Experimental Autoimmune Panencephalitis and Uveoretinitis Transferred to the Lewis Rat by T Lymphocytes Specific for the S100β Molecule, a Calcium Binding Protein of Astroglia

By Kimikazu Kojima,* Thomas Berger,‡§ Hans Lassmann,‡‖ Dunja Hinze-Selch,* Yiping Zhang,* Jochen Gehrmann,¶ Konrad Reske,**Hartmut Wekerle,* and Christopher Linington*

*From the *Department of Neuroimmunology, Max-Planck Institute for Psychiatry, 82152 Martinsried, Germany; the ‡Neurological Institute; the §Department of Neurology, University of Vienna; the ‖Research Unit for Experimental Neuropathology, Austrian Academy of Sciences, A-1090 Vienna, Austria; the ¶Department of Neuromorphology, Max-Planck Institute for Psychiatry, 82152 Martinsried; and the **Institut für Immunologie, Johannes Gutenberg Universität, D-55101 Mainz, Germany*

Summary

The pathogenic potential of autoimmune T cell responses to nonmyelin autoantigens was investigated in the Lewis rat using the astrocyte-derived calcium binding protein S100β, as a model nonmyelin autoantigen. The Lewis rat mounts a vigorous RT1B[1] (major histocompatibility complex class II) restricted autoimmune response to an immunodominant S100β epitope (amino acid residues 76–91). The adoptive transfer of S100β-specific T cell lines induced a severe inflammatory response in the nervous system, but only minimal neurological dysfunction in naive syngeneic recipients. The inability of S100β-specific T cell transfer to induce severe disease was associated with a decreased recruitment of ED1$^+$ macrophages into the central nervous system (CNS) in comparison with that seen in severe experimental autoimmune encephalomyelitis (EAE) induced by the adoptive transfer of myelin basic protein (MBP)-specific T line cells. Moreover, unlike encephalitogenic MBP-specific T cell lines, S100β-specific T cell lines exhibited no cytotoxic activity in vitro. Histopathological analysis also revealed striking differences in the distribution of inflammatory lesions in MBP- and S100β-specific T cell–mediated disease. In contrast to the MBP paradigm, S100β-specific T cell transfer induces intense inflammation not only in the spinal cord, but throughout the entire CNS and also in the uvea and retina of the eye. In view of the distribution of lesions throughout the grey and white matter of the CNS we propose to term this new model experimental autoimmune panencephalomyelitis (EAP) to differentiate it from EAE. These experiments demonstrate for the first time that nonmyelin CNS autoantigens can initiate a pathogenic autoimmune T cell response, although the nature of the target autoantigen profoundly influences the clinical and histopathological characteristics of the resulting autoimmune disease. This is not simply a consequence of the distribution of the autoantigen, as both MBP and S100β are coexpressed in many areas of the CNS, but reflects differences in the capacity of different regions of the CNS to process and present specific autoantigens. This new model of T cell–mediated autoimmune CNS disease exhibits a number of similarities to multiple sclerosis (MS), such as its mild clinical course and the involvement of areas of the brain and eye, which are absent in myelin-mediated models of EAE. Nonmyelin autoantigens may therefore play an unexpectedly important role in the immunopathogenesis of inflammatory diseases of the CNS.

Two basic tenets in cellular immunology were radically revised during the past decade as a direct consequence of studies of experimental autoimmune encephalomyelitis (EAE)[1]. First, the concept of "immunoprivilege" with re-

[1] *Abbreviations used in this paper:* BBB, blood–brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; EAP, experimental autoimmune panencephalitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PPD, purified protein derivative.

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spect to the central nervous system (CNS) was reappraised after the demonstration that not only can activated T lymphocytes migrate through the endothelial blood–brain barrier (BBB) regardless of their antigen specificity (1, 2); but also that the CNS contains numerous cellular elements (astrocytes, microglia, cerebral endothelial cells) that can function as APCs (3–5). Second, the isolation of encephalitogenic T cell lines from both myelin-primed (6) and naive rats (7) demonstrated that potentially autoaggressive T cell clones are not necessarily silenced by either deletion or anergy, but are normal components of the healthy immune repertoire (8, 9).

