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*J Immunol* 1999; 162:6392-6400; ;
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Evidence for Fas-Dependent and Fas-Independent Mechanisms in the Pathogenesis of Experimental Autoimmune Encephalomyelitis

Bonnie N. Dittel, Raina M. Merchant, and Charles A. Janeway, Jr.

To determine whether Fas or Fas ligand (FasL) plays a role in susceptibility to experimental autoimmune encephalomyelitis (EAE), we bred a TCR transgenic mouse specific for the Ac1–11 peptide of myelin basic protein to mice with inactivating mutations in Fas (lpr) or FasL (gld). Disease induction by peptide immunization in such mice produced similar disease scores, demonstrating that Fas/FasL interactions were not necessary to generate EAE. However, adoptive transfer experiments showed evidence that these interactions can play a role in the pathogenesis of EAE, shown most dramatically by the absence of disease following transfer of cells from a normal myelin basic protein TCR transgenic mouse into a Fas-deficient lpr recipient. Furthermore, transfer of cells lacking FasL (gld) into normal or gld recipients gave a diminished disease score. Thus, Fas/FasL interactions can play a role in the pathogenesis of EAE, but they are not required for disease to occur. The Journal of Immunology, 1999, 162: 6392–6400.

Experimental autoimmune encephalomyelitis (EAE) is an animal model for the human demyelinating autoimmune disease multiple sclerosis (1). EAE is clinically characterized by focal areas of inflammation and demyelination and an infiltrate containing large numbers of lymphocytes and macrophages throughout the CNS (2). The disease is acute or chronic relapsing, with a clinical course characterized by a rapid onset of hind limb weakness that commonly progresses to paralysis, followed by spontaneous remission starting 7–10 days after the initial appearance of symptoms. EAE can be actively induced in genetically susceptible animal strains by the injection of myelin basic protein (MBP) in appropriate adjuvants and is mediated by activated CD4+ T cells that are specific for the encephalitogenic portion of MBP (3, 4). Immunization of the B10.PL mouse strain (H-2<sup>d</sup>) with MBP or its encephalitogenic NH<sub>2</sub>-terminal acetylated peptide (MBP Ac1–11) emulsified in CFA leads to the development of acute EAE (5). EAE can also be induced passively by the adoptive transfer of in vitro activated CD4<sup>+</sup> T cells or clones, typically of the Th1 phenotype, into irradiated susceptible recipients (6). In B10.PL mice the quantity of encephalitogenic T cells transferred determines the disease outcome, with the disease course moving from acute to long term chronic to death with transfer of increasing numbers of T cells (6, 7).

The exact mechanism of EAE pathogenesis mediated by encephalitogenic T cells is currently unknown, but undoubtedly involves TNF-α, lymphotoxin-α (LT-α; also known as TNF-β), and FasL (CD95L), members of the TNF family of cytokines (8). The production of TNF-α and LT-α by Th1 clones has been correlated with encephalitogenic potential (9), and Abs to both prevent EAE upon transfer of encephalitogenic clones (10). Upon activation, Th1 cells not only produce TNF and LT, but up-regulate cell surface expression of FasL (11). The subsequent engagement of Fas (CD95), the counter-receptor of FasL, leads to cell death via apoptosis of the Fas-expressing target (12, 13). The Fas/FasL interaction has been linked to regulation of homeostasis within the immune system, dysregulation of which leads to lymphadenopathy, splenomegaly, and autoimmune disorders (14, 15).

Two recent reports investigating the roles of Fas and FasL in EAE induction have shown that a disruption in a functional Fas and FasL interaction has a protective effect on the active induction of EAE using mice carrying the lpr or gld mutations (16, 17). We expanded upon these studies by using an MBP transgenic (tg) mouse to examine the role of Fas/FasL interaction in the active and passive induction of EAE. We found that immunization of MBP mice carrying gld or lpr mutations did not protect against EAE induction, and these animals had a disease course similar to that of MBP-wt mice. In addition, the adoptive transfer of FasL-deficient T cells into wt or gld recipients only led to a partial reduction in EAE disease severity. However, a protective effect was detected when the recipient mice were deficient in Fas. Therefore, it is clear that interaction of Fas with FasL is not necessary to develop EAE, but it can play a role in EAE pathogenesis. Thus, both Fas-dependent and Fas-independent mechanisms contribute to EAE pathogenesis.

Materials and Methods

**Mice**

B10.PL (I-A<sup>b</sup>), C57BL/6-gld/gld, and C57BL/6-lpr/lpr mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MBP-TCR tg mice expressing a TCR transgene specific for the acetylated N-terminal peptide of MBP bound to I-A<sup>b</sup> were generated as previously described (18) and reared in our colony at Yale University (New Haven, CT). B10.PL-gld/gld (B10.PL-gld) and B10.PL-lpr/lpr (B10.PL-lpr) mice were produced in our breeding colony by backcrossing C57BL/6-gld/gld and C57BL/6-lpr/lpr mice onto B10.PL, respectively, and then were crossed...
with the MBP TCR tg (MBP-wt; backcrossed to B10.PL for >10 generations) to generate MBP-TCR-gld/gld (MBP-gld) and MBP-TCR-lpr/lpr (MBP-lpr) mice. All mice were between 5 and 8 wk of age when used.

