamanstein (1978) that CY can induce a transient appearance of "autoreactive" T lymphocytes in spleens of mice. In NZB mice it has been demonstrated that loss of effective suppressive T cell function can lead to autoimmune diseases (Talal 1976). This information support the conclusion that suppressor cells may play a paramount role in both protection and natural resistance to EAE.
The previous findings were of vital importance in planning strategies for treatment of EAE. Numerous attempts have been made to suppress the disease in animals challenged with MBP antigen or with MBP-specific T cell clones by desensitization procedures using the specific antigens relevant to the system (Arnon and Teitelbaum, 1980); by giving monoclonal antibodies to the MHC I region products (Steinman et al., 1981, and Sriram and Steinman, 1983) or by giving monoclonal antibodies to the L3T4 surface marker (Waldor et al., 1985) and by using immunosuppressive treatment with Cyclosporine A (Rifell et al., 1982; Bolton et al., 1982; Fredane et al., 1983; Hinrich et al., 1983; Borel et al., 1986; Schuller-Levis et al., 1986). It has been shown (Alvord et al., 1965; Einstein et al., 1968) that MBP, if given in high doses in incomplete Freund's adjuvant, was highly effective in preventing EAE in guinea pigs when administered before sensitization or in suppressing EAE if given after sensitization. However, not only the encephalitogenic protein was effective in treatment and prevention, but other non-encephalitogenic related peptides or other antigens had a similar protective effect. Thus, the course of EAE was found to be modified by myelin non-encephalitogenic basic proteins (Einstein et al., 1968), altered MBP (Swanborg, 1972; Einstein et al., 1972), and non-encephalitogenic degradation products (Swanborg, 1975) or synthetic fragments of MBP (Teitelbaum
et al., 1971; Teitelbaum et al., 1973; Teitelbaum et al., 1974; Webb et al., 1976; Hashim et al., 1976; Teitelbaum et al., 1977; Lando et al., 1979; Keith et al., 1979).

Reversal of EAE has been attempted in vivo in the mouse by using monoclonal antibodies to Ia antigens or to the L3T4 surface marker. Steinman et al. (1981) showed that in vivo administration of antibodies reactive with I-\textsuperscript{A}\textsuperscript{S} gene products prevented the induction of EAE in the SJL/J mouse when treatment was instituted prior to immunization with MBP. However EAE was delayed, but not prevented, when antibody was administered 5 days after the immunization. The suppressive effect of anti-I-A antibodies on EAE induction may work by blocking APC presentation of the encephalitogenic MBP determinant to T cells. Sriram and Steinman (1983) extended the same study in cases of acute and chronic relapsing EAE by instituting treatment at the onset of paralytic signs and then following the clinical course. In chronic relapsing EAE, animals treated with anti-I-A\textsuperscript{S} antibody had no mortality and fewer relapses when compared with control animals. Similarly, in the acute EAE model, animals treated with anti-I-A\textsuperscript{S} antibody showed a dramatic reversal of paralytic signs and a rapid recovery.

Waldor et al. (1985) gave mice monoclonal antibodies (GKI.5) that recognize the L3T4 marker present on helper T cells and were able to reduce the number of L3T4\textsuperscript{+} cells in the spleen and the lymph nodes. This treatment also con-
sequently prevented EAE when GKI.5 was administered before the onset and shortly after the first symptoms of EAE appeared. However, treatment of quadriplegic or moribund mice (advanced stage) did not ameliorate paralysis or prevent death. The efficacy of monoclonal antibody therapy in treating diseases may, however, be limited by the development by the host of anti-idiotypic antibodies directed towards the therapeutic monoclonal antibody. Consequently, the use of suppressor T cell clones rather than monoclonal antibodies, may be less susceptible to such regulation.

The fungal metabolite cyclosporine A (CSA), which is a very hydrophobic, cyclic endecapeptide (Petcher et al., 1976) is known to exert powerful immunosuppressive effects (Cyclosporine A, Intern. Symposium, 1981). CSA-induced immunosuppression is most effective on T-cell-mediated immune responses (Wiesinger and Borel, 1979). Although the mode of action of CSA is still under study, it has been shown to suppress interleukin-2 (IL-2) production (Thompson et al., 1983; Shidani et al., 1984; Reem et al., 1983) and appears to act selectively by inhibiting IL-2 mRNA (Granelli-Piperno et al., 1984).

CSA has been shown to suppress EAE in rats (Borel et al., 1986, Ryfell et al., 1982, Bolton et al., 1982), guinea pigs (Bolton et al., 1982, Fredane et al., 1983), and rhesus monkeys (Bolton, 1981) if given during the sensitization phase. In addition to this prophylactic
effect, CSA also has a strong therapeutic effect in rats when administration is started at the onset of the symptoms. The treated animals recovered more quickly than the controls, but there was often a relapse when treatment was discontinued. Similar results were obtained in the guinea pig (Fredane et al., 1983).

In the passive transfer of EAE in the rat, if the donors cells are activated in vitro with specific antigen or mitogen in the presence of CSA for 72-hour, proliferation of the donors cells is considerably suppressed, and both clinical and histological disease after passive transfer with such cells is decreased (Ryffel et al., 1982; Bolton et al., 1982; Hinrichs et al., 1983).

Recently, Ellerman et al. (1988) were able to isolate a distinct population of cells with suppressor activity by culturing T cell lines generated from Lewis rats immunized one month before with GP-MBP. These cell lines were grown with GP-MBP and cyclosporine A for 1-7 days and after 5 to 6 cycles of subsequent antigen stimulation, the cells produced mild clinical signs of disease, which decreased with subsequent cycles of antigen stimulation. Most importantly, adoptive transfer using an encephalitogenic T cell line was prevented by the co-transfer of this "suppressor line".

CSA has also been used to treat an induced acute attack after disease onset and remission in chronic relapsing EAE in SJL/J mice (Schuller-Levis et al., 1986).
CSA led to a dose-dependent decrease in the length and severity of attack and significantly lower mortality in the CSA-treated mice compared to the non-CSA-treated mice. In addition, there was decreased production of the lymphokine, lymphocyte-derived chemotactic factor (LDCF), in the cyclosporine treated mice.

Jerne (1974) has developed a "network" theory in which immune regulation results from lymphoid cell receptors recognizing determinants on the other lymphoid cell receptors. According to this hypothesis, it may be possible to generate regulatory suppressor or cytotoxic T cell clones against helper T cells. Lamb and Feldmann (1982) in humans, have shown the feasibility of generating suppressor T cell clones specific for a helper T lymphocyte which recognizes influenza A matrix protein in the absence of APC. Such an autologous suppressor cell clone recognizes and inhibits the activation of this helper T cell clone. Thus, situations in which there are undesirable clones of helper (or Killer) cells such as autoreactive MBP-specific T cell clones may be controllable by suppressor cells of this type, generated in vitro.

Mohagheghpour et al. (1986) showed in humans that CD8+ T cells proliferated upon exposure to autologous, antigen-primed CD4+ T cells, and suppressed the response of fresh T cells to the priming antigen but not to irrelevant antigens. The CD8+ cells inhibited the
response of fresh autologous T cells only to the original allogeneic stimulator cell and to stimulator cells that shared HLA-DR antigens with the priming cell.

