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Presentation of Proteolipid Protein Epitopes and B7-1-Dependent Activation of Encephalitogenic T Cells by IFN-γ-Activated SJL/J Astrocytes


There is controversy regarding the possible role of glial cells as APCs in the pathogenesis of central nervous system (CNS) demyelinating diseases such as multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). Microglia have been clearly shown to present Ag in the CNS, and due to the proximity of activated astroglial cells to infiltrating T cells and macrophages in demyelinating lesions, it is also possible that astrocytes positively or negatively regulate disease initiation and/or progression. We examined the capacity of IFN-γ-treated astrocytes from EAE-susceptible SJL/J mice to process and present myelin epitopes. IFN-γ activation up-regulated ICAM-1, VCAM-1, MHC class II, invariant chain, H2-M, CD40, and B7-1 as determined by FACS and/or RT-PCR analyses. B7-2 expression was only marginally enhanced on SJL/J astrocytes. Consistent with the expression of these accessory molecules, IFN-γ-treated SJL/J astrocytes induced the B7-1-dependent activation of Th1 lines and lymph node T cells specific for the immunodominant encephalitogenic proteolipid protein (PLP) epitope (PLP139–151) as assessed by proliferation and activation for the adoptive transfer of EAE. Interestingly, IFN-γ-activated astrocytes efficiently processed and presented PLP139–151, but not the subdominant PLP56–70 or PLP140–147 epitopes, from intact PLP and a recombinant variant fusion protein of PLP (MP4). The data are consistent with the hypothesis that astrocytes in the proinflammatory CNS environment have the capability of activating CNS-infiltrating encephalitogenic T cells specific for immunodominant epitopes on various myelin proteins that may be involved in either the initial or the relapsing stages of EAE. The Journal of Immunology, 1998, 160: 4271–4279.

The capacity of astrocytes to present myelin epitopes is a major determinant in the progression of EAE. While significant attention has been devoted to elucidating the immunopathology of R-EAE and the potential regimens that can be utilized to prevent and/or treat ongoing disease (10, 15, 18–21), little information exists as to the roles that might be played in disease pathogenesis by the nonprofessional APCs of the CNS. Several reports have demonstrated that microglia are competent to process and present autoantigens in vitro (22–24). In addition, microglia are thought to initiate the effector limb of EAE (25, 26) and express MHC class II in normal white matter and in MS lesions (27). On the other hand, significant controversy exists as to the ability of neural crest-derived astrocytes to process and present protein Ags, let alone participate in the autoimmune pathogenesis (28–31). While it is known that astrocytes play a major role in maintaining the blood-brain barrier, provide potential nutritive functions, and seal off damaged areas in the CNS (32, 33), the ability of astrocytes to process and present Ag, particularly self Ags, is still in dispute (28). Histopathologic examination of the demyelinating plaques of R-EAE shows areas of inflammatory monocytic infiltration that are surrounded by astroglial cells (34). In light of their proximity to the
infiltrating autoimmune T cells, it is a continuing mystery as to the role astrocytes may play in the disease initiation and/or progression. We have recently demonstrated that subsequent to IFN-γ activation, astrocytes isolated from neonatal BALB/c mice up-regulate B7-2 costimulatory molecules and process and present native OVA to naïve transgenic T cells, as well as to Th1 clones and hybridomas (30). In this study, we report that astrocytes from EAE-susceptible SJL/J neonates, activated with IFN-γ, up-regulate all the necessary accessory molecules to initiate and/or perpetuate a T cell response. In addition, we show that SJL/J astrocytes process and present the immunodominant 139–151 epitope from either MP4, a fusion protein containing a recombinant variant of PLP, or whole PLP. In addition, IFN-γ-treated astrocytes were able to process MP4 and activate PLP139–151-specific T cells for the adoptive transfer of R-EAE.

Materials and Methods

Mice

Pregnant (15–17 days) SJL mice and 5- to 6-wk-old female mice were purchased from Harlan Labs, Bethesda, MD. Mice were housed in the Northwestern University animal facility. One- to three-day-old neonates were used for the isolation of astroglial cells.

Antigens

PLP56–70 (DYEYLINVIHAFQYY), PLP104–117 (KTTICCGGSLATV), and PLP139–151 (HSLGKWL) were synthesized on an Applied Biosystems 432A automated peptide synthesizer. PLP139–151 was purchased from Harlan Labs, Bethesda, MD. Mice were housed in the Northwestern University animal facility. One- to three-day-old neonates were used for the isolation of astroglial cells.

Tissue culture flasks were coated from 3 hr to overnight with 10 μg/ml poly-L-lysine (Sigma) and rinsed with PBS-3% FCS before addition of isolated cells. The brains were removed from 1- to 3-day-old neonatal mice, the hind brains were dissected with BSS-3% FCS before addition of isolated cells. The brains were re-cultured and PLP was converted to the water-soluble form by gradual replacement of the organic solvent with water, according to the method of Sherman and Folch-Pi (38).