Peptides and Abs

The N-terminal MBP peptide Ac1–11 (Ac-ASQKRPSQRSK) was synthesized and HPLC purified by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. A clonotypic mAb (19G) specific for the MBP-TCR was produced in our laboratory using standard hybridoma generation techniques (6). Anti-mouse CD4-PE and anti-mouse CD8-R613 were purchased from Life Technologies (Gaithersburg, MD). Anti-mouse IgG-FITC (Fc specific) was purchased from Sigma (St. Louis, MO). Anti-mouse CD49d-biotin, anti-mouse TCR Vβ8/1.2-FFTC, and anti-mouse Fas-PE were purchased from PharMingen (San Diego, CA). Streptavidin-PE was purchased from Caltag (Burlingame, CA).

Cells

MBP-TCR CD4+ T cell lines were generated from the spleens of MBP-wt, MBP-gld, and MBP-lpr mice. Briefly, spleens were minced, and following removal of RBC, 20 × 10^8 total splenocytes were cultured in 25-cm² flasks in the presence of 5 μg/ml Ac1–11 in Click’s Eagle’s Hanks’ amino acid medium containing 5% FCS. After 3–4 d, the cellular contents from each flask were transferred to a 75-cm² flask and maintained in Click’s medium containing 10% FCS and 2 U/ml IL-2. Four days before adoptive transfer, the MBP T cell lines were restimulated with Ac1–11 (5 μg/ml) in the presence of inactivated splenocytes from B10.PL mice. On the day of transfer, live cells were isolated by density gradient separation and washed three times in PBS, and 1 × 10^6 T cells were adoptively transferred in 200 μl of PBS. On the day of adoptive transfer, the cell surface expression of CD4, Vβ8.2, CD49d, and the MBP-TCR (mAb 19G) was confirmed by cell surface staining and flow cytometry, and was ≥99% positive for these markers. In addition, the T cell lines were analyzed for stimulation with Ac1–11 by measuring cell proliferation and cytokine production. All lines produced the Th1 cytokine IFN-γ as detected by ELISA as previously described (19).

Immunofluorescence

Three-color immunofluorescence using anti-MBP-TCR (19G detected with anti-mouse FITC), anti-CD4-PE, and anti-CD8-R613 was performed on single-cell suspensions from thymus and lymph nodes from B10.PL, B10.PL-gld, B10.PL-lpr, MBP-wt, MBP-gld, and MBP-lpr mice at 5 wk of age. Single-color immunofluorescence using anti-CD4-PE, anti-Vβ8/1.2-FFTC, anti-CD49d-biotin detected with streptavidin-PE, and anti-MBP-TCR (19G) detected with anti-mouse Fc-specific IgG-FITC was conducted on T cell lines generated from MBP-wt, -gld, and -lpr mice on the day of transfer. Ab incubations were conducted on ice, and the cells were fixed in 1% paraformaldehyde and analyzed using CellQuest on a FACScan (Becton Dickinson, Mountain View, CA).

T cell activation assay and IFN-γ secretion

For proliferation analysis, 2–4 × 10^6 rested MBP-wt, MBP-gld, and MBP-lpr T cells were restimulated with 5 μg/ml Ac1–11 and inactivated B10.PL splenocytes at least 7 days following primary stimulation. Four days later, dead cells were removed by density separation, washed three times, and plated at 1/2 dilutions of cells starting at 2.5 × 10^5 cells/well. Upon plating the cells were pulsed with [3H]TdR for 18 h and counted on a beta counter to measure proliferation (counts per minute). For Ag-specific proliferation and cytokine production, 20,000 rested T cells were cocultured with 1.5 × 10^5 irradiated B10.PL (H-2b) spleen cells in the presence of 1/10 dilutions of Ac1–11 from 0.001–10 μg/ml. After 24 h, culture supernatants were collected and analyzed by ELISA for the production of IFN-γ as previously described (19), using an anti-mouse IFN-γ and anti-mouse IFN-γ-biotin, both purchased from PharMingen (San Diego, CA). Cultures were pulsed after 48 h with [3H]TdR and were harvested 15 h later.

EAE induction

Groups of four or five MBP-wt, MBP-gld, and MBP-lpr female mice were immunized with 75 μg of MBP Ac1–11 emulsified in CFA containing 4 mg/ml of heat-killed mycobacterium tuberculosis H37Ra (Sigma) s.c. in each internal flank. Two hundred nanograms of pertussis toxin (List, Campbell, CA) in PBS was injected i.v. at the time of immunization and again 48 h later. For experiments requiring adoptive transfer of encephalitogenic T cells, irradiated (600 rad) female B10.PL, B10.PL-gld, or B10.PL-lpr mice were i.v. injected with 1 × 10^6 CD4+ T cells generated from MBP-wt, MBP-gld, or MBP-lpr mice. Individual animals were assessed daily for symptoms of EAE and were scored using a scale from 1–5 as follows: 0, no disease; 1, limp tail and/or wobbly walk; 2, hind limb paresis; 3, hind limb paralysis; 4, hind and fore limb paralysis; and 5, death.