However, these results are all derived from studies in which EAE is mediated by an autoaggressive T cell response against a single myelin antigen, the myelin basic protein (MBP), which until very recently appeared to be the predominant, if not the only, encephalitogenic protein present in the CNS. Analysis of the encephalitogenic, MBP-specific T cell response revealed that it exhibited several unusual properties. In at least two species, the Lewis rat and mice with the H-2^u haplotype (PL/J and B10.PL strains), the primary MBP-specific T cell response is directed against single, strongly immunodominant epitopes, the identity of which is species and strain dependent. Furthermore, encephalitogenic T cells specific for these immunodominant epitopes use a highly restricted repertoire of TCR genes, generally TCR $V_\beta 8.2$ in combination with $V_\alpha 2/V_\alpha 4$ elements (10–13). The potential therapeutic relevance of these observations was rapidly demonstrated in EAE (14–18); however, the molecular characteristics responsible for the encephalitogenicity of the MBP-specific T cell response have still to be elucidated. In particular, it has to be demonstrated whether EAE mediated by myelin-specific T cells provides a general paradigm to study inflammatory responses in the CNS, or is a special case of limited usefulness.

To approach this problem and identify the structural/functional characteristics of CNS proteins necessary to induce EAE, we elected to study the encephalitogenic potential of nonmyelin CNS proteins that differ from MBP with respect to their site of synthesis and physicochemical characteristics. One of these proteins was S100β, a calcium binding protein that was first isolated from the CNS. S100β is a small, highly soluble, acidic protein consisting of 91 amino acids (19, 20). Like MBP, S100β is a major component of the nervous system, accounting for ~0.4% of the total soluble protein in the CNS (21), but unlike MBP which is only present in oligodendrocytes and myelin, expression of S100β in the CNS is restricted to astrocytes. Elsewhere, S100β is also expressed by Schwann cells in the peripheral nervous system and Müller cells in the retina (22, 23). Astrocytes also secrete a biologically active form of S100β and the protein can be detected in the cerebrospinal fluid (24, 25).

To provide a direct comparison with the encephalitogenic MBP-specific T cell response a panel of S100β-specific T cell lines was generated from both bulk, and limiting dilution cultures of lymph node cells obtained from S100β-primed animals. These T cell lines were then analyzed in vitro with respect to their epitope specificity, phenotype, TCR V_β gene usage, cytokine production, and cytotoxicity, and in vivo after adoptive transfer into naive syngeneic recipients to determine their pathogenic potential. S100β-specific, class II MHC–restricted, $CD4^+$ Th1 T line cells were found to mediate an intense inflammatory response throughout the CNS that differs dramatically from MBP-mediated EAE with respect to lesion distribution, structure and cellular composition. Additionally, S100β-specific T cells induce inflammatory changes in both the uvea and retina of the eye, a pathology reported to occur in a subgroup of patients with multiple sclerosis (MS) (26).

Table 1. *Amino Acid Sequences of the Synthetic Rat S100β Peptides Used in This Study*

Designation	Residues	Amino acid sequence
A4	1–14	SELEKAMVAL I DVF
B4	4–18	EKAMVAL I DVFHQYS
C4	10–24	L I DVFHQYSGREGDK
D4	15–20	HQYSGREGDKHKLKK
E4	20–34	REGDKHKLKKSELKE
F4	25–39	HKLKKSELKEL I NNE
A5	31–45	ELKEL I NNELSHFLE
B5	36–50	I NNELSHFLEE I KEQ
C5	41–55	SHFLEE I KEQEVVDK
D5	46–60	E I KEQEVVDKVMETL
E5	51–65	EVVDKVMETLDEDGD
F5	56–70	VMETLDEDGDGECDF
A6	61–75	DEDGDGECDFQEFMA
B6	66–80	GECDFQEFMAFVSMV
C6	71–85	QEFMAFVSMVTTACH
D3	76–91	FVSMVTTACHEFFEHE

Bovine S100β protein differs from rat S100β at residues 7 (Met/Val), 62 (Glu/Ser), 78 (Ser/Ala), and 80 (Val/Ile).

These observations demonstrate that MBP and other myelin proteins are not the only CNS autoantigens able to induce extensive autoimmune-mediated encephalomyelitis/panencephalomyelitis in mammals. A potential role for autoimmune responses to nonmyelin autoantigens will now have to be considered the pathogenesis of inflammatory diseases of the CNS, in particular MS.

Materials and Methods

Animals and Antigens. Inbred Lewis rats aged 6–8 wk were obtained from the animal breeding facilities of the Max-Planck Institute (Martinsried, Germany). Bovine S100β protein was obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic rat S100β peptides were prepared by Dr. N. Groome (Oxford Brookes University, Oxford, UK) on a multiple peptide synthesizer (Abimed, Dusseldorf, Germany) using standard Fmoc chemistry (Table 1). Peptide purity was monitored by amino acid analysis, reverse-phase HPLC and, when appropriate, mass spectrometry.