Media

T cell lines were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 2 mM l-glutamine (Life Technologies, Gaithersburg, MD), 100 U/ml penicillin (Life Technologies, Gaithersburg, MD), 100 μg/ml streptomycin (Life Technologies), 10 μg/ml gentamycin (Life Technologies), 5 × 10−3 M 2-ME (DMEM-7% FCS). Astroglial cells were cultured in the presence or absence of mouse rIFN-γ (100 U/ml) at 37°C and 5.5% CO2 for the indicated amount of time. Cells were washed with PBS, and the cells were gently removed with cell scrapers. The brains were dissected with BSS-3% FCS before addition of isolated cells. The brains were re-cultured and PLP was prepared from chloroform-methanol-soluble PLP was converted to the water-soluble form by gradual replacement of the organic solvent with water, according to the method of Sherman and Folch-Pi (38). Tissue culture flasks were coated from 3 hr to overnight with 10 μg/ml poly-L-lysine (Sigma), 2 mM l-glutamine (Life Technologies, Gaithersburg, MD), 100 U/ml penicillin (Life Technologies, Gaithersburg, MD), 100 μg/ml streptomycin (Life Technologies), 5 × 10−3 M 2-ME, 0.1 mM nonessential amino acids (Sigma), 1 mM sodium pyruvate (Life Technologies), MEM essential vitamins (Life Technologies), 0.1 mM asparagine (Life Technologies), 0.1 mg/ml folic acid (Life Technologies), 0.8% ST (Collaborative Biomedical Research, Bedford, MA), and reduced glutathione (Sigma). Glutathione and hybridomas were propagated and proliferation assays were performed in DMEM supplemented with 7% FCS, 2 × 10−5 M l-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10−3 M 2-ME (DMEM-7% FCS). Astroglial cultures were maintained in DMEM/F12 (1:1) (Sigma) supplemented with 10% FCS and adjusted to a final concentration of 6 g/L glucose, 2.4 g/L NaHCO3, 0.37 g/L l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (DMEM-F12). Media and fetal bovine serum were free of endotoxin contamination.

Astrogial cell isolation

Tissue culture flasks were coated from 3 hr to overnight with 10 μg/ml poly-L-lysine (Sigma) and rinsed with balanced salt solution supplemented with BSS-3% FCS before addition of isolated cells. The brains were removed from 1- to 3-day-old neonatal mice, the hind brains were dissected away and the meninges were removed. The left and right hemispheres were transferred to a nylon mesh bag and gently dissociated. Cells in suspension were passed through No. 60 and No. 100 stainless steel screens (Sigma) to remove large pieces of debris and tissue. Cells were pelleted, resuspended in DMEM-F12 complete medium, and seeded in the poly-L-lysine-coated tissue culture flasks and incubated at 37°C and 7.5% CO2. Fresh medium was added every 3 to 4 days. After 12 to 14 days, microglia and oligodendrocytes were removed from the astroglial bed layer by shaking the flasks on an orbital shaker for 1 h at 100 rpm and 24 h at 300 rpm. Astrocytes remaining adhered to the flask were treated with trypsin and replated.

The purity of normal and IFN-γ-treated astrocytes was determined by intracellular staining with Abs to glial fibrillary acidic protein (GFAP). Cells were adhered overnight to LabTek chambered glass slides (Nunc, Glenrock, Denmark) at 2 × 102 cells/chamber. Cells were fixed for 10 min at room temperature with 10% neutral buffered formalin (Sigma), rinsed with PBS, and permeabilized with PBS-0.2% fish skin gelatin (FSG, Sigma)-0.5% saponin for 20 min. Cells were rinsed with PBS, and nonspecific binding was blocked with PBS-0.2% FSG for 30 min and then incubated with primary anti-GFAP (1:200, Dako, Carpinteria, CA) for 1 h at 37°C. Cells were rinsed with PBS-0.2% FSG and incubated with FITC-conjugated goat-anti-rabbit Ig (1:200, Calbiochem, La Jolla, CA) for 1 h at 37°C. Cells were washed with PBS-FSG, covered with glass coverslips, and examined under a fluorescent microscope. Astrocyte cultures were >98% GFAP+, and showed no staining with I-Aα indicating no contamination with microglia cells.

Cell surface staining

Astrogial cells were incubated in the presence or absence of mAb 34 (100 U/ml) for 6 to 24 h. Cells were scraped from the flasks, washed with PBS, and solubilized with 1 ml of 4 M guanidium thiocyanate. Chromosomal DNA was sheared using a 1-ml syringe equipped with a 23-gauge needle. Total RNA was isolated from the extract using the RNeasy Total RNA Kit (Qiagen, Germany). One microgram of total RNA was typically used per RT reaction. Oligo(dT)12–18 primers were used for cDNA synthesis using RevertAid M-MLV RTase, and 100 ng of total RNA was used. One microgram of total RNA was typically used per RT reaction. Oligo(dT)12–18 primers were used for cDNA synthesis using RevertAid M-MLV RTase, and 100 ng of total RNA was used. One microgram of total RNA was typically used per RT reaction. Oligo(dT)12–18 primers were used for cDNA synthesis using RevertAid M-MLV RTase, and 100 ng of total RNA was used.