Cytokine mRNA synthesis

RT-PCR was used to detect cytokine synthesis by CD4+ T cell lines generated from MBP-wt, MBP-gld, and MBP-lpr mice. Total RNA was isolated from 5 × 10^6 T cells on the day of transfer using Trizol (Life Technologies). cDNA was synthesized as previously described (19) from 5 μg of RNA using Superscript II reverse transcriptase (Life Technologies). PCR reactions were performed using 1 μl of cDNA for all primers except IL-5, with one amplification cycle of denaturation at 94°C for 5 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; followed by 30 or 35 cycles of 94°C for 1 min, 72°C for 1 min, and 94°C for 1 min; and ending with a final extension at 72°C for 15 min. The annealing temperature for the IL-5 primers was 60°C.

Primer sequences for hypoxanthine phosphoribosyltransferase (HPRT), IFN-γ, TNF-α, IL-2, and IL-4 (20) and primers for perforin (21) were previously described. The forward primer for IL-1α was TGGCTGGGAA CAGGGGAAGGTTGAGC; the reverse primer was CGTGCTTTCTTCTA GAACCCCCTTG (22). The forward primer for IL-5 was GAAAGAGAC CTTGACACAGCTG; the reverse primer was GAAACTCTTGCGAGTTA AATCCAGG. The forward primer for IL-10 was CCAGTTCCTTTCCACT GTGTAAGGATG; the reverse primer was AACACGAGACCG TGGACTCCTGGATCTG. The forward primer for FaLa was GAGT GTGCGCCATTTAACAG; the reverse primer was CTTTTAAACCTT ATACAAAGCGAAAAGGT.

Results

Positive selection of the MBP-TCR in MBP-gld and MBP-lpr mice is identical with selection observed in MBP-wt mice

To analyze positive selection of the MBP-TCR transgene containing the rearranged α- and β-chains of a TCR that recognizes the acetylated N-terminal peptide of MBP in mice carrying the gld or lpr mutation, we crossed MBP-wt mice to B10.PL-gld/gld (B10.PL-gld) or -lpr/lpr (B10.PL-lpr) mice to generate B10.PL-gld/gld and B10.PL-lpr/lpr mice with and without the MBP-TCR. The transgenic mice are called MBP-wt, MBP-gld, and MBP-lpr. FACS analysis showed that CD4 and CD8 populations were not altered in the lymph nodes or thymus of B10.PL mice. The MBP-TCR transgenic mice were analyzed for expression of MBP-TCR with the MBP TCR tg (MBP-wt; backcrossed to B10.PL for 10 generations) or MBP-gld (Fig. 1A) or MBP-lpr (Fig. 1B) mice. This is shown by identical CD4 and CD8 populations in the lymph nodes of MBP-wt mice when compared with either MBP-gld (Fig. 1A) or MBP-lpr (Fig. 1B) mice. Further analysis of MBP-TCR tg mice showed that ≤90% of CD4+ lymph node cells expressed the MBP-TCR as assessed by staining with a clonotypic mAb for the MBP-TCR (19G). Similarly, in the thymus the CD4 and CD8 single-positive (SP) and CD4 CD8 double-positive T cell populations were similar in MBP-wt, -gld, and -lpr mice. In addition, examination of thymic CD4 SP cells showed that essentially all cells expressed the MBP-TCR. These data demonstrate good positive selection of the MBP TCR transgene in MBP-wt, -gld, and -lpr mice with no accumulation of any T cell populations.

MBP-gld and -lpr mice are as susceptible to EAE induction by immunization with Ac1–11 as MBP-wt mice

To examine the role of the Fas/Fasl mechanism in EAE, we immunized both MBP-wt and MBP-gld mice simultaneously with the MBP peptide Ac1–11. The mice were evaluated daily for 40 days for clinical signs of EAE. Fig. 2A shows that the disease course for the MBP-wt and MBP-gld mice was almost identical for the two groups with a day of onset as early as days 7–8 postimmunization. The data shown in Fig. 2 are summarized in Table 1, which shows that both the MBP-wt and MBP-gld groups had a high incidence of
EAE (93 and 88%, respectively). Mortality was also similar in the MBP-gld (59%) and MBP-wt (67%) groups. These high rates of mortality suggest that MBP mice that developed EAE were generally unable to recover from its debilitating effects. Additionally, the average day of onset and peak day of disease were identical for the two groups. The average peak disease scores for the MBP-wt (3.8) and MBP-gld (3.5) mice were also comparable. The high incidence, elevated peak disease score, and early onset of EAE in MBP-gld mice suggest that the gld mutation disrupting functional pairing of the FasL with Fas does not alter the induction of or the