Such T-T cell interactions have not been extensively studied in the mouse. Suzuki et al. (1986) showed that resting T cells could proliferate in response to T cell blasts, but were inhibited by anti-Ia antibodies. This inhibition was surprising in that: a) these T-T cell interactions could occur in the absence of APC, and b) murine T cells do not express Ia antigens. These authors postulated that perhaps the T cells were passively acquiring Ia antigens on their surface and that these Ia antigens were then serving as determinants being recognized either alone or in conjunction with other surface determinants.

Sun et al. (1988) isolated homogeneous T lymphocyte lines from spleens of Lewis rats that had recovered from T-cell-mediated EAE caused by the MBP-specific T cell line S1. These spleen-derived anti-S1 T cell lines expressed the CD8-determinant and proliferated against the S1 line. The anti-S1 lines were strictly specific for markers on S1 line cells and were not reactive to MBP. In addition, the anti-S1 cells lysed the encephalitogenic S1 T line in vitro and efficiently neutralized their encephalitogenic capacity in vivo.

The generation of cells with suppressor activity in EAE has been described in rats, but the generation of
murine T cell anti-T cell clones or lines with suppressor activity has not been previously demonstrated in murine EAE.
OBJECTIVES OF THIS STUDY

I. General Objectives

There is substantial evidence in the literature that EAE is mediated by myelin basic protein (MBP) specific T cells, and that, at least in the rat, the severity or recovery from the clinical syndrome is regulated, at least in part, by the generation of a population of "suppressor" T cells.

Therefore, this study sought to generate murine clones or lines of T cells that could regulate the mediation of EAE by MBP-specific lines or clones. The sensitization protocol, cell cloning and passive transfer techniques are well established and feasible. However, the generation of murine T cell anti-T cell clones or lines with "suppressor" activity has not previously been accomplished. The suppressor clones or lines raised against the MBP-reactive clones could be directed against the MBP antigen-receptor, or against another cell surface antigen. In any case, the "regulatory" T cell lines or clones generated in vitro, will also be tested in vivo for the ability to inhibit the passive transfer of EAE by MBP-specific T cells.
II. **Specific Objectives**

1. To generate, "in vitro", murine T cell clones or lines specific for MBP-specific T-cell lines capable of mediating experimental allergic encephalomyelitis.

2. To characterize these T anti-T cells for cell surface phenotype.

3. To examine "in vivo", regulatory effects of murine T-clones or lines specific for EAE-mediating T cell lines.
MATERIALS AND METHODS

I. Cloning of EAE-inducing Cells

a) Myelin basic protein-sensitization

Male and female SJL/J mice, ages 4 to 8 weeks (obtained from the National Institutes of Health), were injected in the hind foot pads with 0.1 ml containing 100 to 250 micrograms (total per mouse) of porcine MBP (Calbiochem, La Jolla, CA) emulsified in an equal volume of complete Freund's adjuvant (supplemented with 6 mg/ml of desiccated Mycobacterium tuberculosis H37 RA (Difco Laboratories, Detroit, MI). This sensitization was followed at 24 and 72 hours by an i.v. injection of 2.2x10\textsuperscript{10} formalin fixed, washed Bordetella pertussis organisms (Michigan Department of Health). Sensitized mice were sacrificed by exsanguination via cardiac puncture under ether anesthesia 10 days after the injection of MBP.

b) Lymph nodes cell culture

Popliteal and inguinal lymph nodes were removed from SJL/J mice 10 days after immunization with porcine MBP and CFA in both hind foot pads. These lymph nodes were put into single cell suspension over nylon mesh screens and subsequently cultured for 3 days with porcine myelin basic protein (PMBP) (10 µg/ml) or PPD (15 µg/ml)
in the absence of interleukin-2 (IL-2). These cultures were established in complete media: RPMI 1640 (Biofluids, Bethesda, MD) with penicillin (100 mg/ml), streptomycin (100 µg/ml), gentamicin (10 µg/ml), 10% fetal calf serum, and 5x10^{-5}M 2-mercaptoethanol.

c) Subculturing of T cell lines

After 3 days, T cell lines were subcultured by plating them at 10 cells/well in round bottom 96-well plates (Costar) along with recombinant IL-2 (10 u/cc) (Amgen, Thousand Oaks, California), 2x10^5 irradiated SJL/J splenocytes and porcine MBP or PPD. These subcloning cultures were fed twice a week with IL-2, and once every other week with irradiated SJL/J splenocytes and porcine MBP or PPD. As T cells became morphologically apparent in the cultures, they were expanded into 24 well plates as needed. In addition, the heterogeneous lymph node-derived cells were grown as an IL-2 dependent T cell line using the approach outlined above without the initial plating at 10 cells per well.

d) Proliferation assay

Antigen reactivity of the MBP-specific IL-2 dependent T cell lines (8BO, F128, A11, D47) and other antigen-specific IL-2 dependent T cell lines (OVA-0-15, B10S-6-20, PPD-13-28, PPD-12 and 9PPD) were assessed via ³H-thymidine incorporation proliferation assays. In these assays, 3x10^4 line cells were added to 3x10^5 irradiated SJL/J splenocytes in 0.2 ml volume of RPMI 1640 with 10%
fetal calf serum in the absence of IL-2 and in the presence or absence of optimal concentrations of antigens (Porcine myelin basic protein, PMBP; Purified protein derivative, PPD and Ovalbumin, OVA). These cultures were maintained at 37° in 10% CO₂ for 48 hours. 2 μCi of ³H-thymidine were then added, and the amount of ³H-thymidine incorporated into cellular DNA assayed 24 hours later with the aid of semiautomated cell harvested and beta-scintilography. The results were expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.

II. Generation of T-anti T Cell Lines

The T-anti T cell lines or responder T cell lines were generated from the spleen of an SJL/J mouse immunized 7 days earlier with the irradiated (6,000 rad; ¹³⁷Cs) EAE-inducing line-8BO (0.9cc i.p. at 95.8x10⁶ cells/cc). Seven days post-immunization, the spleen was removed and cultured at 1.5x10⁶ cells/well with the irradiated (7,500 rad; ¹³⁷Cs) EAE-inducing line 8BO at 1.5x10⁶ cells/well in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol (complete media, CM) in a 24 well tray (Costar). After 7 days of primary culture, the viable cells were isolated on Ficoll-Hypaque and cloned in IL-2 (10 u/cc) in a 96 well round tray (Costar), at 50 cells/well. In addition, these cultures received the irradiated 8BO line (5x10⁴ cells/well for the first
carried out for previous experiment. Lines generated by this experiment were termed line B1 to B30.

a) Proliferation assays

1) Proliferation assay in response to two different concentrations of EAE-inducing line (8BO)

In the preliminary screen for relevant lines, antigen specificity was examined in a 72 hour proliferation assay. 3x10^4 cells/well of responder T cell lines were plated in CM with 3x10^5 cells/well autologous irradiated (2,500 rad; ^{137}Cs) SJL/J splenocytes and with 1x10^5 cells/well or 5x10^5 cells/well of the irradiated (7,500 rad; ^{137}Cs) EAE-inducing line (8BO) in 96 well flat-bottomed tray (Costar) at 0.2 ml final volume. In addition, irradiated splenocytes and the irradiated 8BO line were tested alone as a control using two different concentrations of cells for 8BO (1x10^5 cells/well and 5x10^5 cells/well). After 48 hours, culture were pulsed with 0.2 μCi $^3$H-thymidine and cells were harvested 24 hours later. Incorporation of $^3$H-thymidine were measured by liquid scintillation spectroscopy. The results were expressed as arithmetic means of counts per minute (c.p.m.) from triplicate cultures ± standard deviations.

