The generation of encephalitogenic T cell lines from experimental allergic encephalomyelitis-resistant strains of mice

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Abstract

While only a few strains of mice are susceptible to the primary induction or passive transfer of experimental allergic encephalomyelitis (EAE), the basis of EAE resistance remains unclear. In the present studies, we have defined two approaches that allow for the generation of encephalitogenic, myelin basic protein-reactive, T cell lines from EAE-resistant strains of mice. The first approach, based on the putative relevance of apoptosis to autoimmune disease, involves repeat antigenic stimulation of recently initiated T cell lines. The second approach involves the initiation of lymph node cultures in the absence of exogenous splenocytes as antigenic-presenting cells and the use of a higher antigen concentration. Both approaches lead to the generation of encephalitogenic T cell lines from EAE-resistant mouse strains and will be useful for identifying factors relevant to the pathogenesis of EAE.

Introduction

Experimental allergic encephalomyelitis (EAE) is a well-studied animal model for multiple sclerosis (MS). While our knowledge of the pathogenesis of EAE is extensive, there are still significant gaps in our understanding. One major area of lack of understanding in EAE, that may also be very relevant in understanding MS, is that of ‘resistant’ strains of animals. Thus, in mice, rats and other species, EAE can be induced in only a very few strains, though the basis of EAE resistance remains unclear.

In the present report, we now describe two approaches that allow for the in vitro generation of encephalitogenic T cell lines and clones from resistant strains of mice. One approach was initially based on an attempt to overcome the T cell apoptosis reported to play an important role in down-regulating the autoimmune response in the CNS in EAE and MS (1–6). Our second approach evolved from the first approach through variations in culture parameters. These two approaches will now allow for further identification of mechanisms underlying EAE resistance. Furthermore, because the great majority of gene ‘knockout’ mice and mutant mice are in strains of mice that are EAE resistant, our ability to generate encephalitogenic T cell lines and clones from resistant mice will greatly facilitate the use of knockout and mutant mice in the study of EAE.

Methods

Mice

C57BL/6, C57BL/6-lpr and C3H/HeJ mice were purchased from Jackson Laboratories (Bar Habor, ME), and were 4–10 weeks old when studied.

Antigens

Porcine myelin basic protein (PMBP) was obtained as a gift from Eli Lilly (Indianapolis, IN). Murine myelin basic protein (MMBP) was prepared in our laboratory as previously described (7).

Generation of MBP-specific T cell lines

MBP-specific T cell lines were generated using methods that we have previously described (8) but with differences described in the Results section. In general, mice were immunized in both hind footpads using a total of 100 µg of PMBP emulsified in complete Freund’s adjuvant (CFA)
containing 2.5 mg/ml H37RA mycobacteria (Difco, Detroit, MI). At 8–10 days after this immunization, draining lymph nodes were removed and single-cell suspensions prepared. These lymph node suspensions were initially cultured in ‘mouse media’ (MM) with or without IL-2, along with 13–25 µg/ml of PMBP and in the presence or absence of irradiated syngeneic splenocytes as a source of additional antigen-presenting cells (APC). MM is RPMI 1640 with 10% FCS and 5×10^(-5) M 2-mercaptoethanol. After 3 days, recombinant human IL-2 (16–20 U/ml; Genzyme, Cambridge, MA) was added to the cultures and continued for another 4 days. Cultures were then ‘rested’ by replacing the media with fresh MM in the absence of IL-2 for the next week. At the end of this week, the 2 week cycle was then restarted by stimulating the cultures with PMBP (13–25 µg/ml), irradiated (2600 rad) syngeneic splenocytes (3.1×10^6/24-well culture) and IL-2 (16–20 U/ml). Cultures were fed with fresh media and split as necessary every 3–4 days during the 2 week cycle.