**FIGURE 1.** Positive selection of the MBP-TCR transgene in B10.PL, B10.PL-gld, and B10.PL-lpr mice. Lymph node cells and thymocytes from B10.PL (A and B), MBP-wt (A and B), B10.PL-gld (A), MBP-gld (A), B10.PL-lpr (B), and MBP-lpr (B) mice were stained by three-color immunofluorescence for the expression of CD4, CD8, and the MBP-TCR as described in Materials and Methods. In the first (lymph node) and third (thymus) columns, dot plots are shown comparing total numbers of CD4 (x-axis) and CD8 (y-axis) T cells. In the second column, lymph node cells were gated on CD4+ T cells and analyzed for the expression of the MBP-TCR as shown in the histograms. In the fourth column, CD4 SP thymocytes were analyzed for the expression of the MBP-TCR as shown in histograms. Animals used in the analysis were sex and age matched. The percentages of CD8 SP (upper left quadrant), CD4 SP (lower right quadrant), and CD4CD8 double-positive (upper right quadrant) are indicated.
recovery from EAE in gld/gld mice carrying the MBP transgene. The comparison of the EAE disease course in MBP-wt vs MBP-lpr mice yielded similar results, with the incidence and mortality rate being equal in the two groups (Fig. 2B and Table I). However, a delay in disease onset was observed in the MBP-lpr group, shown as a reduction in the average cumulative disease score from 91 in the MBP-wt to 72 (Fig. 2B and Table I). The decrease observed in the MBP-lpr group was not statistically significant compared with that in the MBP-wt group.

**Table I. Summary of the EAE disease course in MBP-wt vs MBP-gld and -lpr mice**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>No. of Mice</th>
<th>Incidence (%)</th>
<th>Mortality (%)</th>
<th>Average Day of Onset</th>
<th>Average Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP-wt</td>
<td>15</td>
<td>93 (14/15)</td>
<td>67 (10/15)</td>
<td>14.4</td>
<td>95.5 ± 11.2</td>
</tr>
<tr>
<td>MBP-gld</td>
<td>17</td>
<td>88 (15/17)</td>
<td>59 (10/17)</td>
<td>14.1</td>
<td>90 ± 11.1</td>
</tr>
<tr>
<td>MBP-wt</td>
<td>15</td>
<td>100</td>
<td>73 (11/15)</td>
<td>13.8</td>
<td>90.8 ± 10.1</td>
</tr>
<tr>
<td>MBP-lpr</td>
<td>13</td>
<td>92 (12/13)</td>
<td>67 (8/12)</td>
<td>20.2</td>
<td>72 ± 12</td>
</tr>
</tbody>
</table>

* Graded disease score as described in Materials and Methods.
* Represents the percentage of mice that developed a clinical score of at least one.
* Represents the percentage of mice that died or were sacrificed for humane purposes.
* The cumulative disease score was calculated by adding the disease score from the day of onset to day 40. The values shown are the mean ± SE of all the mice with disease in each group.
* Represents the number of mice that got sick vs the total number of mice in each group.
* Represents the number of mice that reached a clinical score of 5 vs the total number of mice in each group.

**FIGURE 2.** Comparison of EAE clinical course in MBP-wt, MBP-gld, and MBP-lpr mice. A, Fifteen MBP-wt and 17 MBP-gld mice were immunized with Ac1–11 for EAE as described in Materials and Methods in three separate groups. The individual mice were scored for the severity of EAE using the following scale: 0, no disease; 1, limp tail and or wobbly walk; 2, hind limb paresis; 3, hind limb paralysis; 4, hind and fore limb paralysis; and 5, death. Mice were examined daily from days 5–40, and the daily scores from all MBP-wt (○) and MBP-gld (●) mice were averaged. B, Fifteen MBP-wt (●) and 13 MBP-lpr (○) mice were immunized and were scored in identical fashion as those in the experiment shown in A.

**MBP-TGR T cell lines generated from wt, gld, and lpr mice have similar cell surface expression patterns and secrete Th1 cytokines**

To further examine the role of Fas/Fasl interactions in the pathogenesis of EAE, we generated T cell lines from MBP-wt, MBP-gld, and MBP-lpr mice. As shown in Fig. 3A, T cell lines generated from the three strains of mice are CD4+ (Fig. 3, A–C) and express the MBP-TCR (Fig. 3, D–F). The MBP-TCR expression was further confirmed by positive staining for the β-chain (Vβ8.2) contained in the MBP-TCR (Fig. 3, G–I). Since we have previously shown a correlation between encephalitogenic potential and the expression of α3 (CD49d)-containing integrins on T cell clones (6), we examined our T cell lines for expression of CD49d and found all three lines to express high levels of the integrin α subunit (Fig. 3, J–L). The efficiency in Fas expression in the MBP-lpr lines was confirmed by lack of Fas cell surface expression (Fig. 3O) compared with that in MBP-wt (Fig. 3M) and MBP-gld (Fig. 3N) lines.

