

# Expression of Costimulatory Molecules B7-1 (CD80), B7-2 (CD86), and Interleukin 12 Cytokine in Multiple Sclerosis Lesions

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## Summary

Resting autoreactive T cells are present in the circulation of normal individuals without pathologic consequences. In autoimmune animal models, stimulation of these self-reactive T cells in the presence of costimulatory molecules B7-1 results in T cell-mediated autoimmune disease, whereas B7-2 stimulation generates regulatory autoreactive T cells that abrogate disease severity. Thus, reactivation in the brain of myelin-autoreactive T cells by antigen with costimulatory molecules may be a critical event in the pathophysiology of multiple sclerosis (MS), a putative autoimmune disease of central nervous system (CNS) myelin. We investigated the expression of cytokines and costimulatory molecules in a panel of 41 histologically characterized CNS specimens from 15 MS and 10 control cases using semiquantitative reverse transcriptase-polymerase chain reaction and immunocytochemistry. In four cases, vascular CNS infarcts with inflammation were compared with MS plaques from the same brain. We observed increased expression of B7-1 and interleukin (IL) 12p40 in acute MS plaques, particularly from early disease cases but not in inflammatory infarcts. B7-1 staining was localized predominantly to the lymphocytes in perivenular inflammatory cuffs but not the parenchyma. In contrast, B7-2 was expressed predominantly on macrophages both in MS lesions of varied time duration and in inflammatory infarcts. These findings indicate that an early event in the initiation of MS involves upregulation of B7-1 and IL-12, resulting in conditions that maximally stimulate T cell activation and induction of T helper 1-type immune responses.

Autoimmune disease is presumably mediated by activated, autoantigen-reactive T cells (1–8). Two distinct signals are required to induce differentiation of naive to activated, effector T cells: an antigen-specific signal mediated through the T cell receptor, and a second non-antigen-specific “costimulatory” signal (9, 10). Interactions between CD28 and its counterreceptors, B7-1 (CD80) and B7-2 (CD86), are important T cell-costimulatory signals (11, 12). Essentially all CD4<sup>+</sup> and most CD8<sup>+</sup> T cells express CD28 constitutively, and CD28-deficient T cells show a markedly reduced response to antigen stimulation (13). A second ligand for B7 is CTLA-4, which is expressed on T cells after activation (14) and also may regulate T cell function (15). The B7 molecules regulate IL-2 secretion and costimulate T cell proliferation (16–18). Blocking of the B7-CD28 pathway *in vitro* results in T cell anergy (19–21) whereas *in vivo* blocking of the B7-CD28 pathway results in immunosuppression (22–24).

Aberrant expression of B7-costimulatory molecules is

important in experimental autoimmune diabetes. Double-transgenic mice with T cell receptors recognizing a viral antigen expressed on pancreatic islet cells do not develop diabetes. Triple-transgenic mice that additionally expressed B7-1 on pancreatic islet cells, however, developed massive tissue destruction and diabetes (25). Moreover, double transgenic mice expressing both MHC class II and B7-1 molecules on pancreatic islet cells developed diabetes (26). These findings suggest that autoreactive T cells, which are a part of the normal T cell repertoire, are usually maintained tolerant to peripheral self-antigens because of the regulated absence of B7-costimulatory molecules on parenchymal cells.

Multiple sclerosis (MS)<sup>1</sup> is a chronic inflammatory disease characterized by lymphocyte infiltration and demyelination in the central nervous system (CNS) (27). Experi-

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<sup>1</sup> Abbreviations used in this paper: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; RT-PCR, reverse transcriptase PCR; SSPE, subacute sclerosing panencephalitis.

mental autoimmune encephalomyelitis (EAE), a model for autoimmune CNS disease, shows pathologic similarities to MS (28). EAE is mediated by CD4<sup>+</sup> T cells secreting the cytokines IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and lymphotoxin, which are defined as Th1 cells, whereas recovery is associated with the secretion of IL-4, IL-10, and TGF- $\beta$ 1, known as Th2 cells (29, 30). The type of effector T cell is determined by the cytokine microenvironment at the time of primary stimulation and by signals induced by costimulatory molecules (31). IL-12 and B7-1 costimulation is important for differentiation into Th1 cells, whereas IL-4 and perhaps B7-2 induce a Th2 response (32–40). The IL-12p40 subunit is transcriptionally regulated, and expression correlates with secretion of the active cytokine. Previous studies have suggested a pathogenic role for IFN- $\gamma$  and TNF- $\alpha$  in patients with MS and other autoimmune diseases (41–47).

A critical link between human autoimmune diseases and their experimental counterparts involves understanding events associated with the activation and differentiation of autoreactive T cells. Here, we investigated expression of the B7-1- and B7-2-costimulatory molecules in relationship to cytokine mRNA expression using a sensitive semiquantitative PCR method and immunohistochemistry. 41 plaques from 15 MS brains, including difficult-to-obtain tissue from two subjects with clinical MS for <2 yr duration, were compared with tissue from inflammatory infarcts, chronic viral-induced inflammatory lesions, and normal control brain. We observed increased expression of B7-1 by reverse transcriptase (RT)-PCR and immunohistology and IL-12p40 by RT-PCR in acute MS plaques but not in either inflammatory infarcts or normal control brains. In contrast, B7-2 was expressed predominantly on macrophages both in MS lesions and in inflammatory infarcts. These data indicate that an early event in the initiation of effector cells in MS involves upregulation of B7-1 and IL-12, resulting in conditions that promote T cell clonal expansion and IFN- $\gamma$  secretion with the breakdown of tolerance.