In vivo mediation of EAE

T cell lines were tested for the ability to mediate EAE in vivo as we have previously described (8,9). Syngeneic, unirradiated, naive, mice were used as recipients. T cells were injected via intracardiac puncture and, in a few cases, additional cells were also injected i.p. T cells were injected 4 days after the last stimulation with APC, PMBP and IL-2, and 1 day after the next feed with fresh IL-2. The dose of T cells injected was 2–5×10^7 cells per mouse. Mice received one injection of T cells, and then received no other treatments or interventions. All recipient mice were 4–10 weeks old when studied. All mice were followed for signs of EAE for a minimum of 3 months after the single injection of T cells. EAE was graded as previously described (8,9). Grade 1, flaccid tail; grade 2, leg weakness and gait abnormality; grade 3, hind leg dragging; grade 4, hind leg dragging with front leg weakness; grade 5, death.

Evaluation of CNS histopathology

The pathological specimens were obtained and the CNS histopathology evaluated as we have previously described (7,9). The histopathology was evaluated without knowledge of the clinical status of the mice.

Antigen-specific proliferative assays

T cell lines were assayed for antigen-specific proliferation as previously described (7–10). T cells were assayed only after they had been rested in the absence of IL-2 for 1 week. T cells were plated in 96-well plates in the absence of IL-2 and co-cultured either with irradiated splenocytes alone (i.e. APC alone) or irradiated splenocytes and PMBP or MMBP (25 µg/ml). After 48 h in culture, [3H]thymidine (2 µCi/well) was added to the cultures and the wells harvested with the aid of a semiautomated cell harvester 18 h later. The incorporation of [3H]thymidine into cellular DNA was assayed using a β scintillation counter, and the results expressed as mean and SD of triplicate wells.

Assay for cytokine secretion

T cell lines were tested after they had been rested in the absence of IL-2 for 1 week. T cells (5×10^5/ml) were plated in MM in the absence of IL-2 in 24-well plates that had been coated with 10 µg/ml anti-murine CD3 mAb (Pharmingen, San Diego, CA). After 3 days, supernatants from these cultures were harvested, filtered and frozen until assayed. Assays for murine IFN-γ and murine tumor necrosis factor (TNF)-α were performed using cytokine-specific ELISA (Genzyme). Results are expressed as µg or ng of cytokine/ml of supernatant generated by 5×10^5 cells.

Assay for expression of Fas and α4 integrin

Surface Fas expression by T cell lines was determined using the FITC-conjugated monoclonal anti-Fas antibody Jo-2 (Pharmingen) and FACS analysis using a FACScan flow cytometer. The surface expression of VLA-4 (α4 integrin) was assayed using FITC-labeled anti-α4 integrin monoclonal antibody (Pharmingen) and FACS analysis.

Results

Generation and EAE mediation of wild-type C57BL/6 T cell lines

We generated our initial T cell line from a wild-type C57BL/6 mouse immunized with PMBP in CFA. Nine days later, the draining lymph node cells were harvested and placed into culture. These lymph nodes were initially cultured with 13 µg/ml of PMBP and in the absence of added irradiated splenocytes. A T cell line (termed LNC 2) was subsequently generated from these cultures through biweekly stimulation with PMBP (13 µg/ml), APC (i.e. irradiated syngeneic splenocytes) and IL-2, followed by 1 week of culture in the continued presence of IL-2. This was followed by a 1 week period of ‘rest’ in the absence of PMBP, APC and IL-2. Over the next 4 months we assessed the ability of LNC 2 to mediate EAE after transfer to naive, unirradiated C57BL/6 mice. In agreement with studies from other laboratories (11,12), we found that LNC 2 was consistently incapable of mediating clinical EAE. With the transfer of 2–3×10^7 cells via an intracardiac route, no clinical signs of EAE were ever noted (Fig. 1). All injected mice were monitored for signs of EAE for up to 4 months.