RT-PCR analyses

Astrogial cells were incubated in the presence or absence of IFN-γ (100 U/ml) for 6 to 24 h. Cells were scraped from the flasks, washed with PBS, and solubilized with 1 ml of 4 M guanidium thiocyanate. Chromosomal DNA was sheared using a 1-ml syringe equipped with a 23-gauge needle. Total RNA was isolated from the extract using the RNeasy Total RNA Kit (Qiagen, Germany). One microgram of total RNA was typically used per RT reaction. Oligo(dT)12–18 primers were used for cDNA synthesis using RevertAid M-MLV RTase, and 100 ng of total RNA was used. One microgram of total RNA was typically used per RT reaction. Oligo(dT)12–18 primers were used for cDNA synthesis using RevertAid M-MLV RTase, and 100 ng of total RNA was used.

Western blot analysis of invariant chain

Astrogial cells (4 × 105) were incubated in the presence or absence of IFN-γ (100 U/ml) for 24 h. Cells were scraped from the flasks, washed with PBS, and solubilized with 1% Nonidet P-40 supplemented with the following protease inhibitors: PMSF, N-tosyl-l-phenylalanine chloromethyl ketone (both from Boehringer Mannheim); and leupeptin (Sigma). Lysates were centrifuged for 20 min at 14,000 rpm in a refrigerated Eppendorf centrifuge, and supernatants were collected, leaving behind the nuclear pellet. Proteins were analyzed by 12% SDS-PAGE and were transferred from SDS-PAGE gels onto nitrocellulose membranes at 70 V for 5 h in Towbin transfer buffer. Membranes were blocked for 1 h at room temperature in 5% dry milk and rinsed with Tris-buffered saline containing 0.1% Tween-20 (TBST). Invariant chain (Ii) molecules were detected by an overnight incubation of the membrane in a 1:20 dilution of the anti-Ii mAb In-1 (the kind gift of Dr. Andrea Sant, University of Chicago, Chicago, IL). Primers for the CIITA and H-2 trans-activator (CIITA) (sense, 5′-CAGT CCT GCT GAA GGA TGT GGA-3′; anti-sense, 5′-AGG TCC ACC GGG AGG GAC-3′), H-2Mα (sense, 5′-GTG CTC GAA GCA TCT ACA CC; anti-sense, 5′-GAC ACT GTC TGC TCT AAC TG-3′), B7-1 (sense, 5′-TGC TGT CTT TGC TCT GCA TGC AAC T-3′; antisense, 5′-CTG AGT TCA GAA CAG AGC-3′), anti-sense, 5′-GGG TCC ATG GTC AGC TCT GGA-3′) and β-actin (sense, 5′-GTG GGC GCG TCT GAG CAC CAA-3′; anti-sense, 5′-CCT TTT GAT GTC AGC CAT GAC CAT TTC-3′). Primers for B7-1, B7-2, and β-actin were synthesized at the Northwestern University Biotechnology Center, Chicago, IL. Primers for the CIITA and H-2Mo were synthesized by distillation water containing 2.5% dry milk for 1 h. Membranes were then washed extensively and visualized by enhanced chemiluminescence (Amersham ECL).
Lines were propagated by repeated in vitro stimulation of fresh spleen cells and Ag of the respective peptide. T cells were restimulated every 3 to 4 wk with presence or absence of rIFN-γ. Culture wells were pulsed with [3H]TdR (1 μCi/well) and incubated for 48 h before assay. Immediately preceding the addition of T cells or hybridomas and Ag, the plates were irradiated with 3000 rads and then gently, but extensively, washed with BSS-3%FCS to remove residual rIFN-γ. Supernatants was assayed using the IL-2 sensitive CTLL-2 cell line. With T cell hybridomas, activity was detected using a Packard Topcount microplate scintillation counter (Packard Instruments, Meriden, CA) for the final 24 h of a 72 h incubation period. Radioactivity was detected as described above.

**Adoptive transfer of R-EAE**

Astrocytes (2.5 × 10^6) were plated onto T-75 tissue culture flasks (coated overnight with poly-lysine) in the presence or absence of rIFN-γ (100 U/ml) for 48 h. The astrocytic cultures were irradiated with 3000 rads and washed with BSS-3%FCS to remove residual rIFN-γ. PLP139–151-specific T cells (35 × 10^6), isolated from the draining lymph nodes of SJL/J mice primed 10 days before with PLP139–151, were added to each of the flask in a total of 12 ml of DMEM-7%FCS in the presence of either 50 μM PLP139–151 peptide or 25 μg/ml MP4. Following a 4-day incubation at 37°C and 7.5% CO₂, T cells were harvested and washed twice, and 5 × 10^6 blasts were injected i.p. into naive SJL/J mice. As a control, 35 × 10^6 PLP139–151 T cells were activated with 18 × 10^7 irradiated syngeneic spleen cells (3000 rads) in the presence of 50 μM PLP139–151 peptide, and 5 × 10^6 blast cells from these cultures were also transferred into naive SJL/J recipients. Recipient mice were observed for clinical signs of disease.