Since encephalitogenicity of transferred T cells has been associated with the production of Th1 cytokines, we examined our T cell lines for cytokine expression on the day of transfer. As shown in Fig. 3P, all three lines express high levels of mRNA for the Th1 cytokines IFN-γ, TNF-α, and LT-α. Only low level expression of the Th2 cytokines IL-4, IL-5, and IL-10 was detected. Interestingly, the MBP-gld line seemed to produce more IL-10 mRNA than the MBP-wt and MBP-lpr T cell lines, and the MBP-lpr line produced less IL-2 (Fig. 3P). Since the PCR shown in Fig. 3P was not quantitative, we performed semiquantitative RT-PCR, as we have previously described (19), on two additional T cell lines generated from each mouse. This analysis did not demonstrate an increase in IL-10 mRNA in the MBP-gld T cells lines compared with that in the MBP-wt and MBP-lpr T cell lines (data not shown). Similarly, IL-2 levels were not different in the three groups of mice. The small amount of IL-2 mRNA is consistent with the lines being polarized effector cells and not activated naive cells. In addition, we were able to detect message for perforin, but not for TGFβ1 or TGFβ2 (data not shown). Since FasL is only expressed following activation of T cells, we wanted to confirm that the T cell lines used in this study were expressing FasL. As shown in Fig. 3P, all three lines produce FasL mRNA. The point mutation of the gld mice affects ligand binding but not mRNA transcription, was confirmed in our colony. The robust expression of the housekeeping gene HPRT is shown as a control for RNA isolation and cDNA synthesis.

It has been previously reported that T cells isolated from gld and lpr mice have elevated proliferative responses and increased production of IFN-γ and TNF-α when stimulated through the TCR.
The increased proliferation is attributed to a lack of T cell death due to a disruption in Fas/FasL interactions. We also observed this phenomenon in proliferation assays conducted with multiple T cell lines. T cells from MBP-gld and MBP-lpr mice stimulated with Ac1–11 consistently gave increased proliferative responses compared with T cells from MBP-wt mice (Fig. 4A). This increased proliferation was accompanied by increased secretion of IFN-γ measured by ELISA (Fig. 4B). In addition, using semiquantitative RT-PCR we observed increased mRNA for both IFN-γ and TNF-α in MBP-gld and -lpr T cell lines compared with that in wt control T cells (data not shown). Since T cell death via Fas/FasL does not occur in proliferation assays with MBP-gld and MBP-lpr T cells, the increased cell proliferation probably reflects increased cell numbers, which could account for increased levels of IFN-γ. To address whether MBP-gld and MBP-lpr T cell lines have an intrinsic capacity for increased proliferation, we examined the rate of proliferation of T cells 4 days following Ag stimulation. In Fig. 4C we show that when equal numbers of T cells are replated in medium alone, MBP-lpr and MBP-gld T cells do not proliferate at a higher rate that MBP-wt T cells.

**FIGURE 3.** Cell surface expression of CD4, MBP TCR, Vβ8.2, CD49d, and Fas and cytokine production by MBP-wt, MBP-gld, and MBP-lpr T cell lines. A–O, Histograms represent fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. The horizontal bar in each histogram defines positive staining, with the percentage of positive cells indicated on each histogram. P, RT-PCR reactions were conducted as described in Materials and Methods using 1 µl of cDNA prepared from total RNA isolated from 5 × 10⁶ MBP-wt, MBP-gld, and MBP-lpr T cells on the day of adoptive transfer. For all primers except HPRT, 35 PCR cycles were used. For HPRT, 30 PCR cycles were used.

MBP-TCR T cells lacking a functional FasL lead to reduced disease severity upon adoptive transfer

To further examine the role of Fas/FasL in the pathogenesis of EAE, we adoptively transferred the T cell lines from MBP-wt and MBP-gld mice into B10.PL or B10.PL-gld recipient mice. Because the number of transferred encephalitogenic T cells affects the severity and duration of the EAE disease course, we performed the
experiment using $1 \times 10^6$ T cells to examine whether a less severe disease course than that observed in Fig. 2 is dependent upon Fas/FasL. As shown in Fig. 5A B10.PL-gld mice receiving MBP-wt T cells had an earlier day of onset of disease (day 7), an earlier peak day of disease (day 13), and a longer, more severe disease course compared with B10.PL mice receiving MBP-wt T cells, which had a day of onset on day 12 and a peak day of disease on day 15 and had essentially recovered by day 24. In contrast, B10.PL and B10.PL-gld mice receiving MBP-gld T cells, had a slight delay in disease onset (days 13 and 14, respectively) and a delayed peak day of disease (day 18) resulting in an overall less severe disease course (Fig. 5A). To further examine the observed differences between disease courses shown in Fig. 5A, we calculated the total disease score obtained for each of the four groups of mice in three separate experiments. The data shown represent the average cumulative disease score $\pm$ SE for each group (Fig. 5B). A small increase in the cumulative disease score was observed in the B10.PL-gld mice transferred with MBP-wt T cells compared with that in the control group of B10.PL mice receiving MBP-wt T cells. The increase was 112% of the control value and was not statistically significant. In contrast, wt and gld recipient mice transferred with MBP-gld T cells had reduced disease scores that were 38 and 68% of the control group value, respectively (Fig. 5B). However, a statistically significant decrease ($p < 0.001$) from the control group was only observed in the B10.PL recipient mice. These data show that in the absence of a functional FasL on the transferred T cells a reduced severity of disease is observed.