mAbs and Anti-sera. The following mAbs were used in this study: OX6 (RT1.B, rat I-A), OX17 (RT.1D, rat I-E), and OX18 (MHC class I), W3/13 (pan T cell/granulocyte); W3/25 (CD4); OX8 (CD8); OX39, IL-2 receptor; OX22 (CD45RO). The suc-

ceeding rat T cell receptor-specific mAbs were used: R73 (constant region of TCR-α/β [27]); R78 (TCR V_β 8.2); B73 (TCR V_β8.5); and G101 (TCR V_β10) (28) and HIS42 (TCR V_β16). Rat macrophages and microglia were differentiated using the mAbs TRPM3 (29), ED1, ED2, ED3 (30), and MUC102 (31). Rabbit anti-S100β and anti-GFAP antisera were purchased from Sigma Chemical Co. W3/13, W3/25, OX8, OX39, and MUC102 were purified in our laboratory from the culture supernatants of the appropriate hybridoma cell lines; ED mAbs, OX22, OX42, and HIS42 were purchased from Serotec (Oxford, UK) and TRPM3 from Dianova (Hamburg, Germany).

Antigen-specific T Cell Lines. Lewis rats were immunized in the hind foot pads with either 50 μg native antigen (S100β protein, guinea pig MBP, or purified protein derivative [PPD]), or 35 μg of synthetic peptide, in complete Freund's Adjuvant (CFA) containing 4 mg/ml *Mycobacterium tuberculosis* H37a (Difco Laboratories, Inc., Detroit, MI).

Two different protocols were used to establish antigen specific T cell lines. 10 d after immunization single cell suspensions were prepared from the draining lymph nodes. The primed cells were either cultured at a concentration of 10^7 cells/ml to establish bulk T cell lines (LS) (6), or alternatively serially diluted to provide a large number of pauciclonal T cell lines (prefixed; S1 [32]). In the latter case, lymph node cell suspensions were cultured in 96-well round-bottom microtiter plates at cell numbers ranging from 2×10^5 to 100 cells/well. The wells were supplemented with antigen and irradiated (4,000 rad) syngeneic thymus cells to give a final cell number of 2×10^5/well. After 3 d, the media was removed and the remaining T cells expanded for a further 7–10 d in media supplemented with IL-2. The T cells were then restimulated with antigen in the presence of 2×10^5 irradiated (4,000 rad) syngeneic thymus cells/well. After visual inspection of the cultures, those positive for growth were expanded in the presence of IL-2 and antigen-specific S1 T cell lines selected as described for the bulk culture-derived LS cell lines. The LS and S1 T cell lines were then subsequently propagated as described previously (6, 33).

Proliferation and Lymphokine Assays. Lymph node cells (2×10^5/well), or 2×10^4 T line cells plus 0.9×10^6 irradiated (4,000 rad) syngeneic thymocytes were cultured in 0.2 ml medium in 96-well round-bottomed microtiter plates in the presence or absence of the appropriate antigens. After 56 h, 1 μCi/well [^{3}H]thymidine was added and the cells were harvested 16 h later. The mean cpm were determined for triplicate cultures using a solid phase gas scintillation counter (Matrix 96 Direct Beta Counter; Packard, Frankfurt, Germany). It should be noted that the counts obtained by this method are approximately five times lower than with methods using scintillation fluid.

The ability of either syngeneic astrocytes or the mouse L cell line, RT3.3, which is transfected with functional RT1B^l to act as APCs was determined using irradiated cultures (5,000 rad) seeded into flat bottomed 96-well plates at a density of 2×10^4 cells/well. T line cells (2×10^4/well) were added to give a total volume of 0.2 ml media in the presence or absence of antigen. T cell proliferation was determined at the end of 3 d culture as described above. The cell line RT3.3 was established using the mulcos system (34) (a kind gift from Dr. I. Saito, National Institutes of Health, Toyko, Japan). Briefly, cDNA clones pLRβ118 (35) and pLRα6 (36) were inserted into EcoRI and HindIII sites respectively of the cassette plasmid pmoRH, excised by Sfi1 digestion and ligated with the Sfi1 linearized cosmid vector dCHD2L (34). MHC class II and invariant chain negative rat-2 cells (10^6) were transfected with 5 μg of cosmid DNA employing the DOTAP procedure (Boehringer Mannheim, Mannheim, Germany) following the supplier's instructions. Transfectants were selected using Hygromycin B at 200 μg/ml added 48 h after transfection.

Cytokine assays were performed on the culture supernatants of the various T cell lines as described previously (37) after 48 h of antigen-specific restimulation. IFN-γ levels were measured using a rat IFN-γ ELISA kit (Holland Biotechnologies, Leiden, The Netherlands). The IL-2-dependent CTLL cell line was used to assay IL-2 activity using mouse rIL-2 as a standard. IL-6 activity was determined using the IL-6-dependent 7TD1 hybridoma cell line. TCR-α/β activity was assayed using TNF-α/β-sensitive LM cells and a polyclonal TNF-α-specific goat antimurine antibody (Genzyme Corp., Cambridge, MA). The 7TD1 and LM cells were generously provided by Dr. K. Frei (Universitätespital Zurich, Switzerland).