**Clinical evaluation**

Mice were observed daily for clinical signs of disease for ~30 to 35 days posttransfusion and, thereafter, every 1 to 7 days for the duration of the experiment. Mice were scored according to their clinical severity as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness (waddling gait); grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; and grade 5, moribund. The scores of the asymptomatic mice (score = 0) were included in the calculation of the daily mean clinical score for each group.

**Statistical analyses**

Comparison of the percentage of animals showing clinical disease were analyzed by χ² and comparisons of T cell proliferative responses were analyzed by Student’s t test. p < 0.05 was considered significant.
Results

CNS astrocytes up-regulate expression of MHC class II, Ii, and H2-M following IFN-γ treatment. To study the effects of a proinflammatory environment on the cell surface expression of MHC class II and the expression of its chaperone molecules, Ii and H2-M, we cultured astrocytes isolated from the brains of SJL/J neonates in the absence or presence of 100 U/ml rIFN-γ for varying periods of time. Following the incubation, cells were washed and stained with a monoclonal anti-I-As Ab. As can be seen in Figure 1A, which is representative of three separate experiments, there is a slight constitutive expression of I-As on the astrocytes, which may be due to partial activation of the cells by adherence to plastic. However, incubation of the astrocytes for increasing periods in rIFN-γ results in a significant increase in MHC class II expression. This is initially visualized by the appearance of a small I-As bright population followed by a general shift in the class II brightness of entire population. Maximal expression of class II is reached between 24 and 48 h and persists up to 72 h postinduction.

The Ii is an important chaperone molecule that participates at many levels in the processing and presentation of Ag in traditional APCs (39–41). In light of our previous observations indicating that proinflammatory cytokines can enhance Ag presentation functions of astrocytes (30), we examined, by Western blotting, the effects of rIFN-γ on the expression of Ii in astrocytes. Figure 1B indicates that in the absence of IFN-γ, there is little or no detectable Ii in the SJL/J astrocytes. However, following treatment with 100 U/ml IFN-γ for 24 h, there is a dramatic increase in the amount of detectable p31 and p41 isoforms of Ii. Treatment with a combination of 100 U/ml IFN-γ and 500 U/ml TNF-α resulted in even higher levels of Ii expression. Additionally, while there does not appear to be any alteration in the ratio of the p31-p41 isoforms with IFN-γ treatment, it appears that there is more of the p41 isoform than the 1:9 ratio of p41-p31 isoforms reported for traditional APCs (41, 42). Regardless, rIFN-γ treatment increases the expression of Ii and MHC class II, suggesting that they may be competent for MHC class II peptide loading.

We next examined the effects of IFN-γ on the expression H2-M. This class II homologue is important in catalyzing the removal of the Ii CLIP peptide from the MHC class II Ag-binding groove or serves as a unique molecular chaperone for MHC class II, thereby facilitating the loading of foreign antigenic peptides (43, 44). IFN-γ treatment of SJL/J astrocytes resulted in an increase in the message levels for the gene for the α-chain of H2-M, Ma. Ma message first appeared at 6 h IFN-γ treatment and peaked at 24 h IFN-γ treatment (Fig. 1C).

Finally, we examined the mRNA levels for CIITA. This protein is involved in the activation of both class II and Ii transcription. As seen in Figure 1C and in support of the data presented above, there is an increase in the message levels of CIITA, which peaks at 6 h of rIFN-γ incubation. These levels appear to decline by 24 h. Thus,
To confirm the effects of IFN-γ on the cell surface expression of B7-1 and B7-2 in SJL/J astrocytes, we examined the steady-state mRNA levels of B7-1 and B7-2 following a 6- or 24-h incubation with IFN-γ. As can be seen in Figure 3, there is detectable message for B7-1 in the absence of rIFN-γ treatment, and incubation in

FIGURE 3. mRNA expression of B7-1 and B7-2 costimulatory molecules in IFN-γ-treated SJL/J primary astrocytes. Primary SJL/J astrocytes were incubated in the absence or presence of 100 U/ml IFN-γ for 6 or 24 h before the isolation of total RNA. Total RNA (1 μg/ml) was used in RT reactions using oligo(dT)12-18 primers. cDNA was amplified (30 cycles) using primer pairs to B7-1, B7-2, and β-actin. PCR products were visualized by ethidium bromide on 2% agarose gels. The results are representative of RNA isolated from four separate astrocyte cultures.

the increase in I-Aα and Ii expression correlates with an earlier increase in the levels of the MHC CIITA mRNA. Thus, a proinflammatory environment as simulated by rIFN-γ results in the up-regulation of all the components necessary for the loading of antigenic peptide onto MHC class II.