The lack of a functional Fas molecule in recipient mice results in protection from EAE

Because the reduction in disease severity shown in Fig. 5 was only partial in the presence of a mutated FasL protein, we performed the analogous experiment using mice with a disrupted Fas gene (lpr) to more fully understand the role of Fas in EAE pathogenesis. Fig. 6A illustrates that a disrupted Fas molecule on the transferred T cells, regardless of the recipient, has no effect on the disease course observed. This is illustrated by the almost identical disease courses of B10.PL recipient mice transferred with MBP-wt or MBP-lpr T cells. In contrast, the B10.PL-lpr mice adoptively transferred with MBP-wt T cells did not exhibit signs of EAE over a 35-day time course (Fig. 6A). Nevertheless, the lpr recipients transferred with MBP-lpr T cells had a disease course similar to that of the wt control group, suggesting an important role for the Fas molecule on the encephalitogenic T cells. The cumulative disease scores for the four groups of mice shown in Fig. 6A were calculated for five separate experiments and are shown in Fig. 6B. Similar disease scores were observed for the wt control group and for B10.PL and B10.PL-lpr mice transferred with MBP-lpr T cells. The lack of disease in the lpr recipient mice transferred with MBP-wt T cells is shown by a cumulative disease score of 1.5, representing a statistically significant decrease ($p < 0.001$) from the control group. This indicates that cells lacking Fas can use alternative mechanisms to generate EAE, whereas cells with competent Fas appear to be biased toward the use of FasL to cause pathology.

Discussion

We have examined the role of Fas and FasL in the induction and pathogenesis of EAE. We found that MBP tg mice carrying deficiencies in Fas or FasL were not protected from EAE upon active immunization with the MBP peptide Ac1–11 (Fig. 2). These results are in contrast to the study by Sabelko et al., demonstrating protection in lpr mice immunized with gpMBP (16). However, an important difference between the two studies is the number of autoreactive T cells at the time of immunization. In the MBP-TCR transgenic mice up to 90% of the CD4$^+$ T cells express the transgene. In nontransgenic mice only a small population of T cells specific for myelin Ags exists. The activation and expansion of a large pool of autoreactive T cells in the MBP transgenic mouse results in a rapid and often fatal disease course (Fig. 2). However, in B10.PL mice immunized with the same Ag an acute disease course occurs with little mortality (20). Unchecked lymphoproliferation of the MBP tg T cells in vivo seems not to be a factor in the EAE observed in transgenic animals, since all T cell populations in the thymus, lymph nodes, and spleen (data not shown) of MBP-gld and MBP-lpr are comparable to those in MBP-wt at the

FIGURE 4. Proliferation and IFN-γ production in MBP-wt, MBP-gld, and MBP-lpr T cells. A and B, T cells were cocultured with B10.PL spleen cells in the presence of 1/10 dilutions of Ac1–11 from 0.001–10 μg/ml. Proliferation and subsequent secretion of IFN-γ of the MBP-wt (●), MBP-gld (■), and MBP-lpr (▲) T cell lines within the same culture are shown. Secretion of IFN-γ was detected by harvesting one-half of the culture supernatant 24 h after Ac1–11 stimulation, was assayed by ELISA as described in Materials and Methods, and was graphed as picograms per milliliter of cytokine detected in the supernatant. C, Four days following Ag stimulation, MBP-wt (●), MBP-gld (■), and MBP-lpr (▲) T cells were replated at 1/2 dilutions starting at 2.5 $\times$ 10$^6$ cells/well in medium alone. The data shown are the average of four separate experiments. A and C, Proliferation was measured by [3H]TdR incorporation and is presented as counts per minute.
The discrepancies in these two studies may be due to differences in EAE induction protocols used. Malipiero et al. reported almost complete protection from EAE in both B6-gld and B6-lpr mice, with wt mice exhibiting a monophasic disease course accompanied by demyelination and a possible role for autoantibody occurrence. In contrast, Waldner et al. reported almost complete protection in both studies.

Protection reported by the Waldner group may partially reflect differences in EAE induction protocols used. The protection reported by the Malipiero group may partially reflect disease induced in older male mice, which are less susceptible to disease than female mice. The lack of substantial protection in the Malipiero study may also reflect differences in EAE induction protocols used in MBP vs MOG. An acute monophasic disease course ensues following MBP immunization in B10.PL mice, while a chronic disease course accompanied by demyelination and a possible role for autoantibody occurrence in MOG immunization in C57BL/6 mice.