Activation-induced cell death (AICD) selection

Next, we hypothesized that MBP-reactive T cell lines from such EAE-resistant C57BL/6 mice might be able to mediate EAE if they were to become relatively or absolutely resistant to apoptosis—specifically to AICD. To attempt to select in vitro for cells that were resistant to AICD, we generated an MBP-reactive T cell line from a second immunized C57BL/6 mouse. This second T cell line (termed LNC B), generated from the draining lymph node cells of a C57BL/6 mouse 8 days after immunization with PMBP in CFA, was initially cultured with the addition of irradiated C57BL/6 splenocytes (2×10^5/well) in addition to PMBP and IL-2. The irradiated splenocytes were added theoretically to serve as an additional source of APC in the initiation of this T cell line. At the end of the initial 3 days of in vitro culture, a portion of the cells was re-stimulated with APC (5×10^5/well irradiated syngeneic splenocytes) and PMBP (25 µg/ml) in IL-2. We postulated that the T cells undergoing a second stimulation via the TCR complex while already actively
Encephalitogenic T cell lines in EAE resistance

Fig. 1. Mediation of EAE after T cell line transfer. C57BL/6-derived (or C57BL/6-lpr-derived) T cell lines were adoptively transferred into naive, unirradiated wild-type C57BL/6 mice. C3H/HeJ-derived T cell lines were adoptively transferred into naive, unirradiated C3H/HeJ mice. Each solid circle represents an individual wild-type C57BL/6 mouse or C3H/HeJ mouse that has been injected via an intracardiac route with 2–3×10^7 cells of the specified cell line. Each open circle represents an individual C57BL/6 wild-type mouse injected with 2–2.5×10^7 cells via an intracardiac route along with 1.5–2×10^7 cells injected i.p. (i.e. 3.5–4.5×10^7 total cells). Signs of EAE were graded using the following scale: grade 1, tail weakness; grade 2, hind leg weakness and abnormal gait; grade 3, hind leg dragging; grade 4, hind leg dragging with front leg weakness. One-half units indicate clinical signs that are mid-way between two clinical grades.

Fig. 1. Mediation of EAE after T cell line transfer. C57BL/6-derived (or C57BL/6-lpr-derived) T cell lines were adoptively transferred into naive, unirradiated wild-type C57BL/6 mice. C3H/HeJ-derived T cell lines were adoptively transferred into naive, unirradiated C3H/HeJ mice. Each solid circle represents an individual wild-type C57BL/6 mouse or C3H/HeJ mouse that has been injected via an intracardiac route with 2–3×10^7 cells of the specified cell line. Each open circle represents an individual C57BL/6 wild-type mouse injected with 2–2.5×10^7 cells via an intracardiac route along with 1.5–2×10^7 cells injected i.p. (i.e. 3.5–4.5×10^7 total cells). Signs of EAE were graded using the following scale: grade 1, tail weakness; grade 2, hind leg weakness and abnormal gait; grade 3, hind leg dragging; grade 4, hind leg dragging with front leg weakness. One-half units indicate clinical signs that are mid-way between two clinical grades.

Dividing should undergo apoptosis (AICD) (13). We further postulated that any T cells remaining viable and able to be expanded in culture after such a re-stimulation should be relatively resistant to AICD. Only one round of this 'AICD selection' was undertaken with LNC B. The T cell line growing out after this selection, using the usual 2 week growth cycle, was termed 'LNC C-Apop'. After this day 3 re-stimulation, both the parent line (i.e. LNC B) and the AICD-selected line (i.e. LNC C-Apop) were subsequently propagated similarly and in the usual fashion but with 25 µg/ml rather than 13 µg/ml PMBP.