Cytofluorographic Analysis. The surface phenotype of the T cell lines was investigated by indirect immunofluorescence staining. 2×10^5 viable cells were washed with PBS containing 0.2% BSA and 10 mM NaN_3 and incubated with the primary mAb for 1 h on ice. The cells were washed to remove the primary antibody and stained with fluorescein conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) for 1 h on ice. Cells were then washed and immediately analyzed using a FACScan® (Becton Dickinson, Heidelberg, Germany). A live gate was obtained by incubating the cells in PBS containing propidium iodide.

Induction of EAE. Lewis rats were injected into the tail vein with varying numbers of T cell blasts harvested after 72 h of antigen driven restimulation. Animals were cared for in accordance with German federal regulations and weighed and examined for clinical signs of EAE on a daily basis. EAE severity was scored as: 0, no disease; 0.5, partial loss of tail tonus; 1, complete tail atony; 2, hind limb paraparesis; 3, hind limb paralysis; 4, moribund; 5, death. Demyelination was induced 5 d after the injection of T cells by the intravenous injection of 4 mg of the mouse mAb 8-18C5 specific for the myelin oligodendrocyte glycoprotein (MOG) (38).

Histology. Rats were perfused through the heart with 4% paraformaldehyde in PBS and tissue postfixed in the same fixative for 3 h at 4°C. Alternatively, tissues were removed and immediately snap frozen. Multiple sections were stained with hematoxylin–eosin and Luxol fast blue. Immunocytochemistry was performed on both paraffin embedded and frozen sections as described previously (39).

The frequency of inflammatory infiltrates in the cortex, periventricular white matter, diencephalon, mesencephalon, cerebellum, medulla oblongata, and spinal cord was determined by counting the number of perivenous inflammatory cuffs in multiple hematoxylin–eosin-stained sections in standardized regions of a total area of 375 mm^2/animal. The results are expressed as the number of inflammatory infiltrates/mm^2. The total number of T cells and macrophages was determined in sections immunostained with the appropriate antibodies using a 100-point morphometric lattice placed in the microscope's ocular (ocular × 10, objective × 100). The total area quantitated was 0.6 mm^2 in the spinal cord and 0.2 mm^2 in the sciatic nerves and spinal roots and the results are presented as the number of cells/mm^2 of tissue area.

Astrocyte Culture. Astrocytes were prepared from newborn Lewis rat cerebra. The meninges were removed and a single cell suspension prepared by trypsinization and seeded into plastic culture flasks (4). The nonadherent cells were removed after 4 h and cultured in 5% CO_2 at 37°C. The medium was first replaced after 24 h and subsequently every 3–4 d. After 10 d the primary cultures were shaken for 3–5 h to remove microglial cells. The remaining adherent astrocytes were removed by trypsinization and transferred to flasks at a density of 3×10^4/cm^2. After a further 2–3 wk in culture, the cells were again harvested by trypsinization before use

Table 2. *Antigen Specificity of S100β-specific T Cell Lines*

Line	No. Ag	Response to antigens			
		D3	S100β	PPD	Con A
			mean cpm		
LS1	28	10,517	12,651	13	10,876
LS2	100	5,642	10,764	82	11,440
SP-D3	524	10,583	8,274	36	10,722
S1-D1	3	4,310	7,710	2	8,869
S1-E12	410	2,968	5,212	171	9,242
S1-F5	5	2,643	7,082	2	10,176
S1-D2	6	2,457	10,354	3	9,955
S1-G5	7	2,133	5,533	3	9,663
S1-F12	5	2,096	7,300	3	11,699
S1-D12	21	1,160	7,173	7	9,336
S1-E8	6	1,037	4,111	4	8,749
S1-E2	6	1,096	8,450	8	10,284
S1-G12	4	755	4,788	6	10,087
S1-F4	124	552	4,512	7	11,233
S1-E3	6	452	2,590	0	9,701
S1-F6	28	375	6,746	8	11,288
S1-F8	4	318	10,205	3	12,203
S1-C7	3	218	6,049	3	9,219
S1-G7	8	131	9,079	5	10,614
S1-G6	3	103	10,574	2	12,408
S1-G9	1	54	2,588	2	8,281
S1-G8	6	8	3,232	3	5,817

Antigen specific T line cells (2×10^4/well) were cultured in triplicate with irradiated (4,000 rad) syngeneic thymocytes (0.9×10^6/well) in the presence or absence of either the synthetic rat S100β peptides (Table 1), bovine S100β, PPD (final concentrations 10 μg/ml), or Con A (2.5 μg/ml) for 3 d. Cultures were pulsed with [^{3}H]TdR for the last 16 h of the 72-h culture and the incorporation of isotope determined as described. The results are presented as the mean cpm obtained, standard deviations were no more than 16%. In addition to the response to peptide D3 the following responses to peptides C6 (LS1, 305 cpm; LS2, 410 cpm; S1-F4, 264 cpm) and A5 (S1-E2, 324 cpm) were observed.