CNS astrocytes up-regulate expression of costimulatory molecules following IFN-γ treatment. To study the effects of a proinflammatory environment on the cell surface expression of costimulatory molecules necessary for T cell activation, neonatal SJL/J astrocytes were cultured in the absence or presence of 100 U/ml rIFN-γ for varying periods of times. The cells were then washed and stained with Abs against B7-1, B7-2, and CD40. As can be seen in Figure 2, which is representative of three separate experiments, there is constitutive expression of B7-1 on a subpopulation of CNS astrocytes, which increases steadily with increasing incubation periods in rIFN-γ (Fig. 2A). Optimum up-regulation of cell surface expression of B7-1 occurs at ~48 h of rIFN-γ treatment. As seen in Figure 2B, B7-2 constitutive cell surface expression is undetectable in untreated astrocytes and increases only slightly during the incubation periods examined. This is in contrast with our previous studies in BALB/c astrocytes, which show little constitutive or induced expression of B7-1 but an increase in B7-2 expression with increasing exposure to IFN-γ (30). CD40 expression is not apparent in untreated astrocytes, but with increasing incubation periods in rIFN-γ, there is a dramatic enhancement of CD40 expression that is first visible at 6 h IFN-γ treatment peaking at 24 h and then rapidly declining (Fig. 2C). This is interesting in light of reports that CD40 ligation may play a role in the events that regulate expression of B7-1 and B7-2 in APCs and may also participate directly in the activation of distinct subsets of T cells (45–47). Finally, ICAM-1 and VCAM-1 show large increases in cell surface expression, reaching an optimum at ~24–48 h of IFN-γ treatment (data not shown). Thus, it appears that in the presence of a proinflammatory cytokine like IFN-γ, astrocytes up-regulate expression of the requisite costimulatory molecules necessary for activation of naive and memory T cells.

mRNA levels of costimulatory molecules in CNS astrocytes increase IFN-γ treatment

To study the effects of IFN-γ on the cell surface expression of B7-1 and B7-2 in SJL/J astrocytes, we examined the steady-state mRNA levels of B7-1 and B7-2 following a 6- or 24-h incubation with IFN-γ. As can be seen in Figure 3, there is detectable message for B7-1 in the absence of rIFN-γ treatment, and incubation in

rIFN-γ results in an increase in the mRNA levels for B7-1 which is visible by 6 h of incubation and maximal by 24 h (Fig. 3A). B7-2 mRNA levels are also visible at 6 h and peak by 24 h (Fig. 3B) but never reach those of B7-1 consistent with the FACS data showing higher constitutive and induced levels of cell surface expression of B7-1 than those of B7-2. Thus, there is correlation in mRNA levels of B7-1 and B7-2 with the cell surface expression levels of the proteins. Taken together with the data for MHC class II expression, SJL/J astrocytes appear to properly up-regulate/express all of the necessary molecules required for the processing and presentation of Ag for the initiation and maintenance of T cell activation.

SJL/J CNS astrocytes process and present intact PLP and MP4 to PLP epitope-specific cell lines and hybridomas

To functionally test the T cell activating capacity of SJL/J astrocytes, we examined the ability of astrocytes treated for 24 or 48 h with 100 U/ml IFN-γ to process and present encephalitogenic PLP epitopes from purified PLP and from the recombinant fusion protein, MP4, to a panel of PLP epitope-specific T cell lines and hybridomas. MP4 has been demonstrated to be encephalitogenic in SJL/J mice (36). Representative results compiled from six experiments using separate astrocyte isolations are shown in Figure 4. As can be seen, IFN-γ-activated astrocytes processed intact MP4 and whole PLP for activation of a T cell line specific for the dominant encephalitogenic PLP139-151 determinant. However, astrocytes failed to present either intact MP4 or PLP to several different T cell lines specific for the subdominant PLP56-70 and

\[ \Delta \text{CPM} \times 10^3 \]

\[ \text{T Cell Line} \]

\[ \begin{array}{cccc}
\text{PLP56-70} & \text{PLP104-117} & \text{PLP139-151} & \text{PLP178-91} \\
(2.5) & (2.9) & (2.8) & (2.9) \\
(5.2) & (5.8) & (5.7) & (5.8) \\
(11.7) & (11.3) & (11.5) & (11.7) \\
(18.3) & (18.5) & (18.4) & (18.6) \\
(24.9) & (24.7) & (24.8) & (24.9) \\
(25.5) & (25.7) & (25.6) & (25.8) \\
(26.1) & (26.3) & (26.2) & (26.4) \\
\end{array} \]

\(*P<0.05\)
PLP<sub>178–191</sub> epitopes. In contrast, LPS-activated splenocytes efficiently process the dominant and both the subdominant PLP epitopes. Since the 178–191 epitope has been implicated in the phenomenon of epitope spreading in R-EAE induced by priming with PLP<sub>139–151</sub> (14), it would appear that astrocytes, at least under the proinflammatory conditions that we have examined, may not be capable of efficiently processing subdominant epitopes from intact PLP but still may be able to process proteolyzed myelin fragments available in the CNS inflammatory milieu.