After a number of weeks of in vitro growth, the T cell line LNC C-Apop as well as its parent line LNC B were tested for encephalitogenicity via intracardiac injection (2–3×10^7 cells) into naive, unirradiated C57BL/6 mice. In addition to the intracardiac injection, a few mice also received additional cells via an i.p. injection. As expected, the parent line, LNC B, was incapable of mediating EAE (Fig. 1). In contrast, the AICD-selected subline, LNC C-Apop, although derived from the non-encephalitogenic line LNC B, was able to induce significant clinical EAE (Fig. 1). We also attempted a second round of AICD selection with the LNC C-Apop T cell line using the same re-stimulation protocol. The resulting T cell line, termed LNC Apop C-2, also was an efficient mediator of EAE, though not significantly better than the LNC C-Apop line (Fig. 1). These two AICD-selected T cell lines were found to transfer EAE after a single injection and did not require any treatment of the naive recipient mice.

Generation and EAE mediation of C57BL/6-lpr T cell lines

In light of the apparent success of our AICD selection approach, we next attempted to confirm the relevance of AICD to EAE susceptibility. To do this we studied mice with the lpr mutation bred onto a C57BL/6 background. The T cells from such mice are deficient in their ability to undergo AICD as a result of their lack of Fas expression (13). We postulated that T cells from lpr mice might be similar to our AICD-selected population but without having to be selected in vitro. We immunized two C57BL/6-lpr mice with PMBP and CFA, and generated T cell lines using either the 8 day (LPR 2B) or 15 day (LPR 3C) draining lymph node cells. It is important to note that we did not add irradiated C57BL/6 splenocytes at the initiation of the culture and we used a slightly higher concentration of PMBP (25 versus 13 µg/ml) from the start. We did not select these lpr-derived T cell lines using AICD selection. We generated one PMBP-reactive T cell line from each of the C57BL/6-lpr mice. The two lpr-derived lines (LPR 2B and LPR 3C) were expanded using the same in vitro growth protocol as the T cell lines generated for generating the LNC C-Apop line.
from wild-type C57BL/6 mice. Despite not being AICD selected, both LPR 2B and LPR 3C were found to consistently transfer clinical EAE to naive, unirradiated wild-type C57BL/6 mice (3×10⁷ T cells injected via an intracardiac route) (Fig. 1).

Second approach for generating C57BL/6-derived T cell lines

To determine whether it was the lpr phenotype or the alteration in protocol that allowed for the generation of encephalitogenic T cell lines from C57BL/6-lpr mice, we next generated a T cell line (LNC 3) from a wild-type C57BL/6 mouse using the protocol used for the C57BL/6-lpr T cell lines. As with the C57BL/6-lpr lines, in this case we did not use AICD selection. In this protocol, the draining lymph nodes were initiated in culture with the increased concentration of PMBP (25 µg/ml) and no irradiated splenocytes were added to the initiating cultures. Surprisingly, in this case, the unselected (i.e. not AICD selected) LNC 3 T cell line was capable of mediating EAE in ~85% of the recipient mice injected (3×10⁷ T cells transferred via an intracardiac route to naive, unirradiated wild-type C57BL/6 mice) (Fig. 1). We also selected a subline from LNC 3, termed ‘3Apop2’, using the AICD selection process. The AICD-selected line, 3Apop2, also mediated EAE in ~85% of the recipient mice injected. The EAE mediated by 3Apop2 was slightly, but not significantly, more severe than that mediated by the unselected parent line, LNC 3 (Fig. 1).