## Materials and Methods

**Patients.** 41 brain tissue specimens were included in this study. These samples were obtained at autopsy from 15 clinically and neuropathologically definite cases of MS and 10 control cases, 8 without neurological disease, 1 with a 2-yr history of subacute sclerosing panencephalitis, and 1 with a stroke (48). Brain specimens were also obtained in 4 cases from areas of infarct in patients who had MS and died from a stroke. The median age of patients was 50 yr (range 20–76 yr). Duration of disease was 1–50 yr, including two patients who had very short disease durations of 1 and 2 yr, respectively (Table 1). Tissue sections were selected from areas of either plaques or tissue damage on the basis of gross examination of the CNS. For each subject, all tissue blocks available were included in the study.

**Immunohistology.** Histology was performed as previously reported (49). Immunohistology was performed to confirm the RT-PCR results on all frozen blocks that contained tissue that could be investigated, and all of these results are reported. Acetone-fixed (4°C, 10 min) sections on gelatin-coated slides were incubated with mouse mAbs directed against CD68 (EBM/11), CD4, CD22 (Dako, High Wycombe, UK), IL-12 (Genetics In-

stitute, Cambridge, MA), B7-1 (PharMingen, San Diego, CA), and B7-2 (PharMingen). Sections were immunoperoxidase stained using a biotinylated anti-mouse IgG antibody and an avidin-biotin complex (Vector Laboratories, Inc., Burlingame, CA) as previously reported (49). All pathology was reviewed by two independent observers masked for both the tissue diagnosis and PCR results. Scoring of pathology is presented in the table legends.

**RNA and cDNA Preparation.** Total cellular RNA and cDNA was extracted and prepared as described previously (49). 5–10  $\mu$ l was used for each PCR reaction.

**Standardization and PCR Amplification.** Each 25- $\mu$ l PCR contained 0.5  $\mu$ g of forward and 0.5  $\mu$ g of reverse primers, 1.2 U of Taq polymerase, 0.08  $\mu$ l of [<sup>32</sup>P]dCTP, and 10  $\mu$ l of a mix of dNTPs and Taq buffer, which was prepared as a master mix for each set of samples to ensure the same reaction conditions in each sample. To avoid intraexperimental variation, samples were tested at the same time with the same PCR mix for each cytokine. Thermal cycling was performed at 94°C for 1 min, 55°C or 60°C for 1 min, and 72°C for 1.5 min for 28–35 cycles. Primer sequences were derived from the Gen Bank using the OLIGO 4.0 computer program (National Biosciences, Inc., Homel, MN). Primers were made by Oligos, Etc. (Wilsonville, OR). The primer sequences are shown in Table 2. To ensure linearity of the PCR and allow comparison of the efficiency of different primer combinations, an external standard curve for each cytokine was generated using a dilution series of standard DNA. This PCR standard was obtained by purification of amplified PCR product over a minicolumn (Wizard minipreps; Promega Corp., Madison, WI) and concentration was determined by OD 260 nm reading. Samples were normalized for their cDNA concentration by comparison of  $\beta$ -actin mRNA levels. Radioactive PCR products were separated on 5% polyacrylamide gels and analyzed on a phosphorimager (Betascope 603 Blot Analyzer; Betagen, Waltham, MA). Radioactivity in each band was counted, and results were expressed as relative units calculated as cpm cytokine/cpm actin  $\times$  100. mRNA expression is listed as relative units to normalize for  $\beta$ -actin concentration, and tissue sections were considered positive for mRNA expression if the relative units were greater than twice the standard deviation of the average value from non-inflammatory control brains. Statistical comparisons among the groups for B7 and IL-12 expression were performed using a two-tailed Fisher's exact test. Comparison of ages between the groups was performed using a two-tailed Mann-Whitney test, as indicated.

For IL-12p40 mRNA measurement expression, we used a competitive PCR method. We constructed a competitor DNA fragment (MIMIC) that had the same primer annealing sites and amplification kinetics as the cDNA, following the method described by Siebert and Larrick (50). The PCR products from the MIMIC and target differed by length to allow separation by gel electrophoresis. Known molar quantities of the competitor were spiked into a series of PCR reactions. The cDNA concentration of the sample was determined by comparing the relative intensity of PCR product bands from competitor and target cDNA. For the PCR analysis all samples were coded, and the pathology of the samples was not known at the time of the experiments.

## Results

IL-12p40, B7-1, and B7-2 mRNA expressions were examined in a panel of histologically characterized tissue specimens from MS patients and normal control subjects. mRNA expression is listed as relative units normalized to

**Table 1.** MS Patients and Normal Controls

Pathologic diagnosis	*Histology cell infiltration/ oil-red O	Tissue location	Age	Disease duration yr	Sex	Cause of death	Kurtzke scale	Death to freezing time h
Control cases								
1	1/0	Parietal	60	–	M	Pneumonia	NA	45
2	0/0	Parietal	42	–	M	Cardiac arrest	NA	21
3	0/0	Cervical cord	20	–	M	Asthma	NA	33
4	0/0	Cerebellum	24	–	M	Cardiac arrest	NA	27
5	1/0	Parietal	43	–	M	Pneumonia	NA	37
6	2/0	Temporal cortex	65	–	F	Pneumonia	NA	30
7	0/0	Periventricular	43	–	M	Renal failure	NA	33
8	1/0	Parietal	40	–	F	Aortic rupture	NA	42
SSPE 9	5/3	Parietal cortex	11	2	F	SSPE	NA	–
Infarct 10	0/2	Occipital	52	–	M	Cardiac arrest	NA	20
MS 1	1/1	Frontal cortex	53	22	M	Sepsis	9	56
Infarct-MS 2	0/0	Occipital pole	41	17	F	Pneumonia	9	52
MS 2.1	5/0	Occipital						
MS 2.2	5/0	Temporal						
MS 3	5/0	Cervical cord	50	15	M	ARDS	6	29
MS4	1/0	Parietal	62	14	M	Cardiac arrest	9	7
MS 5.1	2/1	Occipital	37	10	F	Pneumonia	9	24
MS 5.2	2/1	Parietal						
MS 6.1	1/3	Cervical cord	36	10	F	MS	9	33
MS 6.2	1/3	Cervical cord						
MS 7.1	1/1	Pons	52	9	M	Sepsis	9	29
MS 7.2	3/3	Pons						
MS 7.3	3/3	Pons						
MS 8.1	3/5	Parietal	29	7	F	Pneumonia	8	14
MS 8.2	2/4	Frontal pole	29	7	F			
MS 9.1	3/5	Cervical cord	26	3	M	Pneumonia	9	18
MS 9.2	3/5	Periventricular						
MS 9.3	2/4	Pons						
MS 10	3/5	Cerebellum	22	2	F	Brain stem plaque	9	11
MS 11.1	5/5	Lumbar cord	35	1	M	Asphyxia	4	28
MS 11.2	4/2	Thoracic cord						
MS 11.3	1/1	Corpus callosum						
Normal-MS11	2/0	Periventricular						
MS 12.1	4/3	Lumbar cord	29	8	F	Pneumonia	9	11
MS 12.2	5/4	Occipital	29					
MS 13.1	3/1	Frontal	71	50	F	Stroke	8	42
Infarct-MS 13	1/5	Cerebellum						
MS 14.1	0/0	Cervical cord	74	16	F	Stroke/pulmonary embolus	8	35
Infarct-MS 14	1/0	Occipital						
MS 15.1	1/0	Lumbar cord	76	41	M	Stroke	8	28
Infarct-MS	2/4	Thalamus						