Table 3. *Surface Phenotype of LS and S1 S100β-specific T Cell Lines*

Line	W3/13	W3/25	OX8	OX39	OX22	R73	R78	B73	G101	HIS42
LS1	99.0	96.9	2.5	98.7	0.3	99.0	4.1	4.4	11.3	15.7
LS2	98.9	98.1	4.5	95.8	0.4	99.7	1.8	2.5	3.0	7.8
S1-D1	99.7	99.2	1.2	99.6	0.4	99.9	0.3	0.4	9.8	7.8
S1-D2	99.4	98.3	1.8	98.8	0.47	100	0.2	0.4	4.8	82.0
S1-C7	99.3	97.4	1.1	97.3	0.4	99.9	0.4	0.4	0.3	50.0
S1-G7	98.1	99.3	1.4	100	0.6	99.6	0.4	0.4	0.4	7.8
S1-G9	99.6	99.3	2.9	99.9	0.5	99.9	0.2	0.5	0.2	98.8
SP-D3	99.7	96.3	2.2	96.4	0.3	99.9	1.8	8.9	3.5	14.4

Viable lymphoblasts (2×10^5) were incubated with the appropriate mouse mAb and subsequently stained with FITC-conjugated goat anti-mouse IgG and analyzed by FACScan®. Controls were stained with the second Ab alone. The results are indicated as the percent of positive cells.

Table 4. *Cytokine Profiles of S100β- and MBP-specific T Cell Lines*

Cell line	Supernatant activities			
	IFN-γ	IL-2	IL-6	TNF
		mean ± SE		
LS1	>10,000	6.8 ± 0.3	1358 ± 612	308 ± 82
S1-D2	>10,000	1.8 ± 0.1	1325 ± 632	158 ± 39
SP-D3	>10,000	2.2 ± 0.2	1813 ± 750	108 ± 23
CP1	>10,000	2.9 ± 0.4	127 ± 63	193 ± 87
Z85	>10,000	ND	490 ± 270	705 ± 79
34LMR	>10,000	0.9 ± 0.1	1313 ± 575	220 ± 77
APC control	<250	ND	150	ND

in cytotoxicity and proliferation assays. These cultures contained >97% GFAP$^+$ cells of which >80% were S100β$^+$. Microglia were not detected.

Astrocyte Cytotoxicity Assays. Astrocytes harvested by trypsinisation were cultured in 96-well flat-bottom plates in complete basal medium (Eagles) (BME) (10^4/well) in the presence or absence of 100 U/ml of rat rIFN-γ (Holland Biotechnologies) for 48 h before use in either cytotoxicity or proliferation assays.

Cytotoxicity was determined by ^{51}Cr release (40). The cells were labeled with ^{51}Cr (1 μCi/10^4 cells) for 2 h at 37°C. Excess ^{51}Cr was removed by washing the cells three times, followed by incubation at 37°C for 5 h and a second round of washing. Freshly harvested T cell blasts were added to the labeled astrocytes in complete DME to give different E/T ratios in the presence or absence of the antigen (10 μg/ml). Radioactivity was measured in triplicate using a gamma counter (Cobra Auto-gamma 5003; Packard) after a 12-h incubation. Maximal lysis was determined after incubation of the astrocytes with 2.5% Triton X-100.

Results

Characterization of S100β-specific T Cell Lines. Lewis rats immunized with bovine S100β mounted a vigorous T cell response to this antigen and of a total of 61 S100β-specific T cell lines generated during this study, 21 were investigated in more detail; 2 T cell lines derived from bulk cultures (LS1 and LS2); and a further 19 S100β-specific S1 T cell lines derived from microcultures. After three restimulation cycles the T cell lines were specific for bovine S100β protein and exhibited no residual response to PPD (Table 2).

The eight S100β-specific T cell lines analyzed by FACS® all exhibited the classical W3/13$^+$, OX8$^-$, CD25$^+$ and TCR-α/β$^+$ phenotype of CD4$^+$ rat T cell lines. However they are clearly differentiated from encephalitogenic CD4$^+$ MBP-specific rat T cell lines and clones by their TCR V_β gene usage (Table 3). Unlike the MBP-specific T cell lines, S100β-specific T cell lines do not preferentially use TCR V_β8.2.