Generation of encephalitogenic T cell lines from C3H/HeJ mice

Based on the findings with LNC 3, it became clear that differences in the initiating culture conditions (i.e. no initially-added APC, higher antigen concentration), in addition to our AICD selection procedure, could lead to the successful generation of encephalitogenic T cell lines from C57BL/6 mice. To further study this, we generated T cell lines from a second EAE-resistant strain of mice, C3H/HeJ mice. As with C57BL/6 mice, C3H/HeJ mice have previously been reported to be completely resistant to both primary and secondary EAE. C3H/HeJ mice were immunized in the usual fashion with PMBP and CFA, and the draining lymph node cells were taken at day 8. To determine the relevance of the initially added APC, these lymph node cells were initiated in culture in one of two ways: with 25 µg/ml of PMBP and no added irradiated splenocytes (resulting in the T cell line, LNC HeJ F) or with 25 µg/ml of PMBP together with the addition of irradiated C3H/HeJ splenocytes (3×10⁶ irradiated splenocytes per 3×10⁶ lymphocytes) (resulting in the T cell line, LNC HeJ E). After the first 3 days in culture, these T cell lines were propagated similarly using the standard in vitro propagation protocol used with our C57BL/6-derived T cell lines. Neither LNC HeJ E nor LNC HeJ F underwent AICD selection. These lines were then tested for the mediation of EAE by being adoptively transferred (3×10⁷ T cells via an intracardiac route) into naive, unirradiated C3H/HeJ mice. As can be seen in Fig. 1, LNC HeJ E (added spleen) was found to be incapable of mediating EAE. In contrast, LNC HeJ F (no added spleen) consistently mediated severe EAE. These studies suggested that although an increase in PMBP concentration used in the initiation and propagation of the T cell lines is important, the addition of irradiated splenocytes to the initial cultures leads to a loss of encephalitogenicity in the T cell line generated. Overall, we concluded that we could generate encephalitogenic T cell lines from EAE-resistant mouse strains either through AICD selection when the PMBP concentration used is lower and exogenous splenocytes are added to the initiating cultures or alternatively by initiating the T cell lines in the absence of additional APC and using a higher concentration of antigen.

Characterization of EAE in resistant mice

The recipient mice in all of our in vivo transfer studies were naive wild-type C57BL/6 or C3H/HeJ mice, were not irradiated and did not receive any treatment other than the one injection of the T cell line. The time-course of the development of clinical signs of EAE after T cell transfer was the same whether wild-type C57BL/6 or C3H/HeJ or lpr-derived T cell lines were used. Furthermore, this time-course was identical to that we and others have previously described in passively transferred EAE in SJL mice (8,9). We noted the onset of clinical signs usually between day 6 and 8 after the transfer of cells. The signs of EAE usually progressed to their maximal level between day 10 and 12. Subsequent to this, the mice either stayed at the same clinical level or improved. Mice have been observed for up to 4 months and we have not noted a second ‘exacerbation’ of disease.

The progression of clinical signs after the transfer of our C57BL/6-, C57BL/6-lpr or C3H/HeJ-derived encephalitogenic T cell lines was essentially identical to that seen in EAE mediated by T cell lines generated from EAE-susceptible strains of mice. Thus, in most of our recipient mice, the first abnormality noted was tail weakness (grade 1), and (to various degrees) this was followed in progression by hind leg weakness and gait abnormality (grade 2), hind leg dragging (grade 3), and finally front leg weakness (grade 3.5–4.5). However, in a small proportion of our mice, the first abnormality noted was hind leg weakness with a gait abnormality and leaning to one side, and this was followed in 1–2 days in these mice by tail weakness and then a normal progression of signs.

Histopathological evaluation

The CNS histopathology of the mice injected with our C57BL/6-derived T cell lines was examined and graded for CNS inflammation without knowledge of the clinical status of the mice (data not shown). The T cell lines that did not mediate clinical EAE did not mediate histological evidence of EAE. All of the T cell lines that mediated clinical signs of EAE were found to mediate classical histological changes of EAE with lymphocyte perivascular cuffing, and variable amounts of meningeal and tissue lymphocytic infiltration. In all instances, there was a direct correlation noted between the degree of histological inflammation seen and the grade of clinical EAE observed. There was no qualitative or quantitative difference among the histological abnormalities seen after the transfer of AICD-selected, wild-type non-AICD-selected or lpr-derived encephalitogenic T cell lines (data not shown).

In vitro antigen-specific proliferative responses of T cell lines

All of our T cell lines were tested for in vitro proliferative responses to both PMBP and to MMBP. It has previously been
demonstrated that encephalitogenic T cells cross-react with MMBP as well as with the xenogeneic MBP used as the immunogen (7). Our T cell lines were generated through immunization with PMBP and grown in vitro with PMBP. In these proliferative assays, syngeneic irradiated splenocytes derived from wild-type C57BL/6 or C3H/HeJ mice were used as APC in testing all wild-type or lpr-derived T cell lines.