The active induction of EAE in gld or lpr mice does not definitively reveal the cell populations in which the presence of Fas and FasL INTERACTIONS IN THE PATHOGENESIS OF EAE

age of EAE induction (Fig. 1). In this study the susceptibility to EAE in MBP-gld and MBP-lpr mice is presumably due to a large resident population of autoreactive T cells that can enter the CNS upon activation, leading to cell destruction by mechanisms other than the binding of Fas by FasL.

Two separate groups investigated active EAE induction using MOG<sub>35-55</sub> in C57BL/6 mice carrying the lpr and gld mutations with contrasting results. Waldner et al. reported almost complete protection from EAE in both B6-gld and B6-lpr mice, with wt mice exhibiting a relapsing-remitting disease course. In contrast, the study performed by Malipiero et al. showed only partial protection in disease severity, with lpr and gld mice exhibiting a monophasic disease course, and the wt mice exhibiting a chronic disease course.

The cumulative disease score was calculated by adding the disease score from the day of onset to day 35. The values shown are the mean ± SE of all mice in each group. The mean cumulative disease score shown is the average of mice from five separate experiments. The control transfer of MBP-wt T cells into B10.PL recipients was shown with light diagonal stripes, B10.PL-lpr mice transferred with MBP-lpr T cells (□) were averaged. The data in A are from one representative experiment with five mice in each group. B. The cumulative disease score was calculated by adding the disease score from the day of onset to day 35. The values shown are the mean ± SE of all mice in each group.
FasL are important in EAE. To examine this question we performed adoptive transfers to passively induce EAE and targeted the deficiency in Fas and FasL to the transferred T cells, the recipient, or both. Because EAE is believed to be mediated by Th1 CD4+ T cells, we generated Th1 T cell lines from MBP-wt, MBP-gld, and MBP-lpr mice. These lines expressed CD4 and the MBP TCR and produced the Th1 cytokines IFN-γ, TNF-α, and LT-α on the day of transfer (Fig. 3). These T cell lines also expressed high levels of very late Ag-4, which we have previously shown to be important for migration into the CNS (6). These experiments revealed that the absence of a functional Fas molecule in the recipient mice was protective against EAE induction by the transfer of MBP-wt T cells compared with transfer into wt recipients (Fig. 6). We attribute this result to a blockage of Fas-induced cell death due to the absence of functional Fas expression in the CNS. This result is supported by the reverse experiment, showing no disease protection when Fas-deficient T cells are transferred into wt recipients. Thus, protection is only observed when Fas is deficient in the CNS. To our surprise, the transfer of MBP-lpr T cells into lpr recipients did not result in any measurable disease protection. This result is probably due to the loss of homeostatic regulation of the transferred T cell population by Fas and FasL (28). The lymphoproliferative disorders observed in MRL mice carrying the gld and lpr mutations led investigators to propose that Fas/FasL interactions are essential for the homeostatic control of T cell populations (29). This was supported by the observation that T cells express both Fas and FasL upon activation and that T cells commit fratricide and perhaps even suicide by this mechanism, controlling the extent of the T cell response (30). Thus, the loss of Fas on the cell surface of the transferred T cells may allow them to accumulate in sufficient numbers to cause EAE by a mechanism that is clearly unrelated to Fas/FasL.

We cannot, however, exclude the possibility that the protection observed upon transfer of MBP-wt T cells into lpr recipients is due to death of the Fas-expressing wt T cells by interaction with FasL-expressing cells in the lpr recipients. This control of the T cell population could occur in the periphery or perhaps in the CNS. This mechanism of controlling the transferred T cell population would not be present when Fas-deficient T cells are transferred into lpr recipient mice. To test whether MBP-wt T cells undergo cell death following transfer into lpr recipients we are performing experiments to determine whether the wt T cells enter the CNS and undergo apoptosis or whether T cell apoptosis occurs in the periphery in T cells that never enter the CNS. Additional evidence for homeostatic control of the transferred T cell population is the increased disease severity observed in gld animals transferred with MBP-wt T cells (Fig. 5b). The absence of a functional FasL to mediate Fas-induced death in the recipient may allow the encephalitogenic T cells to accumulate more rapidly and in larger numbers than in wt recipients, leading to earlier onset and a more severe disease course. The increase in disease severity was consistent in three independent experiments. Since the MBP-gld and -lpr T cell lines do not proliferate at a higher rate in vitro on the day of transfer (Fig. 4c), we are investigating the potential loss of homeostatic control in vivo due to deficiencies in Fas or FasL by measuring the numbers of MBP tg T cells observed in the CNS at specific time points following adoptive transfer. We are also examining whether there is an increase in cell divisions in the transferred T cell population when both the transferred T cells and the recipient mice are deficient in Fas or FasL, and no protection from disease is observed.