The spectrum of cytokines secreted by representative T cell lines specific for either S100β (LS1, S1-D2, SP-D3) or MBP (Z85 [37] CP1 [34], 34LMR) was analyzed during the first 48 h of an antigen-specific restimulation. All T cell lines secreted IFN-γ, IL-6, and TNF-α into the medium and IL-2 was detected at low levels in the supernatants with the exception of Z85 (Table 4). The production of IFN-γ indicates that these cell lines are of the Th1 subtype.

The MHC restriction of the Lewis rat T cell response to bovine S100β was established using the T cell line S1-D2. The anti–rat I-A mAb OX6 (125 μg/ml) reduced the proliferative response of this T cell line to S100β by 70%, whereas the same concentration of mAb reduced the proliferative response of a control MBP-specific T cell line, C1-C9, by 97%. The mAbs OX17 and OX18 had no significant effect on the proliferative response of either T cell line. These results were confirmed using the transfected rat-2 mouse L cell line RT3.3, which expresses both the α and β chains of Lewis rat I-A. This mouse fibroblast cell line was capable of acting as an APC in vitro inducing an antigen-specific proliferative response in both T cell lines tested (Table 5).

Identification of the Target Epitopes and V_β TCR Usage. A panel of sixteen overlapping synthetic rat S100β peptides (Table 1) was used to identify T cell epitopes recognized by the S100β-specific T cell lines. The T cell lines LS1 and LS2 both recognized an immunodominant epitope common to both rat and bovine S100β located within the COOH-terminal sequence of the protein, amino acid 76-91, peptide D3 (Fig. 1). Analysis of the S1 T cell lines selected from serial dilutions of the original lymph node cell preparations confirmed that the rat S100β sequence 76–91 was a dominant epitope with respect to the T cell response to bovine S100β (Table 2). However, although all S1 T cell lines proliferated strongly in response to native bovine S100β, the proliferative response to this epitope was significantly lower than that seen in the bulk-derived T cell lines LS1 and LS2. Proliferation in response to peptide D3 ranged from 20 to 55% of the response to bovine S100β for eight of the S1 T cell lines analyzed, while the remaining T cell lines responded poorly to this rat epitope. In the case of the T cell lines such as S1-G8 and S1-G9

Table 5. *MHC Restriction of the S100β-specific T Cell Line S1-D2 and the MBP-specific T Cell Line C1-C9*

T cell line	APC	Ag	mAb added			
			0	OX6	OX17	OX18
					125 μg/ml	
S1-D2	Thy	+	9534 ± 380	2832 ± 364 (70.3)	8268 ± 413	7846 ± 471
	Thy	−	4 ± 2	ND	ND	ND
	RT3.3	+	1085 ± 430	ND	ND	ND
	RT3.3	−	6 ± 2	ND	ND	ND
C1-C9	Thy	+	4602 ± 920	134 ± 105 (97.1)	5425 ± 109	3272 ± 262
	Thy	−	4 ± 2	ND	ND	ND
	RT3.3	+	1136 ± 511	ND	ND	ND
	RT3.3	−	4 ± 4	ND	ND	ND

The MHC restriction of the T cell lines S1-D2 (S100β-specific) and C1-C9 (MBP-specific) was determined using the mAbs OX6, OX17, and OX18. Confirmation that these responses were I-A restricted was obtained using the transfected mouse L cell line, RT3.3, that expresses Lewis rat RT1B[l]. Expression was confirmed by both FACS® analysis and biosyntheic labelling. The data are presented as the mean cpm of triplicate cultures ± SD. The percent inhibition obtained in the presence of the mAb OX6 is given in brackets.

there was no response to any of the peptides tested indicating that they recognize bovine S100β-specific epitopes (Table 2). The T cell line, SP-D3, established from Lewis rats immunized with the peptide D3 (amino acid 76–91), as anticipated, proliferated vigorously in response to both the immunizing peptide and native bovine S100β (Table 2). All subsequent studies were performed using the T cell lines LS1, LS2, S1-D2, S1-C7, S1-G7, S1-G9, and SP-D3, which exhibit differing degrees of reactivity with the immunodominant peptide amino acid 76-91.