We also repeated the above assay using T cell hybridomas specific for the encephalitogenic 139–151 and 178–191 epitopes of PLP. A representative experiment is shown in Figure 5. The data follow the trend observed for activation of conventional T cell lines. Only a hybridoma specific for PLP<sub>139–151</sub> responded to both autologous peptide and intact MP4 or PLP. In contrast, PLP<sub>178–191</sub>-specific hybridomas failed to respond to intact MP4 or PLP presented by IFN-γ-activated astrocytes but responded well to autologous peptide. This phenomenon does not appear to be dependent on the dose of intact Ag since when equimolar concentrations of intact PLP and autologous peptide were used, similar results were obtained (data not shown). These data and those shown in Figure 4 may therefore reflect a deficiency in the astrocytes to effectively process MP4 and PLP into the encephalitogenic 56–70, 104–117, and 178–191 determinants. Thus, epitope spreading may be due to the presentation of subdominant epitopes on other CNS (e.g., microglia or more likely, infiltrating macrophages) or peripheral APCs. It is also possible that the local CNS microenvironment that may result in astrocyte processing and presentation of the “spread” 178–191 determinant is not mimicked by the in vitro proinflammatory conditions examined in this study.

**Activation of T cell lines specific for PLP encephalitogenic determinants is dependent on B7/CD28 costimulatory interactions**

We have previously demonstrated that activation of naïve OVA-specific TCR-transgenic T cells and Th1 lines by BALB/c astrocytes is predominantly B7-2 dependent. In light of the current data suggesting that SJL/J astrocytes can process and present Ag to T cell lines, we examined whether the observed differences in B7-1 and B7-2 expression in the SJL/J astrocytes, as compared with BALB/c astrocytes, would translate into a different costimulation dependence. In Figure 6, we assessed the ability of anti-B7-1 and anti-B7-2 mAbs to inhibit SJL/J astrocyte-induced T cell activation. As can be seen, anti-B7-1 almost completely inhibited proliferative responses of the PLP<sub>139–151</sub>, PLP<sub>104–117</sub>, and PLP<sub>178–191</sub> specific Th1 lines and partially inhibited the response of the PLP<sub>104–117</sub>-specific Th2 line. Anti-B7-2 was generally less effective than anti-B7-1 for inhibiting the Th1 responses and the combination of anti-B7-1 and anti-B7-2 completely eliminated responses of the Th1 lines. Activation of the PLP<sub>104–117</sub> Th2 cell line was only partially inhibited by anti-B7-1 and/or anti-B7-2 reflecting the reduced costimulatory dependency of this Th2 line. Thus, these results suggest that B7-1 is the dominant functional costimulatory molecule for SJL/J astrocyte-induced activation of Th1 lines. This is consistent with the predominant cell surface expression of B7-1 on SJL/J astrocytes activated for 48 h with rIFN-γ.

**IFN-γ-activated SJL/J astrocytes can activate PLP<sub>139–151</sub>-specific T cells for adoptive transfer of R-EAE**

Since SJL/J astrocytes can process PLP and MP4 leading to activation of a PLP<sub>139–151</sub>-specific Th1 line, we tested whether IFN-γ-treated SJL/J astrocytes had the capacity to process and present MP4 in vitro to activate PLP<sub>139–151</sub>-primed T cells for the adoptive transfer of R-EAE. As can be seen in Figure 7, IFN-γ-activated astrocytes pulsed with 25 μg/ml MP4 were able to activate PLP<sub>139–151</sub>-primed LN T cells to efficiently transfer EAE to 100% of naïve recipients. MP4-activated T cells were somewhat more efficient for disease induction than T cells activated with IFN-γ-activated astrocytes or naive splenic APCs pulsed with the autologous peptide.
The minimal constitutive level of I-As which we observed was significantly up-regulated surface expression of I-A\(\text{d}\) (Fig. 1). These results are representative of two separate signs of disease for 49 days. Disease incidence in each group is indicated (100\(\text{cells}\) for adoptive transfer. Therefore, it appears that under the proper inflammatory conditions, SJL/J astrocytes may participate in the disease process.

The lack of disease relapses during the observation period in recipients of T cells activated with PLP\(_{139-151}\)-pulsed, IFN-\(\gamma\)-treated astrocytes likely reflects the protracted day of onset and reduced severity of acute disease. On the other hand, astrocytes not treated with IFN-\(\gamma\) were incapable of activating the PLP\(_{139-151}\)-primed T cells for adoptive transfer. Therefore, it appears that under the proper inflammatory conditions, SJL/J astrocytes may participate in the disease process.