2) Proliferation assay in the absence of antigen presenting cell (APC)

Antigen specificity was examined in the absence of antigen presenting cells (APC) in a 72 hour proliferation assay. 3x10^4 cells/well of responder lines,
stimulation cycle and $1 \times 10^5$ cells/well for further stimulation cycles) and autologous irradiated splenocytes (2,500 rad; $^{137}$Cs) at $3 \times 10^5$ cells/well as a source of antigen presenting cells. The wells demonstrating positive growth were transferred to a 48 well plate (Costar) with irradiated 8BO line ($0.5 \times 10^6$ cells/well) and autologous irradiated splenocytes ($1.5 \times 10^6$ cells/well) in the presence of interleukin-2 (10 u/cc). Following further culture, the lines with positive growth were transferred to a 24 well plate (Costar) with the irradiated 8BO line ($1 \times 10^6$ cells/well) and autologous irradiated splenocytes ($3 \times 10^6$ cells/well in the presence of interleukin-2 (10 u/cc). All 96, 48 and 24 well plates, were fed every other week with the irradiated 8BO line and autologous irradiated SJL/J splenocytes in the presence of interleukin-2 (10 u/cc) alone. In addition, these cells were fed twice a week with interleukin-2 (10 u/cc). Lines generated by this experiment were termed line A1 to A30.

Additional responder T cell lines were generated in a similar manner, except that, 7 days post-immunization, spleen cells from immunized an mouse were cultured ($2 \times 10^6$ cells) with the irradiated 8BO line ($4 \times 10^6$ cells) in a flask (10 cc final volume) for 3 days. After 3 days, the viable cells were isolated on Ficoll-Hypaque and cultured with interleukin-2 (10 u/cc) for 4 days. After 4 days, the viable cells were cloned using the same procedure.
were plated in CM alone or with $5 \times 10^5$ cells/well of the irradiated (6,000 rad; $^{137}\text{Cs}$) 8BO or with lines of different antigen specificities (PPD 13-28 or 9PPD), but without autologous irradiated splenocytes, in 96 well flat bottomed plate (Costar) at 0.2 ml total volume. Cultures were pulsed and harvested as previously described.

3) Proliferation assay in response to EAE-inducing line (8BO) and lines with different antigen specificities (PPD-13-28)

In the preliminary screen for relevant lines, antigen specificity was examined in a 72 hour proliferation assay. $3 \times 10^4$ cells/well of responder T cell lines were plated in CM with $3 \times 10^5$ cells/well of autologous irradiated SJL/J splenocytes and $5 \times 10^5$ cells/well of the irradiated 8BO line. In addition each line were also tested for stimulation with autologous irradiated splenocytes ($3 \times 10^5$ cells/well) alone or with an irradiated line (6,000 rad; $^{137}\text{Cs}$) with a different antigen specificity (PPD-13-28) at $5 \times 10^5$ cell/well. Irradiated autologous splenocytes with irradiated 8BO line or with the PPD-13-28 line were used as controls. Each group was tested in triplicates in a 96-well-flat-bottomed-tray at 0.2 ml final volume. Cultures were pulsed and harvested as previously described.
4) Antigen specificity of the in vitro proliferation of the responder line A4 in mouse serum

This assay was carried out the same way as assay a.3, except that, line A4 was used and plated with 0.5% of mouse serum in the absence of 10% fetal calf serum.

5) Proliferation assay of the responder T cell lines in 0.5% of mouse serum

This assay was carried out the same way as assay a.3., except that, the responder T cell lines were plated with 0.5% mouse serum, and tested using an irradiated line with a different antigen specificity (PPD-6-20).

6) Proliferation assay in response to 8BO line and to lines with different antigen specificities in 0.5% of mouse serum

Antigen specificity was examined in a 72 hour proliferation assay. 3x10^4 cells/well of line A4 was plated in CM with 3x10^5 cells/well of autologous irradiated SJL/J splenocytes and 5x10^5 cells/well of the irradiated 8BO line alone or with PMBP at 10 µg/cc. In addition, line A4 was also tested with autologous irradiated splenocyte at 3x10^5 cells/well alone or with PMBP (10 µg/cc), and with irradiated lines/clones (6,000 rad; ^{137}Cs) with different antigen specificities (PPD-12, A11, 8BO-57, F_{128}, OVA-015 and D47) at 5x10^5 cells/well. Irradiated splenocytes with irradiated 8BO line or with an
irradiated irrelevant clone/line (PPD-12; A11; 8BO-57; F128; OVA-015 and D47) were used as controls. Each group were tested in triplicates in a 96-well-flat-bottomed-trays at 0.2 ml final volume. Cultures were pulsed and harvested as previously described.

7) Proliferation assay in response to the 8BO line and to lines/clones with different antigen specificities in 0.5% of mouse serum

Antigen specificity was examined in a 72 hour proliferation assay. 3x10^4 cells/well of line A4 was plated in RPMI 1640 supplemented with 0.5% mouse serum and 2-mercaptoethanol with 3x10^5 cells/well of autologous irradiated SJL/J splenocytes and 5x10^5 cells/well of the irradiated 8BO line alone or together with PMBP at 10 µg/cc. In addition, line A4 was also tested alone or with autologous irradiated splenocytes at 3x10^5 cells/well alone or with PMBP(10 µg/cc), and with the irradiated lines/clones with different antigen specificities with their antigens (PPD clone, PPD clone+PPD, OVA clone, OVA clone+OVA, D10 clone, F128 line, F128 line+PMBP antigen) at 5x10^5 cells/well. Irradiated splenocytes with irradiated 8BO line or with irradiated non-PMBP-specific clones/lines (PPD clone, OVA clone, D10 clone and F128 line) were used as controls. In order to test if line A4 needed antigen presenting cells to respond to 8BO line, all combinations were tested in absence of APC. Each group was tested in triplicates in a 96-well-flat-bottomed
trays at 0.2 ml final volume. Cultures were pulsed and harvested as previously described.

III. In Vivo Effects of the Responder T Cell Line A4 and the Stimulator Cell Line 8BO

To assess the ability of responder T cell line (A4) and the stimulator cell line 8BO to induce or suppress EAE in SJL/J mice, a total of 4 animals received sublethal irradiation (350 rad; \(^{137}\text{Cs}\)). Each animal was injected intraperitoneally as follows: animal #1 received 0.5 cc of line A4 at 18x10^6 total cells and 0.5 cc of the 8BO line at 15x10^6 total cells; animal #2 received 0.5 cc of the 8BO line at 15x10^6 total cells and 0.5 cc of PBS; animal #3 received 0.5 cc of line A4 at 18x10^6 total cells and 0.5 cc of PBS; animal #4 received 0.5 cc of a clone with a different antigen specificity (9PPD) at 18x10^6 total cells and 0.5 cc of EAE-inducing line (8BO) at 15x10^6 total cells.