ARDS, adult respiratory distress syndrome; NA, not applicable.

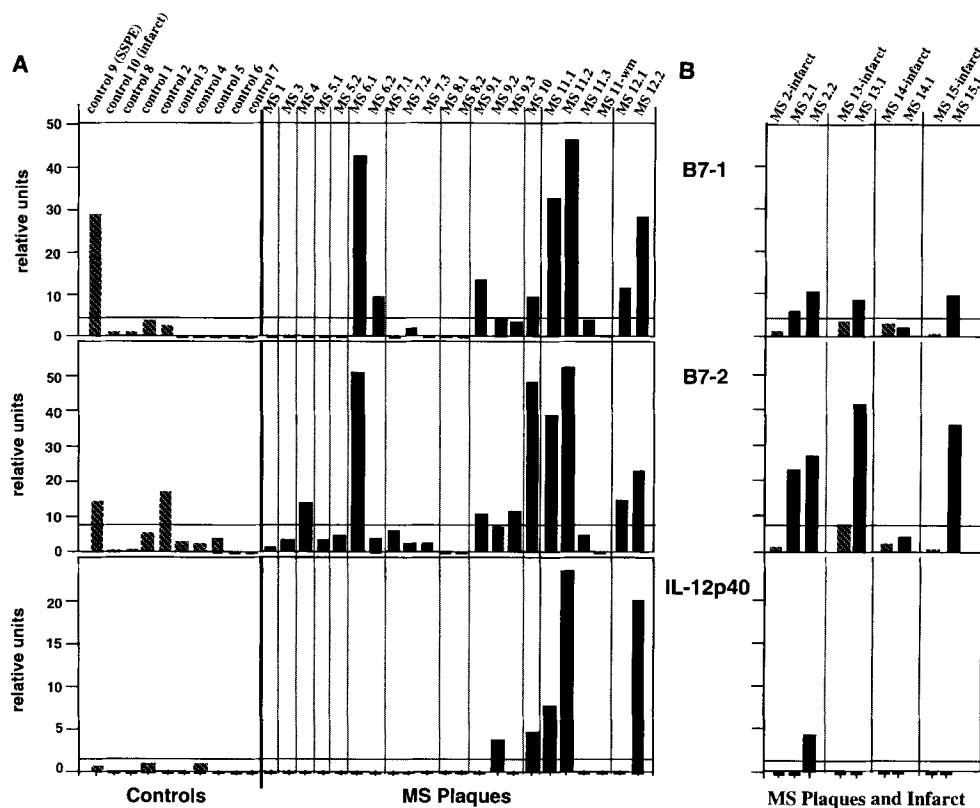
\*See Table 3 legend for scoring methods.

**Table 2.** Sequences of IL-12, B7, and Actin Primers Used for PCR

Cytokine	Length of PCR Product bp	Annealing temperature	5' - - - - -> 3'
β-Actin	406	60°C	Forward AAC CCC AAG GCC AAC CGC GAG AAG ATG ACC
			Reverse GGT GAT GAC CTG GCC GTC AGG CAG CTC GTA
IL-12p40	609	60°C	Forward ATT CAg TgT CAA AAg CAg CAg Agg
			Reverse Tgg gTC TAT TCC gTT gTg TC
IL-12p35	296	60°C	Forward CCT CAg TTT ggC CAg AAA CC
			Reverse ggT CTT TCT ggA ggC CAg gC
B7-1	389	60°C	Forward AAC TCg CAT CTA CTg gCA AAA ggA gAA
			Reverse AgA AAA Tgg TgC TgA CTA TgA TgT CTg
B7-2	409	60°C	Forward TgA TTC ggA CAg TTg gAC CCT gAG AC
			Reverse gTA TCA ATg TAT CAT CCA TCA CAA AAA gCCC