Discussion

The role of astrocytes in the pathogenesis of R-EAE is controversial (31, 48–50). Little evidence exists that directly implicates astrocytes in either the progression of or recovery from clinical disease. There is, however, substantial evidence that suggests that under the proper inflammatory conditions, microglia are competent to activate PLP\(_{139-151}\)-specific T cells to adoptively transfer clinical R-EAE (22, 28). In this study, we addressed the potential capacity of astrocytes to serve as “professional” APCs that may participate in the initiation or progression (epitope spreading) of T cell-mediated demyelination in the EAE-susceptible SJL/J mouse.

We recently demonstrated that BALB/c astrocytes activated for 24 to 48 h with IFN-\(\gamma\), up-regulated cell surface expression of I-A\(d\) (30). Following IFN-\(\gamma\)-treatment, SJL/J astrocytes also significantly up-regulated surface expression of I-A\(d\) (Fig. 1A). As expected, untreated astrocytes expressed only trace amounts of MHC class II. The minimal constitutive level of I-A\(d\) which we observed may be due to interaction of the astrocytes with the plastic culture wells because we noted that their adherence was enhanced after incubation with IFN-\(\gamma\) for extended culture periods. Maximal up-regulation of MHC class II occurred ∼48 h following addition of IFN-\(\gamma\), a time course similar to that for expression of I-A\(d\) on BALB/c astrocytes.

To further examine the capacity of astrocytes to serve as potential APCs, we investigated the effect of the IFN-\(\gamma\) treatment on the expression of two additional molecules required for Ag processing and presentation, Ii and H2-M. Ii is not expressed at detectable levels in untreated astrocytes (Fig. 1B) as determined by Western blotting. However, following a 24-h treatment with IFN-\(\gamma\), Ii expression was dramatically up-regulated. The ratio of the p31 to the p41 isoforms of Ii was clearly lower than the expected 9:1 ratio previously reported for B cells (41, 42). This is interesting in light of speculation that the p41 isoform may play a role in differential Ag processing within endocytic compartments (42, 51). We are currently exploring the possibility that this difference might be related to the heightened susceptibility of the SJL/J strain to R-EAE. Using RT-PCR, we also determined that the mRNA levels of the MHC CIITA, the protein involved in the transcriptional regulation of both MHC class II and Ii, were significantly up-regulated and peaked within 6 h of IFN-\(\gamma\) treatment (Fig. 1C). Thus, as would be expected, CIITA mRNA levels peaked before the maximal expression of I-A\(d\) and Ii. Similarly, transcription of the \(\alpha\)-chain (\(\alpha\text{-}\)) of the H-2 M molecule, which is involved in the removal of the Ii CLIP peptide from the MHC class II binding groove (43), was also up-regulated within 6 h and peaked at 24 h of IFN-\(\gamma\) treatment (Fig. 1C). Thus, it appears that SJL/J astrocytes possess all the necessary molecules required for efficient Ag processing and presentation following IFN-\(\gamma\)-treatment.

As efficient T cell activation requires two signals, TCR occupancy and delivery of the appropriate costimulatory signals (52), we next examined the constitutive and IFN-\(\gamma\)-induced expression levels of B7-1, B7-2, CD40, ICAM-1, and VCAM-1 on primary SJL/J astrocytes. B7-1 is constitutively expressed at low levels and significantly up-regulated on IFN-\(\gamma\)-treated SJL/J astrocytes (Fig. 2A), in contrast to our earlier finding that IFN-\(\gamma\)-treated BALB/c astrocytes up-regulated B7-2 but not B7-1 (30). This is supported by RT-PCR data, which showed detectable constitutive expression of B7-1 mRNA that was significantly up-regulated upon IFN-\(\gamma\)-treatment (Fig. 3A). Additionally, functional activation of Th1 lines specific for several different PLP encephalitogenic epitopes was dependent primarily on B7-1-CD28 interactions, although the combination of B7-1 and B7-2 Abs resulted in maximal inhibition (Fig. 6). On the other hand, B7-2 was not constitutively expressed on SJL/J astrocytes and only marginally expressed following treatment with IFN-\(\gamma\) for 24 h (Figs. 2B and 3B). IFN-\(\gamma\)-treatment of SJL/J astrocytes was also shown to up-regulate expression of CD40 (Fig. 3C) compatible with the findings that CD40-CD40L interactions are involved in inducing the expression of B7-1 and B7-2 (45, 46). Additionally, expression of ICAM-1 and VCAM-1, adherence molecules also critical for T cell activation (53, 54), are also increased following 24 h of IFN-\(\gamma\)-treatment (data not shown). Thus, SJL/J astrocytes in an inflammatory environment such as exists in the CNS of mice with ongoing R-EAE can express the requisite molecules necessary for costimulation of T cell activation.