FACS® analysis using a panel of four murine mAbs recognizing defined rat TCR V_β products revealed that only 2–4% of the LS1 and LS2 S100β-specific bulk culture T cell lines expressed V_β8.2 on their surface (Table 3). The extent of V_β8.5 expression was also very low, whereas V_β10 usage was slightly higher (3–11%), as was usage of V_β16 (8–16%). The SP-D3 T cell line raised against the immunodominant peptide D3 also expressed low levels of V_β8.2 (2%), V_β10 (4%), and V_β8.5 (9%), and again a significant proportion of V_β16 (14%). None of the S1 T cell lines tested expressed either V_β8.2 or V_β8.5, whereas V_β16 usage was ~50% in S1-C7, 80% in S1-D2, and 100% in S1-G9. Within the limits of the technique used these results demonstrate that unlike the T cell response to MBP, the Lewis rat T cell response to S100β does not preferentially use TCR V_β8.2.

S100β-specific CD4+ T Cell Lines Induce an Autoimmune Panencephalitis with Uveoretinitis, Rather Than a Pure Encephalomyelitis. Naive Lewis rats injected with 5×10^6, 10^7, or 2×10^7 LS1 and LS2 lymphoblasts failed to exhibit any dose-related clinical signs of EAE. All animals began to lose weight 4 d post T cell transfer and exhibited a transient loss of tail tone that lasted a further 3–4 d. After this time the animals began to recover weight (Fig. 2). No other clinical signs of disease were noted, in particular hind limb paraparesis, paralysis, and urinary incontinence, which are the clinical hallmarks of MBP-induced EAE, were absent. Similar losses of weight and tail tone were also observed in animals injected with 10^7 T cell blasts obtained from the S100β-specific T cell lines SP-D3, S1-D1, and S1-D2. The adoptive

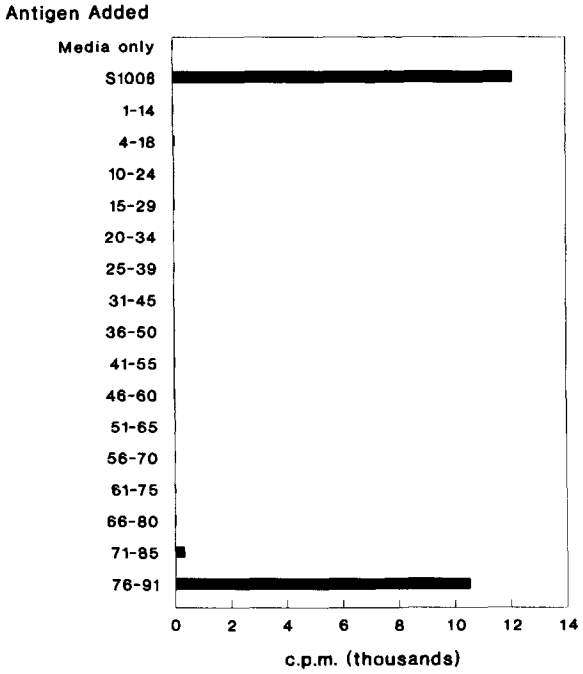

Figure 1. Epitope specificity of the S100β-specific T cell line, LS1. LS1 T line cells were restimulated with either native bovine S100β, or synthetic rat S100β peptides (see Table 1) as described in the text. Only the overlapping COOH-terminal peptides amino acids 71–85 and amino acids 76–91 induced a significant proliferative response of the LS1 T cell line in vitro locating the immunodominant T cell epitope(s) to this domain of the molecule. No other peptides were found to initiate a significant proliferative response in either this, or any other of the T cell lines tested.