Mice were observed at least through day 10 for clinical signs. The clinical status of the mice was graded on a 0 to 5 scale of increasing severity: 0, no abnormality; 1, tail weakness; 2, tail weakness with moderate hind limb weakness; 3, hind leg paralysis and eventually mild forelimb weakness; 4, quadriplegia or premoribund state; 5, death of animal in acute EAE.

A second similar experiment was carried out, except that animal #1 received 0.5 cc of line A4 at 25x10^6 total
cells, line 8BO at $10 \times 10^6$ total cells, PMBP at 20 μg and PBS; animal #2 received 8BO line at $10 \times 10^6$ total cells, PMBP at 20 μg and PBS; animal #3 received a PPD-specific clone (9PPD) at $25 \times 10^6$ total cells, 8BO at $10 \times 10^6$ total cells, PMBP at 20 μg and PBS; animal #4 received 8BO line at $10 \times 10^6$ total cells and PBS.

Mice were observed and graded as previously described.

IV. Surface Phenotype of the Responder Line

Surface marker characterization was carried out to phenotype the responder line A4. Cells were resuspended in a 1 cc of 10% Sodium Azide (SA). The pellet was resuspended and washed 3x with PBS/SA. After the last wash, cells were resuspended in 0.8 cc of PBS/SA. An aliquot of 0.1 cc was taken and added into 3 Eppendorf tubes. To each tube was added 0.1 cc of: a 1:40 dilution of a monoclonal rat anti-L3T4 or a 1:50 dilution of anti-Lyt2. In the third tube no antibody was added (PBS as a control). The cells were incubated for 15 to 30 min at 4°C. After incubation, 3 washes were performed by adding 1 ml of PBS/SA. A second antibody (Goat anti-rat antibody conjugated to FITC) was added to all 3 tubes and incubated for 30 min at 4°C. The cells were washed 2 times with PBS/SA and fixed, then 0.1 ml of PBS/SA was added to 0.1 ml of fixative after diluting 1.5M NaCl 1:10 with 10% formalin.
RESULTS

Of the PMBP responsive lines tested to be the "stimulating" line in the generation of "suppressor or responder" T cell lines, line 8BO was selected. This line was selected based on the criterion of responsiveness to MBP using an in vitro proliferation assay and the in vivo mediation of EAE. To test for antigen reactivity of the EAE inducing line and control lines, an in vitro proliferation assay (shown in Table I) was used. Cells from individual lines were plated with APC in the presence or absence of their respective antigens.

The results in Table I indicated that all T lymphocyte lines proliferated strongly and specifically only with their specific antigens. However, in the absence of their antigens, all T lymphocyte lines had a significant lower response. The proliferative response of T lymphocyte lines towards unrelated antigens was negative, but not shown.

Of the PMBP responsive lines (8BO, F128, D47, A11) tested in vivo, only 8BO line was consistently able to mediate EAE in vivo (in 100% of SJL/J mice, data not shown).
The cells in each tube were further processed for an analysis on the fluorescence-activated cell sorter (FACS) and by using a fluorescence microscope.
I. In Vitro Studies

Tables II to IX show the proliferative response of T cell lines generated from spleens of SJL/J mice that had been immunized with the irradiated MBP-specific T cell line (8BO). These responder lines were grown in vitro with the irradiated stimulator T cell line (8BO) and antigen presenting cell (APC) in the presence of IL-2 containing medium as described in Materials and Methods.

The proliferative assays shown (Tables II to IX) represent typical experiments testing the response and specificity of the responder lines towards line 8BO. Some responder lines were stimulated and others suppressed by 8BO. These responses of either stimulation or suppression were generally greater when higher concentration of 8BO were used. Tables II and III show typical responses of responder lines A1, A7, A12, B2, B3. These assays were all performed in the presence of fetal calf serum.

In Table IV, we examined the requirement for irradiated spleen (as APC) in the responses noted. As can be seen, a number of responder lines were capable of responding to 8BO (eg: line A3 and B3) or to a PPD-specific lines PPD 13-28 and 9-PPD (eg: line A1 A12 and B3) in the absence of APC. Other lines however, were totally unresponsive to either 8BO or 9-PPD in the absence of APC. Of note was the finding that the high background counts seen in Tables II and III were not seen in the
absence of APC. Thus, APC was not necessarily important for some of T-T cells interactions noted above.

In the next experiment, shown in Table V, T cell lines were stimulated with either an irradiated EAE-inducing line or an irradiated PPD-specific line at $5 \times 10^5$ cells/well. Once again, it can be seen that certain responder lines were suppressed by either 8BO or PPD 13-28 (eg: A9, B3, B9), while others were suppressed only by 8BO (eg: A1). In contrast, some lines were stimulated rather specifically by 8BO (eg: A4, A12).

Because of the continued high background counts seen in the presence of APC, it appeared that all the responder lines were at least partially responding in either an autologous mixed lymphocyte reaction (AMLR) or responding to antigenic determinants in fetal calf serum.

To attempt to understand and eliminate the high background counts, all subsequent tests were performed in the absence of fetal calf serum and, instead, in the presence of 0.5% autologous mouse serum. As can be seen in Table VI, with A4, the change to mouse serum greatly decreased the background counts, suggesting that these proliferation responses were in response to fetal calf serum determinants. As can also be seen in Table VI, the responses of A4 towards 8BO and PPD-13-28 persisted despite the absence of fetal calf serum.

In Table VII, a panel of responder lines have been tested in the presence of 0.5% mouse serum and in the
absence of fetal calf serum. As can be seen, a number of responder lines now demonstrated no significant proliferative response to either 8BO or PPD-6-20 (lines A17, B11, B16, B21), while other lines showed responses to both 8BO and PPD-6-20 (lines A1, A4, A12, B3).

In Table VIII, line A4 has been singled out for further study, and its responses to a number of different irradiated stimulator clones is shown. As can be seen in Table VIII, A4 responded well to 8BO, PPD-12, A-11, 8BO-57 (a MBP-specific clone of 8BO), OVA-0.15, D47, but responded poorly to line F128. It should also be noted that in this experiment line A4 itself, showed a response to PMBP (as did the irradiated line 8BO). Of most interest, however, was the finding that the response of A4 to 8BO was significantly decreased in the presence of PMBP.

This surprising finding was investigated further in experiments in which A4 was stimulated with irradiated PPD-specific and ovalbumin (OVA)-specific clones, in the presence or absence of PPD or ovalbumin, respectively. As can be seen in Table IX, A4 responded to the PPD clone, but in the presence of PPD this response increased. This was in contrast to the response to 8BO which again decreased in the presence of PMBP. A4 responded to the OVA-specific clone, and in the presence of ovalbumin this response also decreased but to a much lesser degree than with 8BO and PMBP. As can also be seen in Table IX, A4
again did not respond to the MBP-specific line F_{128}, but the baseline response to F_{128} nevertheless decreased by approximately 50% in the presence of PMBP. A4 itself was again seen to respond to PMBP. Thus, unlike the situation with A4 and 8BO and A4 and F_{128}, PMBP itself did not suppress the proliferation of A4 in the presence of APC alone. Finally, as can be seen in Table IX, A4 did not respond to D10, which is an allogeneic clone specific for ovalbumin and derived from an AKR mouse (a generous gift of Dr. Charles Janeway and Dr. K. Bottomly, Yale University).