The in vitro proliferative responses of the T cell lines to both PMBP and MMBP are shown in Table 1. All of the lines, whether or not they are capable of mediating EAE, respond to both PMBP and MMBP. Furthermore, there is no correlation between the ability to mediate EAE and the magnitude of the proliferative responses to either PMBP or MMBP (Table 1).

**Cytokine secretion by the T cell lines**

All of our T cell lines, generated from either wild-type C57BL/6 or C3H/HeJ mice or from C57BL/6- lpr mice, were phenotypically 100% CD4+ CD8- by FACS analysis (data not shown). To assay for cytokine secretion, the T cell lines were stimulated with immobilized anti-CD3 antibody in the absence of APC, antigen and IL-2, and the supernatants harvested after 3 days. The secretion of IFN-γ and TNF-α by these lines was determined using cytokine-specific ELISA. Results of such assays are shown in Table 2. All of our lines were found to be type 1 helper cells based on their secretion of IFN-γ and TNF-α. We found no correlation between the ability of any of our T cell lines to mediate EAE and the amount of either IFN-γ or TNF-α secreted by these lines after stimulation with immobilized anti-CD3 antibody (Table 2) (or after stimulation with APC and MMBP; data not shown).

**Expression of Fas by the T cell lines**

In light of our success in generating encephalitogenic T cell lines from C57BL/6 mice after AICD selection and from C57BL/6- lpr mice, we measured the expression of the Fas molecule on the surface of those C57BL/6 T cell lines that had been involved in AICD selection. Figure 2 shows an example of flow cytometric analysis of binding of FITC-conjugated anti-murine Fas antibody to our T cell lines. As expected, the T cell lines LPR 2B and LPR 3C, derived from Fas-deficient C57BL/6-lpr mice, showed no staining with the anti-Fas antibody (Fig. 2A and B). The non-encephalitogenic T cell lines LNC B and LNC 2, the AICD-selected encephalitogenic T cell line LNC C-Apop (and LNC Apop C-2, data not shown), and the encephalitogenic line LNC 3 all expressed similar levels of Fas (Fig. 2C–F). For all of our T cell lines, we found no correlation between encephalitogenicity and the level of expression of Fas.

**Expression of VLA-4 by the T cell lines**

It has been suggested that the surface expression of VLA-4 is important for T cell entry into the CNS and thus for T cell encephalitogenicity (14,15). We have assayed for VLA-4 expression using a mAb to the α chain of the VLA-4 molecule. All of our wild-type C57BL/6, C57BL/6-lpr and C3H/HeJ-derived T cell lines express VLA-4 but we have found no correlation between the level of VLA-4 expression as measured by flow cytometry and encephalitogenicity with any of our T cell lines (data not shown). Thus, the differences in encephalitogenicity seen among our T cell lines is not related to a difference in VLA-4 expression.

**Discussion**

While our knowledge of EAE is extensive, there are still significant gaps in our understanding of fundamental pathophysiologic processes involved in this model of autoimmune disease. One significant gap in our knowledge of EAE, that may also be very relevant in understanding MS, is that of ‘EAE resistance’. Thus, in mice, rats and other species, EAE can be induced in only a small number of strains, though the basis for the EAE resistance remains unclear.