It is tempting to speculate that the differential susceptibility to active EAE of SJL/J vs BALB/c mice may, in part, relate to the differential CNS expression of B7-1 and B7-2 costimulatory molecules. There are several salient observations that support this hypothesis. First, we have shown that B7-1 is preferentially up-regulated on F4/80\(^+\) microglia/macrophages and B cells in the CNS of SJL/J mice undergoing active R-EAE and that clinical relapses can be ameliorated by blocking B7-1 during the remission period after the acute phase of disease (55). Secondly, preliminary
experiments with a genome scan of an F2 cross between EAE-susceptible SJL/J and EAE-resistant B10.S mice have revealed a linkage of susceptibility to B7-1/B7-2 on mouse chromosome 16.6

We have previously demonstrated that IFN-γ-treated BALB/c astrocytes had the capacity to process and present OVA to naive transgenic T cells derived from the DOI1.10 mouse which are specific for the immunodominant OVA323-339 epitope and to memory Th1 lines derived from these mice (30). Thus, we also wanted to investigate the functional ability of SJL/J astrocytes to process and present various encephalitogenic PLP epitopes. We used whole PLP purified from bovine brain and MP4 as sources of Ag and a panel of T cell lines and hybridomas specific for the four described SJL/J encephalitogenic epitopes, PLP139-151, PLP56-70, PLP104-117 and PLP178-191. Interestingly, SJL/J astrocytes fed intact PLP or MP4 efficiently activated lines and hybridomas specific for the immunodominant PLP139-151 epitope (Figs. 4 and 5). However, T cell lines specific for the less immunodominant self encephalitogenic peptides (PLP56-70, PLP104-117, and PLP178-191) were not activated by IFN-γ-treated astrocytes fed intact PLP but were activated by astrocytes pulsed with the relevant autologous peptide. Similar results were seen with multiple independently derived PLP peptide-specific T cell lines and hybridomas specific for the less dominant epitopes and upon the use of astrocytes activated with a combination of TNF-α and IFN-γ, which enhances MHC class II and Ii expression above the levels seen for astrocytes stimulated with IFN-γ alone (data not shown). In light of these results, it appears that astrocytes, under the proinflammatory conditions that we have examined, may not play a major role in the phenomenon of epitope spreading wherein relapses in SJL/J mice with PLP19-151-induced R-EAE are mediated primarily by T cells specific for the PLP128-151 determinant (14). However, it is also possible that in the local milieu of the CNS, additional cytokines may play a role in activating astrocytes to more effectively process and present the subdominant PLP epitopes (56-59). Alternatively, in the inflammatory environment within the CNS of mice with acute R-EAE, extracellular proteases may cleave PLP into smaller fragments, which may result in enhanced presentation of subdominant determinants by astrocytes (60–62).

Significantly, IFN-γ-treated SJL/J astrocytes pulsed with either intact MP4 or PLP139-151 were also capable of activating PLP139-151-specific T cells for the adoptive transfer of R-EAE (Fig. 7), indicating that they can induce the up-regulation of the appropriate integrins and cytokines necessary for CD4 T cells to home to the CNS and initiate the demyelinating process. This is consistent with an earlier report showing that rat astrocytes could induce proliferation of encephalitogenic MBP-specific T cell lines (63). Regardless of the roles of astrocytes in initiating or maintaining CNS presentation of myelin epitopes, our recent data indicate that myelin peptides related to the epitope spreading process are generated within the CNS of SJL/J mice undergoing immune-mediated demyelination. CNS MHC class II”, F4/80+ microglia/macrophages isolated from the spinal cords of mice with ongoing PLP139-151-induced R-EAE or from mice with Thelier’s virus-induced demyelinating disease both have the capacity to directly activate T cell lines and hybridomas specific for both immunodominant and subdominant epitopes on myelin and virus proteins in the absence of addition of exogenous proteins/peptides to the in vitro cultures (Y. Katz-Levy, L. J. Tan, and S.D. Miller, unpublished data).

Collectively, these and our previous results (30) clearly indicate that astrocytes exposed to proinflammatory cytokines have the capacity to up-regulate the necessary molecules, including MHC class II, Ii, and H-2M and B7-1, enabling them to efficiently process and present self Ags and activate CD4 T cells. Astrocytes clearly have the functional capacity to process and present immunodominant antigenic determinants of conventional foreign proteins (30, 64) and self myelin Ags (Figs. 4–6) to both naive and memory T cells. Most significantly, T cells specific for the immunodominant PLP139-151 epitope could be activated in vitro by SJL/J astrocytes for the adoptive transfer R-EAE. Astrocytes may thus play a role in the process of chronic demyelination and epitope spreading by presenting dominant encephalitogenic epitopes from endogenous myelin proteins to encephalitogenic T cells. However, it is also possible that in response to different cytokine stimuli in the CNS, or at the activation state of the autoreactive T cells that astrocytes could also participate in down-regulating disease (28, 50). The definitive proof of a pathologic role of astrocytes in generating and presenting myelin epitopes will have to await the conclusion of ongoing experiments examining the Ag presenting capacity of astrocytes directly purified from the CNS of adult mice with ongoing R-EAE.

References


C. Teuscher and E. Blankenhorn. Submitted for publication.