II. In Vivo Studies

The in vivo effects of the anti-8BO cell line A4 on the EAE-inducing line 8BO was next studied (Tables X and XI).

Both lines were injected intraperitoneally into a naive, irradiated (350 rad) SJL/J mouse; A4 at 18x10^6 cells/mouse and 8BO at 15x10^6 cells/mouse. We found that line A4 did not protect the mice from 8BO mediated EAE. As shown in Table X, when A4 and 8BO were injected together, the first signs of EAE developed at day 7, and within 10 days the mouse was dead. 8BO injected alone into a mouse led to the same clinical outcome except that the disease onset was at day 6. However, when the control line 9PPD was injected with 8BO, only mild clinical signs were observed (maximal severity=1) from day 6 through day
10. We have noted such a modulation of signs of EAE with PPD lines previously, but only inconsistently. Line A4 injected alone failed to mediate any signs of EAE.

In Table XI, we asked whether an increase in the amount of line A4 injected or the injection along with PMBP could affect the mediation of EAE described above. The latter question was based on our in vitro observation that PMBP could suppress A4's response to PMBP-specific lines. We postulated that the addition of PMBP might lead to the secretion of suppressor factors by A4. The simultaneous injection of PMBP did not seem to modify the clinical signs of the disease in any cases. However, injecting line A4, at $25 \times 10^6$ cells per mouse along with 8BO at $10 \times 10^6$ cells per mouse together with PMBP seemed to accelerate the disease onset (3 days earlier than the previous experiment). No attempt was made to test A4 alone or with PMBP in these experiments.

III. Analysis of Surface Membrane Markers of the Responder Line

Surface marker characterization was carried out to phenotype the responder line A4. Treatment of line A4 cells with rat antibodies to the determinat Lyt2$^+$ and with a monoclonal antibody to L3T4 followed by a secondary antibody (fluorescein conjugated-goat anti-rat antibody) indicated that the A4 line cells did not express Lyt2$^+$ on their surface, but did express L3T4. The control cells,
which did not receive any primary antibody was negative. The results of fluorescence-activated cell sorter (FACS) analyses are shown in Figures 1, 2, and 3.
Table I. In vitro proliferation assay (c.p.m. $^{3}$H-TdR) of the EAE-inducing line (8BO), and control lines.

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>APC + PMBP</td>
</tr>
<tr>
<td>8BO</td>
<td>977±424</td>
</tr>
<tr>
<td>F128</td>
<td>1,013±293</td>
</tr>
<tr>
<td>OVA 0.15</td>
<td>1,544±977</td>
</tr>
<tr>
<td>B10S-6-20</td>
<td>977±178</td>
</tr>
<tr>
<td>A-11</td>
<td>524±193</td>
</tr>
<tr>
<td>PPD-13-28</td>
<td>310±124</td>
</tr>
<tr>
<td>D47</td>
<td>1,011±505</td>
</tr>
<tr>
<td>PPD-12</td>
<td>293±94</td>
</tr>
<tr>
<td>9 PPD</td>
<td>1.912±182</td>
</tr>
</tbody>
</table>

To test for antigen reactivity, responder T cell lines were plated in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol at $3\times10^4$ cells/well with $3\times10^5$ cells/well autologous irradiated splenocytes (2,500 rad; $^{137}$Cs) in the presence or absence of optimal concentrations of antigens (porcine myelin basic protein – PMBP, purified protein derivative of tuberculin – PPD and ovalbumin – OVA) in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume.
Table I (cont'd)

After 48 h, cultures were pulsed with 2μCi $^3$H-thymidine and cells were harvested 24 h later. Incorporation of $^3$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table II. In vitro proliferation assay (c.p.m. \(^{3}\text{H}-\text{TdR}\)) in response to two different concentrations of EAE-inducing line (8BO).

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator cells</th>
<th>APC (10^5 cells/well)</th>
<th>APC + 8BO (5x10^5 cells/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>APC</td>
<td>51,682 ± 7,223</td>
<td>81,209 ± 2,340</td>
</tr>
<tr>
<td>A1</td>
<td>APC + 8BO</td>
<td>126,900 ± 9,618</td>
<td>45,928 ± 2,162</td>
</tr>
<tr>
<td>A7</td>
<td>APC + 8BO</td>
<td>42,927 ± 16,723</td>
<td>69,110 ± 8,942</td>
</tr>
<tr>
<td>A12</td>
<td>APC + 8BO</td>
<td>833 ± 278</td>
<td>1,263 ± 961</td>
</tr>
</tbody>
</table>

To test for antigen specificity, lines A1, A7 and A12 were plated in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol at 3x10^4 cells/well, with autologous irradiated (2,500 rad; \(^{137}\text{Cs}\)) SJL/J splenocytes 3x10^5 cells/well and 1x10^5 cells/well or 5x10^5 cells/well of the irradiated (7,500 rad; \(^{137}\text{Cs}\)) EAE-inducing line (8BO) in 96-well-flat-bottomed trays (Costar) at 0.2ml final volume. After 48 h, cultures were pulsed with 0.2µCi \(^{3}\text{H}-\text{thymidine}\) and cells were harvested 24 h later. Incorporation of \(^{3}\text{H}-\text{thymidine}\) was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table III. In vitro proliferation assay (c.p.m. $^3$H-TdR) in response to two different 8B0 concentrations.

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator cells</th>
<th>APC</th>
<th>APC + 8B0</th>
<th>APC + 8B0</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td></td>
<td>(10^5 cells/well)</td>
<td>(5x10^5 cells/well)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>211,669± 8,841</td>
<td>190,276±25,798</td>
<td>126,087±13,502</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>65,492±20,085</td>
<td>104,614± 5,713</td>
<td>121,490±17,753</td>
<td></td>
</tr>
</tbody>
</table>

To test for antigen specificity, lines B2 and B3 were plated in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol at 3x10^4 cells/well with 3x10^5 cells/well autologous irradiated (2,500 rad; $^{137}$Cs) SJL/J splenocytes and 1x10^5 cells/well or 5x10^5 cells/well of the irradiated (6,000 rad; $^{137}$Cs) EAE-inducing line (8B0) in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2μCi $^3$H thymidine and cells were harvested 24 h later. Incorporation of $^3$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table IV. In vitro proliferation assay (c.p.m. $^3$H-TdR) in the absence of APC.

<table>
<thead>
<tr>
<th>Responder Stimulator cells</th>
<th>T cell</th>
<th>Media</th>
<th>8BO</th>
<th>PPD-13.28</th>
<th>9-PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>90±34</td>
<td>173±59</td>
<td>2,532±368</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>112±7</td>
<td>918±534</td>
<td>686±147</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>103±37</td>
<td>315±241</td>
<td>-</td>
<td>402±212</td>
<td></td>
</tr>
<tr>
<td>A12</td>
<td>269±</td>
<td>374±95</td>
<td>-</td>
<td>1,073±134</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>445±187</td>
<td>332±166</td>
<td>-</td>
<td>538±97</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>263±130</td>
<td>1,361±199</td>
<td>-</td>
<td>2,903±259</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>411±209</td>
<td>162±54</td>
<td>-</td>
<td>578±43</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>104±32</td>
<td>-</td>
<td>239±75</td>
<td></td>
</tr>
</tbody>
</table>

To test for antigen specificity, lines A1, A3, A8, A12, B2, B3 and B7 were plated in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol at 3x10⁴ cells/well with 5x10⁵ cells/well of the irradiated (6000 rad; $^{137}$Cs) EAE-inducing line (8BO) or 5x10⁵ cells/well of irradiated (6,000 rad; $^{137}$Cs) lines with a different antigen specificities (PPD-13-28 or 9PPD) but without autologous irradiated splenocytes, in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2μCi $^3$H thymidine and cells were harvested 24 h later. Incorporation of $^3$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table V. In vitro proliferation assay (c.p.m. $^{3}$H-TdR) in response to EAE-inducing line (8BO) and a line with different antigen specificity (PPD-13-28).