MBP is the most often studied and most well-characterized autoantigen used in the induction of EAE. In almost all published reports, C57BL/6 mice have been found to be resistant to both the primary induction of EAE induced with MBP and to the adoptive transfer of ‘secondary’ EAE with MBP-sensitized lymph node cells. Despite their documented resistance to both primary and secondary EAE induced with MBP, C57BL/6 mice are capable of mounting a T cell response to MBP. Many investigators have used C57BL/6 mice to study EAE resistance. For example, Skundric et al. used MBP-

### Table 1. T cell proliferative responses to PMBP and MMBP

<table>
<thead>
<tr>
<th>T cell</th>
<th>APC alone</th>
<th>APC and PMBP</th>
<th>APC and MMBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC 2</td>
<td>11,129</td>
<td>75,345</td>
<td>80,436</td>
</tr>
<tr>
<td>LNC C-Apop</td>
<td>945</td>
<td>85,959</td>
<td>78,847</td>
</tr>
<tr>
<td>LNC Apop C-2</td>
<td>1436</td>
<td>115,085</td>
<td>107,110</td>
</tr>
<tr>
<td>LNC B</td>
<td>1217</td>
<td>138,529</td>
<td>104,903</td>
</tr>
<tr>
<td>LNC 3</td>
<td>4316</td>
<td>246,682</td>
<td>26,689</td>
</tr>
<tr>
<td>3 Apop 2</td>
<td>4181</td>
<td>247,340</td>
<td>21,689</td>
</tr>
<tr>
<td>LPR 2B</td>
<td>2562</td>
<td>162,315</td>
<td>155,025</td>
</tr>
<tr>
<td>LPR 3C</td>
<td>40,584</td>
<td>137,547</td>
<td>129,584</td>
</tr>
<tr>
<td>HeJ F</td>
<td>3997</td>
<td>497,178</td>
<td>128,732</td>
</tr>
<tr>
<td>HeJ E</td>
<td>2579</td>
<td>200,583</td>
<td>48,844</td>
</tr>
</tbody>
</table>

*Three-day proliferation assay using 2.5×10⁴ T cells, 2.5×10⁵ irradiated (2600 rad) syngeneic, wild-type C57BL/6 or C3H/HeJ splenocytes (APC) and 25 µg/ml PMBP or MMBP.

*Values represent the mean c.p.m. ([³H]thymidine) of triplicate wells. SD < 20% of the means.

### Table 2. Cytokine secretion of T cell lines

<table>
<thead>
<tr>
<th>T cell line</th>
<th>IFN-γ (µg/ml)</th>
<th>TNF-α (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC 2</td>
<td>1.7</td>
<td>58</td>
</tr>
<tr>
<td>LNC B</td>
<td>1.1</td>
<td>59</td>
</tr>
<tr>
<td>C-Apop</td>
<td>1.6</td>
<td>50</td>
</tr>
<tr>
<td>LPR 2B</td>
<td>1.4</td>
<td>57</td>
</tr>
<tr>
<td>LNC 3</td>
<td>1.2</td>
<td>37</td>
</tr>
<tr>
<td>LPR 3C</td>
<td>4.1</td>
<td>40</td>
</tr>
<tr>
<td>HeJ E</td>
<td>0.55</td>
<td>30</td>
</tr>
<tr>
<td>HeJ F</td>
<td>0.62</td>
<td>34</td>
</tr>
</tbody>
</table>

*The cytokine secretion of T cell lines was determined by ELISA using supernatants harvested 3 days after stimulation of the T cell lines with immobilized anti-CD3 antibody. The results are presented as µg or ng of cytokine/ml of supernatant generated by 5×10⁵ T cells.
Encephalitogenic T cell lines in EAE resistance

Fig. 2. FACS analysis of anti-Fas binding. Surface expression of Fas was determined using the anti-murine Fas mAb, Jo-2, and FACS analysis. Unshaded curves represent background binding and shaded curves represent binding with anti-Fas antibody. (A) LPR 2B. (B) LPR 3C. (C) LNC B. (D) LNC C-Apop. (E) LNC 2. (F) LNC 3.

responsive T cells derived from C57BL/6 mice bearing the Thy-1.2 allele to study the homing of such autoreactive, but non-encephalitogenic cells after adoptive transfer into the Thy-1.1 congenic strain C57BL/Ka (12). These investigators found that the transfer of such cells resulted in no evidence of clinical or histological EAE.