The substantial protection observed when Fas was absent from the CNS was only partially reproduced in the reciprocal experiment examining EAE in the absence of FasL on the transferred T cells (Fig. 5). Inflammatory cytokines produced by the infiltrating T cells are likely candidates for the cause of the paralysis that we observed in the absence of FasL on the transferred T cell population. These include IFN-γ, TNF-α, and LT-α, all abundantly produced by the encephalitogenic T cell lines we generated (Fig. 3). By ELISA, we found that the MBP-gld and -lpr T cell lines secreted a higher level of IFN-γ compared with MBP-wt T cells (Fig. 4b). Increased IFN-γ production may contribute to the lack of total protection observed when FasL-deficient T cells are transferred into B10.PL mice. IFN-γ production is highly correlated with encephalitogenicity and may contribute to disease by up-regulation of adhesion molecules on endothelial cells, facilitating migration of lymphocytes into the CNS; induction of MHC class I and MHC class II molecules on astrocytes, microglial cells, and brain endothelium facilitating Ag presentation in the CNS; and activation of macrophages leading to production of nitric oxide, a potent cytotoxic molecule (1). TNF-α and LT-α, like FasL, are members of the TNF family and cause cell death by apoptosis following interaction with their counter-receptors, the TNFR1 and TNFR2, leading to a cascade of proteolytic events culminating in blebbing of the cytoplasmic membrane, nuclear condensation, and DNA fragmentation (8, 31). In addition to death via a Fas mechanism (32), oligodendrocyte cell death and subsequent myelin damage have been shown to be mediated by both TNF-α and LT-α (33, 34). TNF-α and LT-α share the same receptors (TNFR1 and TNFR2), with TNFR1 shown to be expressed by astrocytes, microglia, and oligodendrocytes, and TNFR2 expressed by microglia in rat brain (35).

In addition to the production of IFN-γ, TNF-α, and LT-α we have shown that our CD4 T cells lines express perforin (Fig. 3). Perforin is contained in cytoplasmic organelles resembling granules and is released into the cell interface between the effector and the target cell, where it inserts into the cell membrane, creating holes that allow the uptake of granzyme, which causes lysis (36). Produced in abundance in CD8 CTL, CD4 T cells have also been shown to mediate cytotoxicity via perforin (37). Although perforin knockout C57BL/6 mice were shown to be susceptible to MOG35-55-induced EAE (25), perforin mRNA was detected before disease onset in MBP-induced EAE in the Lewis rat (38). In addition, Held et al. (39) showed that only 1.6% of IL-2Rα+ cells express perforin mRNA following EAE induced by MBP immunization. In contrast, 25% of the same cell population was shown to produce perforin in EAE induced by adoptive transfer of encephalitogenic T cells, suggesting a greater role for perforin in passive induction of EAE than in active induction. We propose that the early pathogenesis in the CNS is mediated by a small number of CD4+ T cells that express FasL. FasL interacts with Fas on oligodendrocytes, presumably up-regulated by IFN-γ (8) produced by the Th1 encephalitogenic T cell, leading to apoptotic death of oligodendrocytes. This early cell destruction then leads to a full inflammatory response and the recruitment of additional autoreactive T cells, macrophages, and other cell types to the site of immune attack forming an inflammatory lesion. The ensuing myelin destruction would provide ample Ag for the continued activation and expansion of the autoreactive T cell pool. As the autoreactive Th1 cells accumulate, the local production of the pathogenic Th1 cytokines TNF-α and LT-α would also increase, resulting in continued apoptotic death of oligodendrocytes and demyelination. Macrophages present may contribute to oligodendrocyte destruction by the production of nitric oxide (40). The escalating inflammatory response could then be dampened by any number of proposed mechanisms, including T cell apoptosis by Fas and FasL and the emergence of a Th2 response (41).
Thus, only when small numbers of encephalitogenic T cells, allowing only limited production of Th1 inflammatory cytokines in the CNS, are transferred into recipients deficient in Fas, is protection from disease observed. The transfer of 10 times more T cells into Fas-deficient recipients (our unpublished observations) or the presence of large numbers of autoreactive T cells in MBP transgenic mice (Fig. 2) overcomes protection, presumably due to overwhelming production of inflammatory cytokines in the CNS, leading to oligodendrocyte cell damage and circumventing a role for Fas. This is supported by data demonstrating only partial protection when the encephalitogenic T cells lack a functional FasL. Although experimental evidence shows that Fas expression in the CNS is important for EAE induction, it is not essential. This becomes important in the pursuit of therapeutic agents. Therapeutic strategies targeting blocking of Fas in the CNS may be helpful for the prevention of multiple sclerosis exacerbations, but not for the treatment of an acute episode once inflammation is established.

Acknowledgments

We thank the W. M. Keck Foundation Biotechnology Resource Laboratory for peptide and oligo synthesis, Charles Annicelli III for assistance with the mouse colony, Irene Visentin for help with graphics, and Jennifer Li for assistance with screening of the transgenic mice.

References