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>APC</td>
</tr>
<tr>
<td>A1</td>
<td>154,030± 3,987</td>
</tr>
<tr>
<td>A4</td>
<td>99,679±34,286</td>
</tr>
<tr>
<td>A9</td>
<td>134,361±17,749</td>
</tr>
<tr>
<td>A12</td>
<td>98,467± 5,468</td>
</tr>
<tr>
<td>B2</td>
<td>256,258±22,108</td>
</tr>
<tr>
<td>B3</td>
<td>168,011±35,257</td>
</tr>
<tr>
<td>B9</td>
<td>161,315±18,326</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

To test for antigen specificity, T cell lines were plated in RPMI 1640 supplemented with 10% fetal calf serum and 2-mercaptoethanol at 3x10^4 cells/well with 3x10^5 cells/well autologous irradiated (2,500 rad $^{137}$Cs) SJL/J splenocytes and 5x10^5 cells/well of the irradiated (6,000 rad $^{137}$Cs) EAE-inducing (8BO) or 5x10^5 cells/well of the irradiated (6,000 rad; $^{137}$Cs) line with a PPD-specific line (PPD-13-28) in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2µCi $^{3}$H thymidine and cells were harvested 24 h later. Incorporation of $^{3}$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table VI. Antigen specificity of the in vitro proliferation (c.p.m. $^3$H-TdR) of the responder A4 line in mouse serum.

<table>
<thead>
<tr>
<th>Responder</th>
<th>Stimulator cell</th>
<th>APC</th>
<th>APC + 8BO</th>
<th>APC + PPD-13-28</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td></td>
<td>1,525±203</td>
<td>6,582±1,838</td>
<td>32,696±5,017</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>-</td>
<td>417</td>
<td>382</td>
</tr>
</tbody>
</table>

To test for antigen specificity, line A4 was plated in RPMI 1640 supplemented with 0.5% mouse serum and 2-mercaptoethanol at $3 \times 10^4$ cells/well with $3 \times 10^5$ cells/well autologous irradiated (2,500 rad; $^{137}$Cs) SJL/J splenocytes and $5 \times 10^5$ cells/well of the irradiated (6,000 rad; $^{137}$Cs) EAE-inducing line (8BO) or $5 \times 10^5$ cells/well of the irradiated (6,000 rad; $^{137}$Cs) PPD-specific line (PPD-13-28) in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2μCi $^3$H thymidine and cells were harvested 24 h later. Incorporation of $^3$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table VII. Antigen specificity of the in vitro proliferation (c.p.m. \(^{3}\text{H}-\text{TdR}\)) response of T cell lines in mouse serum.

<table>
<thead>
<tr>
<th>Responder Stimulator cells</th>
<th>APC</th>
<th>APC + 8BO</th>
<th>APC + PPD-6-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>475±88</td>
<td>6,063±254</td>
<td>8,292±1,586</td>
</tr>
<tr>
<td>A4</td>
<td>1,847±1,082</td>
<td>33,457±1,180</td>
<td>46,384±9,966</td>
</tr>
<tr>
<td>A12</td>
<td>631±305</td>
<td>9,119±5,544</td>
<td>10,564±1,831</td>
</tr>
<tr>
<td>A17</td>
<td>953±143</td>
<td>1,462±250</td>
<td>1,929±202</td>
</tr>
<tr>
<td>B3</td>
<td>21,530±3,799</td>
<td>92,957±13,475</td>
<td>101,361±23,217</td>
</tr>
<tr>
<td>B11</td>
<td>1,581±47</td>
<td>2,968±3,003</td>
<td>1,186±381</td>
</tr>
<tr>
<td>B16</td>
<td>931±221</td>
<td>826±146</td>
<td>1,189±170</td>
</tr>
<tr>
<td>B21</td>
<td>848±158</td>
<td>1,039±393</td>
<td>1,760±389</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>313±63</td>
<td>363±10</td>
</tr>
</tbody>
</table>

To test for antigen specificity, T cell lines were plated in RPMI 1640 supplemented with 0.5% mouse serum and 2-mercaptoethanol at 3x10\(^4\) cells/well with 3x10\(^5\) cells/well autologous irradiated (2,500 rad; \(^{137}\)Cs) SJL/J splenocytes and 5x10\(^5\) cells/well of the irradiated (6,000 rad; \(^{137}\)Cs) EAE-inducing line (8BO) or 5x10\(^5\) cells/well of irradiated (6,000 rad; \(^{137}\)Cs) PPD-specific line (PPD-6-20) in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2μCi \(^{3}\text{H}\) thymidine and cells were harvested 24 h later. Incorporation of \(^{3}\text{H}\)-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table VIII. In vitro proliferation (c.p.m. $^{3}$H-TdR) in response to EAE-inducing line (8BO) and to lines with different antigen specificity in mouse serum.

<table>
<thead>
<tr>
<th>Responder T cell (A4) and Stimulator cells</th>
<th>Stimulator cells alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>15,131± 4,644</td>
</tr>
<tr>
<td>APC+8BO</td>
<td>37,679± 3,315</td>
</tr>
<tr>
<td>APC+8BO+PMBP</td>
<td>4,749± 730</td>
</tr>
<tr>
<td>APC+PPD-12</td>
<td>38,898±15,257</td>
</tr>
<tr>
<td>APC+A-11</td>
<td>42,053± 6,361</td>
</tr>
<tr>
<td>APC+8BO-57</td>
<td>45,839±16,381</td>
</tr>
<tr>
<td>APC+F$^{1}$28</td>
<td>19,213± 2,049</td>
</tr>
<tr>
<td>APC+OVA-0.15</td>
<td>41,691±16,805</td>
</tr>
<tr>
<td>APC+D47</td>
<td>37,978± 4,278</td>
</tr>
<tr>
<td>APC+PMBP</td>
<td>26,858±11,787</td>
</tr>
</tbody>
</table>

To test for antigen specificity, line A4 was plated in RPMI 1640 supplemented with 0.5% mouse serum and 2-mercaptoethanol at $3\times10^4$ cells/well with $3\times10^5$ cells/well autologous irradiated (2,500 rad; $^{137}$Cs) SJL/J splenocytes and $5\times10^5$ cells/well of the irradiated (6,000 rad; $^{137}$Cs) EAE-inducing line (8BO) or $5\times10^5$ cells/well of irradiated (6,000 rad; $^{137}$Cs) lines with different antigen specificities (PPD-12; A-11, 8BO-57, F$^{1}$18; OVA-15 and D47) with or without PMBP at 10µg/ml cc in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with $2\mu$Ci $^{3}$H thymidine and cells were harvested 24 h later. Incorporation of $^{3}$H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table IX. In vitro proliferation (c.p.m. $^3$H-TdR) of A4 in response to EAE-inducing line (8BO) and to lines with different antigen specificities in mouse serum.