There is only one report in which MBP-driven EAE has been reported to be inducible in C57BL/6 and other resistant strains of mice, and this was accomplished using an unusual induction protocol (11). In this report, Shaw et al. demonstrated that MBP-driven EAE could be induced in C57BL/6 mice but only using a combined approach that included the i.v. injection of MBP-primed (and in vitro re-stimulated) lymphocytes given simultaneously with a s.c. injection of MBP and CFA. This study again confirmed, however, that the MBP-stimulated T cell lines generated from C57BL/6 mice (and C3H/HeJ mice), when used alone, are totally incapable of transferring EAE even at doses of $5 \times 10^7$ cells. The mechanisms underlying the efficacy of this unusual induction protocol for C57BL/6 mice have never been established. In the present report, we now describe two simple and efficient approaches for the generation of encephalitogenic T cell lines from both C57BL/6 and C3H/HeJ mice. These T cell lines mediate EAE when
Given as a single injection and do not require any further or additional treatment of the recipient mice.

Our first approach evolved from an attempt to select for T cell lines that are resistant to AICD (13). A number of studies have reported that T cells found in the CNS in MS and EAE appear to be undergoing apoptosis (1–6). It has been postulated that this T cell apoptosis observed in the CNS may result from either antigen-stimulated AICD, apoptosis-inducing cytokines or the glucocorticoids secreted in response to the autoimmune inflammation. Most investigators have concluded that this apoptosis represents an important means of regulating the autoreactive T cells and the autoimmune inflammatory response in the CNS (reviewed in 4). Apoptosis of T cells in the CNS has not only been seen in EAE, but has also been reported in active lesions of MS (5). Thus, there is significant evidence accruing that suggests that apoptosis of CNS-antigen-reactive T cells may represent an important immunoregulatory mechanism in EAE and MS (1–6, 16).

Given that T cell apoptosis may be an important mechanism in controlling T cell autoimmunity in EAE, we postulated that if CNS-autoreactive T cells were relatively or absolutely resistant to apoptosis, this would result in an increased likelihood of CNS autoimmunity disease. The present studies were initiated to test this postulate using EAE-resistant, C57BL/6 mice. We found that when MBP-immunized lymph node lymphocytes were initially cultured with a relatively low concentration of antigen and with additional APC in the form of syngeneic irradiated splenocytes, the resulting long-term lines were not encephalitogenic (LNC B). However, if subcultures of these lines were immediately re-stimulated with antigen and APC in an attempt to negatively select AICD-sensitive cells, the surviving T cells could be propagated into a long-term line that was consistently encephalitogenic (LNC C-APOP B). We initially believed we had confirmed our AICD selection in wild-type mice (LNC C-APOP C-2). We later demonstrated the effectiveness of this second approach using another EAE-resistant strain, C3H/HeJ mice (LNC C-APOP HeJ F).

It is not as yet clear whether our two approaches are related and whether either or both are related to apoptosis sensitivity. We are presently pursuing studies to determine the AICD sensitivity of all of our encephalitogenic populations. To date we have not been able to document a consistent difference in AICD sensitivity between our encephalitogenic and non-encephalitogenic T cell lines, regardless of the initiating approach used. Therefore, ongoing studies in our laboratory are directed toward identifying all genotypic and phenotypic differences between our encephalitogenic and non-encephalitogenic T cell lines and clones. The two approaches described in the present report will now be useful for identifying factors relevant to the pathogenesis of EAE, and will also greatly facilitate the use of knockout and mutant mice in the study of EAE.

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Abbreviations
AICD activation-induced cellular death
APC antigen-presenting cell
CFA complete Freund’s adjuvant
CNS central nervous system
EAE experimental allergic encephalomyelitis
MM mouse media
MS multiple sclerosis
MBP myelin basic protein
MMBP murine myelin basic protein
PMBP porcine myelin basic protein
TNF tumor necrosis factor

References
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