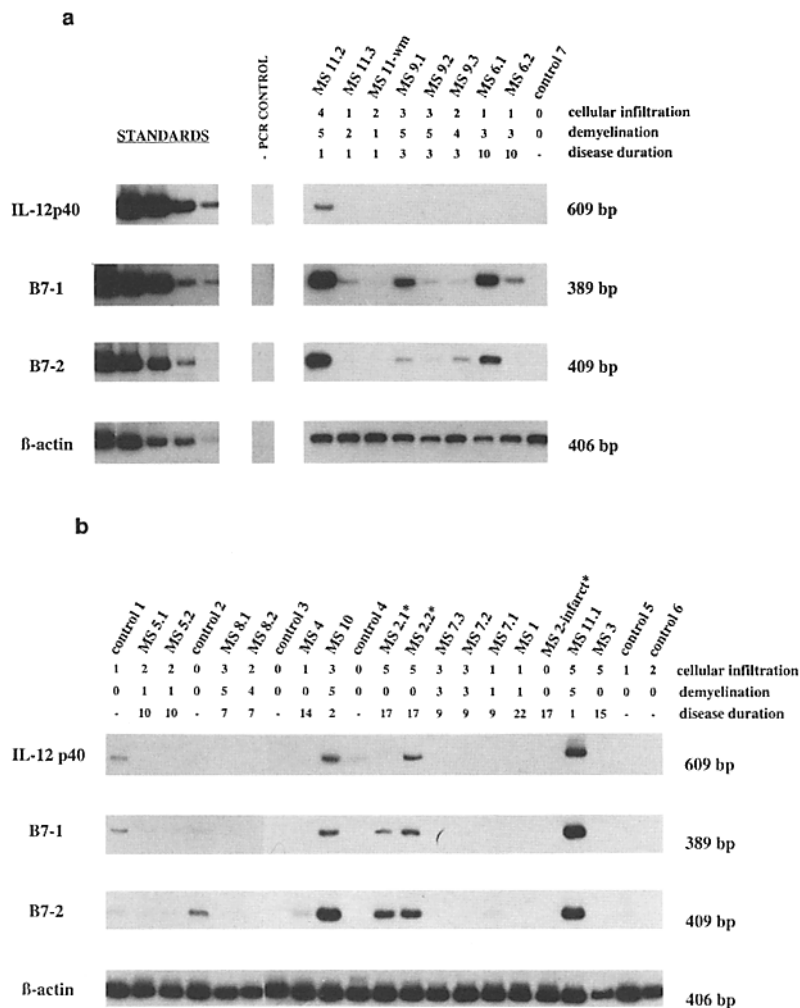
β-actin concentration. Tissue sections were considered positive for mRNA expression if the relative units were greater than twice the standard deviation of the average value from noninflammatory control brains. These results are shown in Fig. 1, and representative autoradiographs af-

ter PCR amplification are shown in Fig. 2. Increased B7-1 expression was observed in 8 of 15 MS cases and in 0 of 9 of the nonviral inflammatory control brains ( $P < 0.009$ ). Although none of the inflammatory stroke lesions expressed B7-1, tissue from a subject with a 2-yr history of



**Figure 1.** mRNA expression of IL-12p40, B7-1 (CD80), and B7-2 (CD86) in all CNS brain specimens. Results are shown as relative units calculated as cpm cytokine/cpm actin  $\times$  100. The mean relative index  $\pm$  2 SD was calculated for the noninflammatory disease control cases, and is designated by the stippled line for B7-1, B7-2, and IL-2. Values  $>$ 2 SD over the mean were considered positive and analyzed by a Fisher's exact test. (a) Solid bars represent patients with MS and cross-hatched bars represent control brain specimens. In cases expressing IL-12p40, the average disease duration was  $6.2 \pm 2.6$  (SE) yr, whereas in the brains with no IL-12 expression, the average disease duration was  $19.4 \pm 4.4$  (SE) yr ( $P < 0.04$ , two-tailed Mann-Whitney test). Increased B7-1 expression was observed in 8 of 15 MS cases and in 0 of 9 of the nonviral inflammatory control brains ( $P < 0.009$ , two-tailed Fisher's exact test). None of the inflammatory stroke lesions expressed B7-1. Tissue from a SSPE case expressed levels of B7-1 comparable to patients with MS. 9 of 15 MS cases

and 1 of 9 nonviral inflammatory control brains expressed increased levels of B7-2 mRNA ( $P < 0.03$ ). The frequency of plaque samples with cell infiltrations  $\geq 3$  correlated with positive mRNA expression of either B7-1, B7-2 ( $P < 0.005$ , Fisher's exact test), or IL-12 ( $P < 0.04$ ). (b) Direct comparison of brain specimens from MS patients with strokes. Solid bars represent MS inflammatory plaques and cross-hatched bars represent stroke lesions from the same brain. There was little perivascular cell infiltration, whereas significant infiltrations of lipid-laden macrophages were observed in the infarcts (see Fig. 4, c and d).