<table>
<thead>
<tr>
<th>Responder T cell (A4) and Stimulator cells</th>
<th>Stimulator cells alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>521±281</td>
</tr>
<tr>
<td>APC</td>
<td>11,055±1,347</td>
</tr>
<tr>
<td>PMBP + APC</td>
<td>16,230±9,604</td>
</tr>
<tr>
<td>APC+PPD clone</td>
<td>23,034±8,349</td>
</tr>
<tr>
<td>APC+PPD clone + PPD</td>
<td>38,300±6,313</td>
</tr>
<tr>
<td>APC+OVA clone</td>
<td>22,693±3,909</td>
</tr>
<tr>
<td>APC+OVA clone + OVA</td>
<td>16,557±1,622</td>
</tr>
<tr>
<td>APC + 8BO</td>
<td>16,636±3,259</td>
</tr>
<tr>
<td>APC+8BO+PMBP</td>
<td>6,779±4,248</td>
</tr>
<tr>
<td>APC+D10</td>
<td>12,051±2,707</td>
</tr>
<tr>
<td>APC+F128</td>
<td>10,259±657</td>
</tr>
<tr>
<td>APC+F128 + PMBP</td>
<td>5,218±34</td>
</tr>
<tr>
<td>8BO (no APC)</td>
<td>301±45</td>
</tr>
<tr>
<td></td>
<td>2,222±203</td>
</tr>
<tr>
<td></td>
<td>278±3</td>
</tr>
<tr>
<td></td>
<td>333±206</td>
</tr>
<tr>
<td></td>
<td>655±194</td>
</tr>
<tr>
<td></td>
<td>403±151</td>
</tr>
</tbody>
</table>

cont'd on next page
To test for antigen specificity, line A4 was plated in RPMI 1640 supplemented with 0.5% mouse serum and 2-mercaptoethanol at 3x10^4 cells/well with 3x10^5 cells/well autologous irradiated (2,500 rad; 137 Cs) SJL/J splenocytes and 5x10^5 cells/well of the irradiated (6,000 rad; 137 Cs) EAE-inducing line (880) or 5x10^5 cells/well of irradiated (6,000 rad; 137 Cs) lines with different antigen specificities (PPD, OVA, D10, F128) with or without (PMBP; PPD; OVA) at 10μg l cc in 96-well-flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 2μCi ³H thymidine and cells were harvested 24 h later. Incorporation of ³H-thymidine was measured by liquid scintillation spectroscopy. The results are expressed as arithmetic means of c.p.m. from triplicate cultures ± standard deviation.
Table X: Ability of responder T cell line A4 to modify 8BO-induced EAE in SJL/J mice.

<table>
<thead>
<tr>
<th>Day of Onset</th>
<th>(n) of mice</th>
<th>Final Severity of clinical disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 7 8 9 10</td>
<td></td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>A4+8BO +</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>8BO alone +</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>A4 alone</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>9PPD+8BO +</td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

To assess the ability of line A4 to modify 8BO-induced EAE in naive mice, 4 animals received sublethal irradiation (350 rad; $^{137}$Cs) and were injected i.p. as follows: animal #1 received A4 line at $18 \times 10^6$ cells and the EAE-inducing line (8BO) at $15 \times 10^6$ cells; animal #2 received EAE-inducing line (8BO) alone; animal #3 received A4 line at $18 \times 10^6$ cells alone and animal #4 a line with different antigen specificity (9PPD) at $18 \times 10^6$ cells and EAE-inducing line (8BO) at $15 \times 10^6$ cells. Mice were observed at least through day 10 for clinical signs. The status of the mice were graded on a 0 to 5 scale of increasing severity: 0, no abnormality; 1, tail weakness; 2, a floppy tail weakness with moderate hind limb weakness; 3, hind leg paralysis and eventually mild forelimb weakness; 4, quadriplegia or premoribund state; 5, death.
Table XI. Ability of the responder T cell line A4 to modify 8BO-induced EAE in SJL/J mice in the presence of PMBP

<table>
<thead>
<tr>
<th>Day of Onset</th>
<th>(n) of mice</th>
<th>Final severity of clinical disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 7 9 10</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>A4+8BO+PMBP</td>
<td>+ 1 +</td>
<td></td>
</tr>
<tr>
<td>8BO+PMBP</td>
<td>+ 1 +</td>
<td></td>
</tr>
<tr>
<td>9PPD+8BO+PMBP</td>
<td>1 +</td>
<td></td>
</tr>
<tr>
<td>8BO alone</td>
<td>+ 1 +</td>
<td></td>
</tr>
</tbody>
</table>

To assess the ability of line A4 to modify 8BO-induced EAE in a naive mouse, 4 animals received sublethal irradiation (350 rad; $^{137}$Cs) and were injected i.p. as follows: animal #1 received A4 line at $25 \times 10^6$ cells, the EAE-inducing line (8BO) at $10 \times 10^6$ cells and porcine myelin basic protein (PMBP) at 20 µg; animal #2 received EAE-inducing line (8BO) at $10 \times 10^6$ cells and porcine myelin basic protein (PMBP) at 20 µg; animal #3 received purified protein derivative line (9PPD) at $25 \times 10^6$ cells, EAE-inducing line (8BO) at $10 \times 10^6$ cells and porcine myelin basic protein (PMBP) at 20 µg; animal #4 received EAE-inducing line (8BO) alone at $10 \times 10^6$ cells. Mice were observed at least through day 10 for clinical signs. The clinical status of the mice were graded on a 0 to 5 scale of increasing severity (see previous table X).
Fig. 1 Surface phenotype of the responder line A4. Line A4 was incubated with a monoclonal anti-Lyt2 rat antibody and then with FITC-conjugated anti-rat antibodies. The amount of binding was assayed using FACS analysis.
Fig. 2 Surface phenotype of the responder line A4. Line A4 was incubated with a monoclonal anti-L3T4 rat antibody and then with FITC-conjugated goat anti-rat antibodies. The amount of binding was assayed using FACS analysis.
Fig. 3. Surface phenotype of the responder line A4. Line A4 was incubated with PBS and then with FITC-conjugated goat anti-rat antibodies. The amount of binding was assayed using FACS analysis.
DISCUSSION

In these studies, we have attempted to isolate T cell lines that were reactive and specific for EAE-mediating T cell lines. We postulated that we could generate T cell lines that would be specific for the antigen receptors on EAE mediating lines or clones. What we in fact have been able to accomplish is the generation of T cell lines that appear to be reactive to most T cell lines or clones.