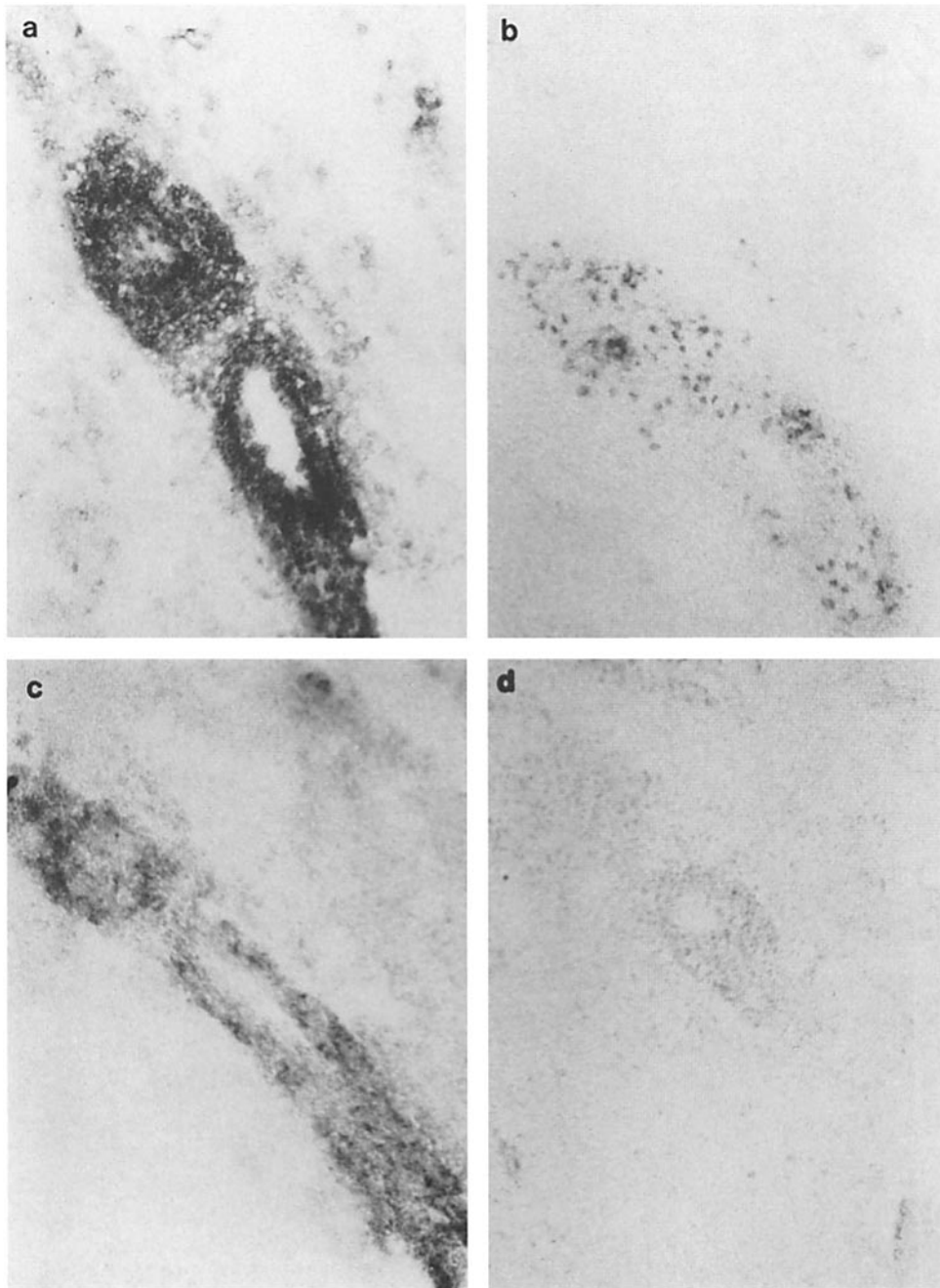
**Figure 2.** mRNA expression of IL-12p40, B7-1, B7-2, and  $\beta$ -actin in 30 CNS tissue specimens from MS patients and normal control cases listed in Table 1. The samples are in a random order since the PCR analysis was done blinded. For each PCR set an external standard curve was generated. The concentrations of the standards were  $2 \times 10^{-1}$ ,  $4 \times 10^{-2}$ ,  $8 \times 10^{-3}$ ,  $1.6 \times 10^{-3}$ , and  $3.2 \times 10^{-4}$  for  $\beta$ -actin;  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  for IL-12p40;  $4 \times 10^{-3}$ ,  $8 \times 10^{-4}$ ,  $1.6 \times 10^{-4}$ ,  $3.2 \times 10^{-5}$ , and  $6.4 \times 10^{-6}$  for B7-1; and  $10^{-1}$ ,  $2 \times 10^{-2}$ ,  $4 \times 10^{-3}$ ,  $8 \times 10^{-4}$ , and  $1.6 \times 10^{-4}$  for B7-2.

subacute sclerosing panencephalitis (SSPE), a chronic CNS infection by defective measles virus, expressed levels of B7-1 comparable to patients with MS. Similarly, 9 of 15 MS cases and 1 of 9 nonviral inflammatory control brains expressed increased levels of B7-2 mRNA ( $P < 0.03$ ). Direct assessment of B7-1 expression showed  $4 \times 10^{-3}$  attomoles/ $\mu$ l for B7-1 and  $2 \times 10^{-2}$  attomoles/ $\mu$ l for B7-2 expression in tissue from MS11.2. As B7 was expressed in only approximately half the MS brains, we examined whether there was a correlation between perivascular cell infiltration and RT-PCR mRNA measurements of B7 expression. The frequency of plaque samples with cell infiltrations of  $\geq 3$  correlated with positive mRNA expression of either B7-1 or B7-2 ( $P < 0.005$ ).

To define the morphologic substrate of B7 protein expression in MS plaques and cerebral vascular infarcts, sections were stained with antibodies for macrophages (EBM/11), CD4<sup>+</sup> T cells, B cells (CD22), and for B7-1 and B7-2. Representative photomicrographs are shown in Figs. 3 and 4, and the results are summarized in Table 3. Immunocytochemistry was performed on 9 control cases (2 with CNS inflammation), 16 MS plaques, and 4 MS plaque/infarct paired samples.

Normal control samples demonstrated characteristic microglial staining pattern with EBM/11 and occasional B and T cells near blood vessels, but no B7-1<sup>+</sup> or B7-2<sup>+</sup> cells were detected. As we observed in the PCR analysis, the MS plaque samples with perivascular cell infiltrations of  $\geq 3$  exhibited B7-1 expression that reflected staining with lymphocyte markers for CD2 (T cells) and CD22 (B cells) and was restricted to the blood vessel area (Fig. 3 *c* and Fig. 4 *a*). This contrasted with the presence of widespread EBM/11<sup>+</sup> perivascular macrophages and parenchymal microglia. The B7-2 staining, however, was clearly detectable on what appeared to be a subpopulation of activated macrophages in the majority of plaques, contrasting with the more restricted B7-1 expression (Fig. 4). Comparison of serially cut sections stained with the different mAbs indicated that the intense B7-1-stained cells were likely to be T cells, and were detected in the inflammatory cuffs amid cells expressing lower levels of B7-1.