We initially noted that our responder lines were stimulated by irradiated splenocytes alone (Table II, III and V). This suggested that the lines might be responding in an autologous mixed lymphocyte reaction (AMLR). In the AMLR, T cells recognize and are stimulated by self Ia displayed by the irradiated autologous antigen presenting cells (APC). The other possibility, however, was that fetal calf serum proteins were acting as antigens and stimulating the responding T cells lines. In other words, either Ia antigens alone on the APC were stimulating our responder T cell lines or fetal calf serum antigens together with Ia antigens were stimulating the responder T cell lines. It is well established that interactions between soluble protein-reactive T cells and either the soluble antigen alone or syngeneic APC alone is not sufficient to induce proliferation (Janeway et al., 1976). However, studies of the AMLR have indicated that
autologous APC can at times, in the absence of added antigen, induce proliferation through their Ia antigens alone among soluble protein reactive T cell (Hausman et al., 1981). In fact, Hausman et al. (1980) and Raff et al. (1980) reported that the AMLR actually represents proliferation among two distinct population of T cells in response to signals from distinct stimulator cells. One population of T cells is activated by stimulators present in autologous B cell- enriched, macrophage-depleted cells, whereas another is induced to proliferate by signals from a subpopulation of autologous macrophages.

To distinguish between the AMLR and a fetal calf serum response, we tested our lines in the absence of fetal calf serum, using autologous mouse serum instead. We initially found that the line A4 showed no AMLR when assayed in mouse serum. However, in later assays, even in mouse serum, the response of A4 to APCs alone was again found to be increasing. This suggests that the response of A4 to APCs alone initially represented a response to fetal calf serum determinants, and perhaps later, a true AMLR.

In the present studies we have found that the reaction between some of the responder T cell lines and stimulator T cell lines did not require antigen presenting cells (Table IV). This is consistent with the studies of Lamb and Feldman (1982), in which they generated human regulatory T cell clones against a T helper cell which
recognizes influenza A matrix protein in the absence of APC. In addition, this finding is consistent with the reports of Damle et al. (1983 and 1984); Mohagheghpour et al. (1984); and Mohagheghpour et al. (1986) who have described (in humans) a form of T suppressor cell which is activated by direct interaction with the antigen receptors on antigen-primed inducer T cells.

In examining the response and specificity of our responder lines, we found that our responder T cell lines appeared to be stimulated by determinants expressed on both the EAE-mediating line and on unrelated lymphoblasts. Suzuki et al. (1986) have reported the only studies of murine T cells reactive to other T cells. They have shown that resting murine T cells can be found to proliferate in response to T cell blasts. These authors did not generate long term lines from their responding resting T cells. Although they found that this response was not dependent on the presence of APC, they nevertheless found that their T-T responses could be inhibited by anti-Ia antibodies. These authors could not explain their surprising findings that APC were not necessary, that murine T cells do not express Ia antigens and yet, anti-Ia antibodies could inhibit their responses. As noted above, we have found that our T-T responses occur both in the presence and absence of APC. Furthermore, we have shown in previous experiments, that there are no residual feeder cells (i.e. Ia-bearing APC) remaining at the time our T cell lines are
assayed (date not shown). This suggests that the responses are also not Ia-dependent. However, based on the findings of Suzuki et al. (1986), it might still be postulated that Ia was playing a role in our responses. As suggested by Suzuki et al. (1986), such a role of Ia determinants may occur by passive acquisition of Ia (secreted by other cells) by T cells. Thus, it is possible that our responder lines were reacting to Ia passively acquired by our irradiated T cell lines/clones. We think this explanation is unlikely in light of our results showing varied patterns of responses of the responder lines towards various stimulators. As seen in Table IV, some of the responder lines were stimulated best by PPD-lines or equally well by both 8BO and PPD-clones, or somewhat better by 8BO. These patterns suggest that, the response of our lines to passively acquired Ia is unlikely to explain all of the responses noted. It is likely that other T cell surface determinants (possibly in combination with passively acquired Ia) are the relevant determinants in the stimulation of our responder lines.

Our studies indicated an unusual pattern of suppression when 8BO or F128 was used as stimulator in the presence of PMBP. When A4 was cultured with PMBP there was a small response noted (Table VIII and IX). This response may have been due to a small contamination of A4 with irradiated 8BO that was nevertheless able to escape the effects of the irradiation and to proliferate in
culture. However, when A4 was cultured with 8BO or F128 together with PMBP, a decreased proliferative response was noted. This surprising PMBP-related suppression was not noted when A4 was stimulated with a PPD-specific clones together with PPD. However, there was a small decrease in proliferation noted when A4 responded to an OVA-clone in the presence of ovalbumin. The mechanisms underlying this suppression are not yet clear. One possible explanation would involve an antigen-induced increase in IL-2 receptors on the irradiated stimulators resulting in a increased adsorption of responder-secreted IL-2 on the irradiated stimulators. This could theoretically decrease the proliferative response of the responder lines. However, the lack of a suppressive response noted with the PPD-clones together with PPD argues against this explanation.

Although we found that line A4 was a phenotypic helper cell, it was still possible that it could have suppressive effects in vivo. In fact, Ellerman et al. (1988) generated T suppressor cells in rats that were strongly CD4+ but became transiently CD8+ when activated. The activation-dependent co-expression of CD4 and CD8 cell-surface markers has been described for both human and rat polyclonal T-cell populations (Blue et al., 1985; Blue et al., 1986; Green and Jotte, 1985). Human T suppressor clones have been described that co-express the CD4 and CD8 antigens (Ottenhoff et al., 1986). We found that the A4
line did not protect syngeneic recipients from 8B0 line mediated EAE when injected simultaneously with 8B0. Despite our in vitro proliferation assays showing that A4 responds to PMBP, the in vivo test indicated that when injected alone, A4 does not mediate EAE.

We found that by increasing the amount of A4 we may have accelerated the disease onset. However, since only a limited number of experiments were performed, we will have to use different concentrations of responder and EAE-mediating lines in future experiments. Because of the surprising suppression noted in vitro in the presence of PMBP, in the second in vivo experiment we injected the lines together with PMBP. We postulated that this addition might lead to the secretion of suppressor factors in vivo. As noted above, this did not yield a suppressive effect, but rather an enhancing effect was seen. The part played by the PMBP in this effect cannot be evaluated at this time. Finally, as seen in Tables IX and X, the PPD-specific line seemed to lessen the clinical signs of EAE when coinfected with 8B0. This effect has been seen occasionally (but variably) by our laboratory and is presently being studied.

In summary, these studies we have generated data suggesting a novel type of T cell-T cell interaction occurring between two IL-2 dependent T cell lines. Furthermore, while such interactions do not appear to be T cell receptor-specific, we have nevertheless demonstrated
that relevant specific antigen is able to inhibit certain, but not all of these lines. Future studies will involve the elucidation of the part played by Ia determinants in these reactions. In addition, the antigen-mediated inhibition of these T-T reactions will be further studied.
CONCLUSIONS

1. We have been able to generate responder T cells that proliferate to both the immunized line 8BO and to other unrelated lines.

2. The results indicate that the responses of some responder T cell lines to stimulator T cell lines did not require antigen presenting cells.

3. While the specific determinant recognized by our responder line has not been determined, we have found that the response of the responder lines to PMBP-specific lines can be inhibited by PMBP. However, the response of the responder line to a PPD-specific line could not be inhibited by PPD.

4. The results demonstrate that the A4 line, which is reactive to the MBP-specific line 8BO, express surface markers typical of helper cells (L3T4+).

5. In vivo, the responder line A4 did not protect syngeneic normal recipients from the EAE-mediating line, 8BO.

6. In the future, additional in vitro studies characterizing the determinant(s) recognized by our responder lines will be performed. Further in vivo studies will also be undertaken.