Based on recent experiments in the EAE model, we postulated that tissue cellular infiltrates associated with MS would have B7-1 expression, whereas inflammation associated with tissue damage, such as that induced by stroke, would express B7-2. This was directly investigated by

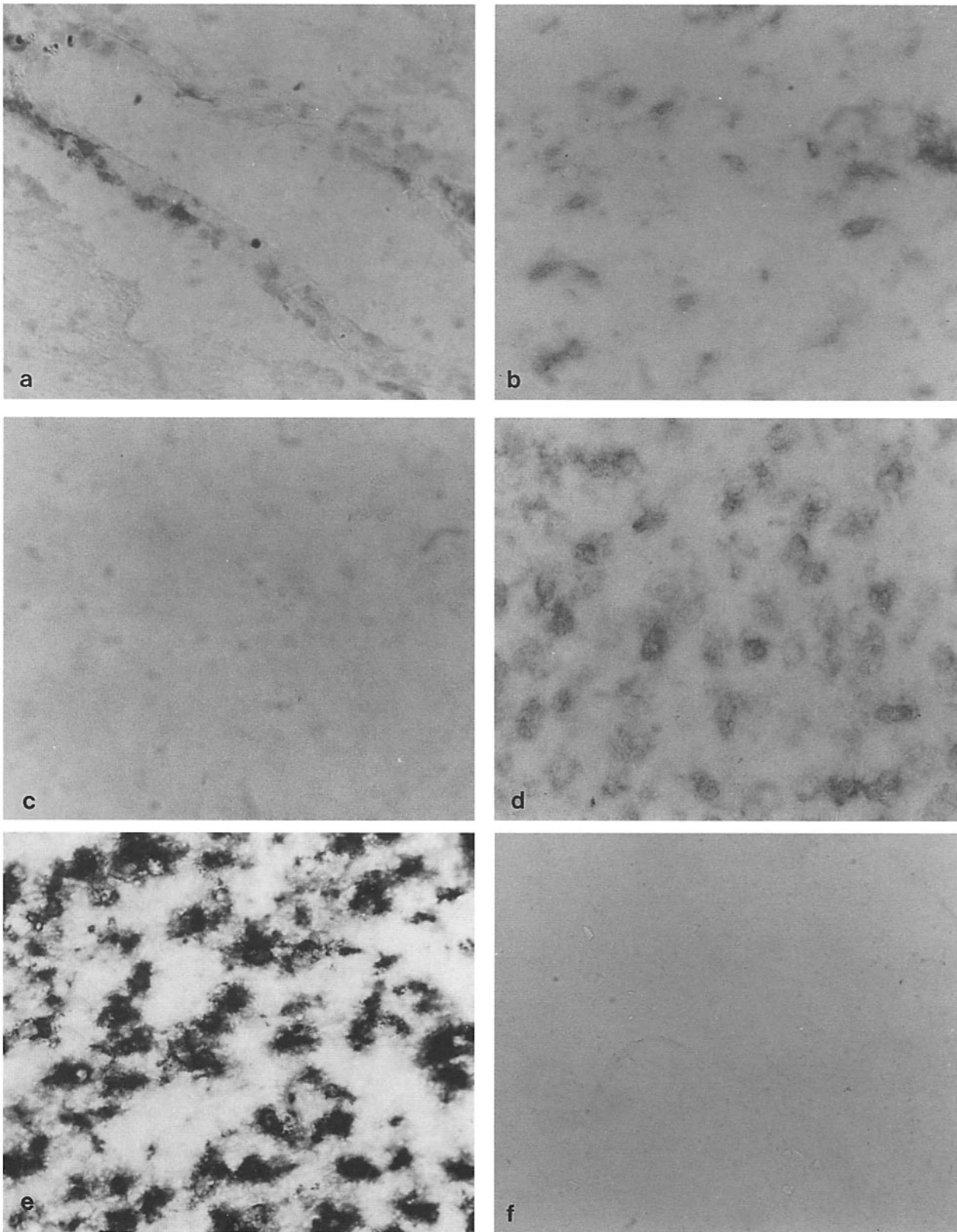


**Figure 3.** Cryostat sections of a perivenular inflammatory cuff and the surrounding tissue in an inflammatory MS plaque from CNS11.1 with disease duration of 1 yr. (a) Anti-CD4; (b) anti-CD22; (c) anti-B7-1; and (d) anti-keratin control.  $\times 98$ .

comparing four cases with both MS and stroke associated pathologically with inflammation. There was no B7-1 staining in the infarcts from patients with MS, whereas the associated plaque tissue in two of four cases contained B7-1<sup>+</sup> perivascular cells (Fig. 4, *c* and *d*, and Table 3). On the other hand, B7-2 expression was observed in both infarct and plaque samples. The cerebrovascular infarct from control 10 had barely detectable staining for lymphocyte markers and B7-1 and B7-2 staining despite the presence of activated macrophages. Interestingly, the SSPE sample, which displayed a strong PCR signal, had only a very small number of B7-1<sup>+</sup> leukocytes, but these were intensely stained

and clearly detectable in a restricted number of blood vessels, which was consistent with the high RT-PCR signal found in this sample (data not shown). As in the MS plaques, the more widespread B7-2 staining appeared to be associated with macrophages.

We observed an increase in expression of IL-12p40 mRNA in at least one plaque from 5 of 15 MS brains. In cases expressing IL-12p40, the average disease duration was  $6.2 \pm 2.6$  (SE) yr, and 4 of 5 had disease for  $\leq 8$  yr, whereas in the brains with no IL-12 expression, the average disease duration was  $19.4 \pm 4.4$  (SE) yr ( $P < 0.04$ , two-tailed Mann-Whitney test), and 9 of 10 cases had disease



**Figure 4.** Immunocytochemical analysis of cryostat sections from MS plaque (*a* and *b*) and infarct tissue (*c-e*) from MS brain 13.1 and 13.2. *a* and *c* are stained with anti-B7-1 (CD80); *b* and *d* are stained with anti-B7-2 (CD86); *e* is stained with the macrophage marker EBM 11; *f* is normal control white matter (control 8) with anti-B7-2 staining. There was no counterstaining. B7-1 staining is localized to perivenular inflammatory cuffs in MS plaques but not infarct areas, whereas B7-2 staining was present in both infarct and MS plaque.  $\times 370$ .

**Table 3.** Summary of Immunohistopathology

	Disease duration	Oil-red O*	Cell infiltration (MNC)‡		B7-1 staining	B7-2 staining	Disease duration	Oil-red O*	Cell infiltration (MNC)‡		
			yr	macrophages					Inflammatory control tissue	B7-1 staining	B7-2 staining
Control							Inflammatory control tissue				
CNS tissue							Cont 9	2			
Case 8		0	1	-	-	SSPE		3	5	++ +	
Case 1		0	1	-	-	Cont 10	-				
Case 2		0	0	-	-	Infarct		0	2	- -	
Case 3		0	0	-	-						
Case 4		0	0	-	-						
Case 5		0	1	-	-						
Case 6		0	2	-	-						
Case 7		0	0	-	-						
MS tissue											
MS 1	22	1	1	-	+						
MS 15	15	0	5	+	-						
MS 4	14	0	1	-	+						
MS 6	10	1	2	-	-						
		1	2	-	-						
MS 7	9	1	1	-	++						
		3	3	+	++						
		3	3	-	++						
MS 12	8	3	4	-	+						
		4	5	++	++						
MS 8	7	5	3	-	++						
		4	2	-	++						
MS 10	2	5	3	++	++						
MS 11	1	5	5	++	+						
		2	4	++							
		0	2	+	ND						
Paired MS											
plaques/infarcts											
MS 2	17										
Infarct		0	0	-	-						
Plaque		0	5	+	+						
		0	5	+	+						
MS 13	50										
Infarct		5	1	-	++						
Plaque		1	3	+	+						
MS 14	16										
Infarct		0	1	-	-						
Plaque		0	0	-	-						
MS 15	41										
Infarct		4	2	-	+						
Plaque		0	1	-	-						

Continued

Data are B7-1 and B7-2 staining of CNS samples, as assessed by two observers masked for disease state and PCR data. B7-1 and B7-2 immunohistochemistry were graded as negative (-), positive with occasional moderate staining of perivascular cuffs (+), or maximally positive with intense staining of cells in perivascular cuffs or parenchyma (++) . Disease duration, cell infiltration on the basis of hematoxylin and eosin staining, and demyelination on the basis of 0.7-red O staining is shown for comparison (see below for scoring methods).

\*Oil-red O staining is a measure of lipid-laden macrophages, and thus correlates with the macrophage infiltration in the tissue and degree of ongoing or recent demyelination. The staining is scored on a scale of 0-5 by estimating the number and density of oil-red O-positive macrophages in both the area of perivascular cuffing and in the zone radiating to the edge of the normal-appearing white matter. (0 is what is expected in normal control white matter and 5 represents maximal macrophage infiltration with oil-red O-containing material).

‡Cell infiltration is scored on a scale of 0-5. Cellular infiltration as judged by hematoxylin staining is scored on the basis of the number of inflammatory cuffs per unit field and their size and thickness, with the range of 0-5 (0 is what is expected in normal control white matter and 5 represents maximal perivascular cellular infiltration). At least five fields are estimated. Inflammatory lesions are defined as perivascular cellular infiltrates >3, whereas perivascular cellular infiltrates of 0-1 in MS brain represent chronic lesions with gliosis and minimal mononuclear cell infiltrates. In infarcts, there was macrophage infiltration in the tissue with little in the way of perivascular cuffing. Pathology was assessed by two observers blinded as to the tissue diagnosis and PCR results.

for ≥8 yr. There was no IL-12p40 mRNA expression in any of the cases from control brains. As with B7 expression, the frequency of plaque samples with cell infiltrations ≥3 also correlated with positive mRNA expression of IL-12 ( $P < 0.04$ ). To further quantitate the amounts of IL-12p40, we performed a competitive PCR analysis for IL-12p40. Although less sensitive than standard RT-PCR, this technique allows a more precise quantification of the amount of mRNA. IL-12p40 was detected at a concentration of  $2 \times 10^{-6}$  attomoles/ $\mu$ l in CNS MS.11, the tissue with the highest level of mRNA detected by semiquantitative PCR (data not shown). As predicted from the quantification using RT-PCR, we were unable to detect IL-12 using the less sensitive methods of immunocytochemistry and in situ hybridization to detect the cellular source of IL-12 mRNA.



## Discussion

We examined the expression of the costimulatory molecules B7-1 and B7-2 and IL-12p40 mRNA in the CNS of patients with MS compared with tissue from inflammatory infarcts, chronic viral-induced inflammatory lesions, and normal control brains. B7-1 and IL-12p40 were expressed in acute MS plaques from early disease cases but not in the inflammatory infarct control tissue. B7-1 staining was localized predominantly to the lymphocytes in perivascular inflammatory cuffs but not the parenchyma. In contrast, B7-2 was expressed predominantly on macrophages both in MS lesions of varied time duration and in cerebral infarcts. These findings suggest that an early event in the initiation of MS involves upregulation of B7-1 and IL-12, resulting in conditions that promote T cell clonal expansion and IFN- $\gamma$  secretion with the breakdown of tolerance.

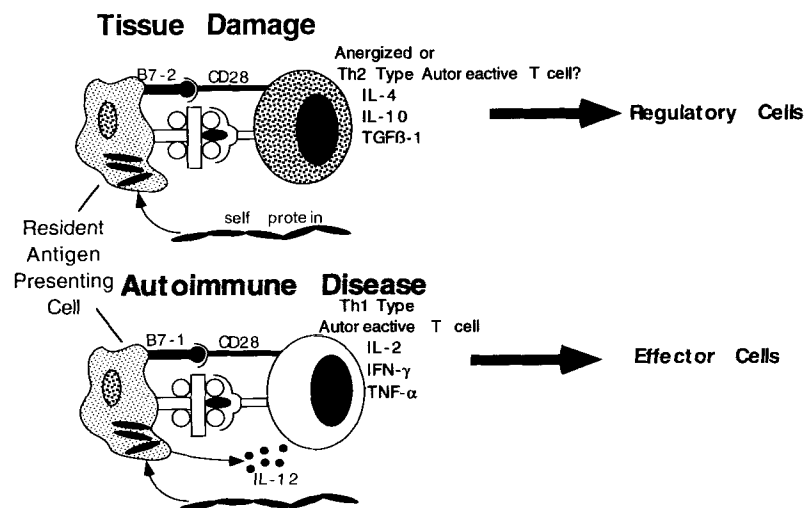
Early MS lesions have predominantly perivascular lymphocytic cell infiltrations followed in time by the accumulation of lipid-laden phagocytic macrophages that express oil-red O staining. Patients with longer disease duration tend to have chronic lesions with sharply demarcated gliotic areas, which lack inflammation. Because B7-1, B7-2, and IL-12 were expressed in some but not all of the MS cases, it was important to examine whether expression of these molecules reflected either the degree of inflammation or the disease duration. Tissue sections graded by blinded observers with perivascular cell infiltrations  $\geq 3$  had significantly higher B7-1, B7-2, or IL-12 mRNA expression. Moreover, patients with shorter disease duration were significantly more likely to express IL-12. In marked contrast, other regulatory cytokines including IL-2 and IL-4 were not observed more frequently in the inflamed MS tissue compared with noninflammatory control brains (data not shown).

There was not a perfect correlation between immunocytochemistry and PCR analysis. In case MS 11-WM, B7-1 was expressed in the section cut for immunocytochemistry, whereas no signal was detected in those cut for PCR, and a high PCR signal was detected in MS 6.1, which on the ba-

sis of immunocytochemistry and histological analysis was not inflammatory. This may relate to technical issues such as tissue storage with mRNA degradation or differences in pathology from different sections of the same tissue block. Alternatively, these differences may relate to posttranscriptional regulation of B7-1 expression in the inflammatory cells infiltrating the lesions.

Perhaps the most convincing data to suggest that B7 expression is associated with MS lesions and is not related simply to macrophage infiltration were obtained by comparing B7-1 and B7-2 expression in inflammatory MS plaques and inflammatory strokes from the same brain. This provided a control for other factors that may be associated with B7 or IL-12 expression in the brain, such as HLA type or other genetic influences. There was significant inflammation in the areas of infarct, as evidenced by either cell infiltration or oil-red O staining of macrophages. In the two MS cases with cell infiltration, B7-1 was expressed predominantly on the lymphocytes in the MS plaques but not in the stroke lesions. In striking contrast, B7-2 expression was observed predominantly on macrophages both in the MS plaques and cerebral infarcts. These data indicate that inflammatory MS plaques but not cerebral infarcts are associated with B7-1 expression. A low level of B7-2 expression was also found in RT-PCR in noninflammatory control brains, which may represent constitutive expression on microglia as B7-2 is expressed on resting monocytes. Our results of increased B7-1 expression in the MS brain are also consistent with recently published results by Antel (51) and Aloisi (52).

Myelin exposure to T cells after a break in the blood-brain barrier after stroke, trauma, or tumor does not result in autoimmune demyelination. Our data suggest that this may relate to the regulated absence of B7-1 expression at the lesion. That is, autoreactive T cells stimulated at tissue sites with B7-2 costimulation do not induce tissue destruction but instead induce scar formation with tissue fibrosis. Thus, autoreactive T cells may function to regulate nonviral-induced tissue destruction, as may occur in the brain



**Figure 5.** Model for the role of B7 costimulatory molecules in the induction of autoimmune disease. Autoreactive T cells stimulated at tissue sites with B7-2 costimulation do not induce tissue destruction but instead lead to scar formation with tissue fibrosis. Thus, autoreactive T cells may function to regulate nonviral-induced tissue destruction, as may occur in the brain with stroke. In contrast, costimulation with B7-1 in addition to B7-2 on organ-specific APC leads to effector-autoreactive T cells. It is postulated that B7-1 expression is induced by either systemically activated T cells inducing B7-1 by gp39-CD40 interactions on CNS APC or by a direct CNS viral infection. Similarly, we postulate IL-12 secretion by CNS APC is induced by IFN- $\gamma$  secretion by systemically activated T cells.

with stroke. In contrast, costimulation with B7-1 in addition to B7-2 on organ-specific APC leads to effector autoreactive T cells (Fig. 5). Interestingly, B7-1 expression has also been found in active skin lesions from psoriasis patients (53), suggesting that B7 might indeed be involved in a variety of autoimmune diseases.

A critical question remains as to what might induce expression of B7-1 and IL-12 in MS brains, and whether this is a primary or secondary event in the disease pathogenesis. Although data from the transgenic diabetes model would suggest that B7 expression in a genetically predisposed individual may trigger the disease, it may not be possible to formally prove this hypothesis in human autoimmune disease. MHC class II is constitutively expressed in the healthy brain and was found to be upregulated in inflammatory MS lesions, and could therefore synergize with B7-1 (54, 55).

We postulate that B7-1 expression is induced by either systemically activated T cells via gp39-CD40 interactions on CNS APC or by a direct CNS viral infection. Similarly, we postulate that IL-12 secretion by CNS APC further induces IFN- $\gamma$  secretion by systemically activated T cells.

In summary, we demonstrate specific increases in expression of the costimulatory molecule B7-1 and the cytokine IL-12 in inflammatory MS brain plaques, whereas B7-2 expression was not specific for MS plaques and was seen in nonautoimmune/viral-mediated inflammation. Thus, expression of B7-1 and IL-12 may be an important event in disease initiation, providing maximal stimulation for an autoreactive, pathogenic Th1-type T cell response. Further investigations will focus on the regulation and interaction of these molecules in vitro and in vivo, which might open new approaches to MS therapy.

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