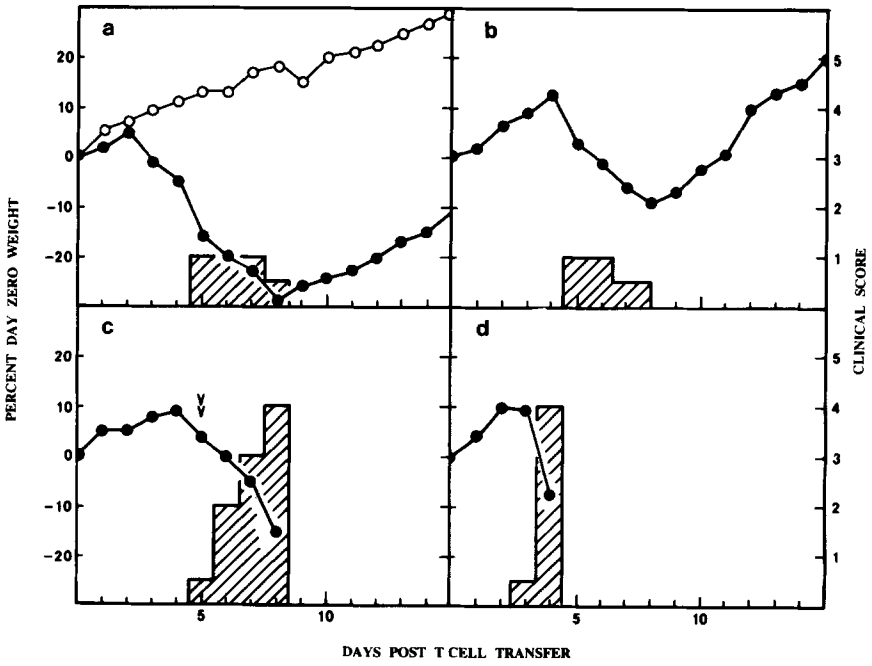

Figure 2. The clinical course of disease induced by the adoptive transfer of CNS-autoantigen-specific T cell lines. Representative results obtained after the adoptive transfer of (*a*) *closed circles*, 10^7 freshly activated S100β-specific T cells (line LS1); *open circles*, 10^7 freshly activated PPD-specific T cells; (*b*) 10^7 freshly activated SP-D3 T line cells specific for the synthetic S100β peptide D3, amino acid residues 76–91; (*c*) 10^7 freshly activated S100β-specific T cells (line LS1) in combination with an intravenous injection of 4 mg of the mAb 8-18C5 5 d after T cell transfer (*arrow*), due to the severity of the disease induced after the mAb transfer animals were perfused on day 8; (*d*) 10^7 freshly activated MBP-specific T cells (T cell line CP1).

transfer of 10^7 S1-G7 or S1-G9 T cell blasts, which respond poorly if at all to the peptide D3 (Table 2) failed to induce any clinical changes in naive recipients. Controls injected with the same doses of PPD-specific T cell blasts showed no loss of weight, or tail tone, whereas Lewis rats injected with comparable numbers of MBP-specific T line cells developed severe clinical signs of acute EAE by day 4 (Fig. 2).

Rats were perfused 6 d after T cell transfer and the CNS (brain, spinal cord, optic nerve), peripheral nervous system (PNS) (nerve roots, sciatic nerve, trigeminal nerve), eyes, heart, lungs, tongue, thymus, kidney, spleen, bowel, adrenal glands, and salivary glands removed for histopathology.

Surprisingly, despite the absence of obvious signs of CNS disease this analysis revealed that, with one exception (T cell line S1-G9), all the S100β-specific T cell lines induced a severe inflammatory response in the CNS (Fig. 3, *a*, *c*, *d*, and *f*). Inflammatory infiltrates were also seen in the peripheral nerve roots in 60% of animals, but only very rarely in the sciatic nerve. In contrast, uveitis was very common, occurring in 49 of the 60 animals examined and was in some cases associated with scleritis, iridocyclitis, and periphlebitis retinae (Fig. 3, *g*, *h*, and *i*). Uveitis was not observed in any animal with MBP-induced EAE. No inflammatory responses were seen in any other of the tissues examined.

The intensity of the inflammatory response induced in the CNS by the S100β-specific T cell lines was qualitatively similar to that seen in animals with severe (grade 2+), EAE induced by a MBP-specific T cell line (Figure 3, *a* and *b*). However, unlike MBP-induced disease, in which the inflammatory lesions are most prominent in the spinal cord and rare in brain, S100β-specific T cell lines induced an inflammatory response throughout the CNS, in which involvement of both brain and spinal cord was common (Fig. 3; Table 6). The difference between the two models was most marked in the cortex and mesencephalon, both of which are strongly involved in the S100β-specific T cell–mediated disease (Table 6). This S100β-specific T cell–mediated inflammatory disease of the CNS thus qualifies as an experimental autoimmune panencephalitis (EAP).

The Clinical Course and Histopathology of S100β-specific T Cell–mediated CNS Inflammation Can Be Modulated by the Cotransfer of MOG-specific Antibody. In view of the extensive inflammation seen in animals receiving S100β-specific T cell lines it was of interest to determine whether these clinically silent lesions could be modulated by cotransfer of the mAb 8-18C5. This mAb recognizes an epitope of the MOG exposed on the surface of the myelin membrane. Intravenous injection of this antibody in animals with MBP-mediated EAE enhances the clinical severity of the disease and induces local demyelination (41, 42).

In the present study, intravenous injection of 4 mg of purified 8-18C5 5 d after the transfer of S100β-specific T cells rapidly induced the classical signs of EAE, paraparesis/paralysis within 24 h (Fig. 2). This was associated with the formation of large plaques of demyelination throughout the CNS (Fig. 3 *e*). Injection of the same dose of a control mouse IgG preparation failed to induce any enhancement in either clinical status or demyelination in animals with S100β-induced CNS inflammation. The intense inflammatory response seen in the CNS of animals injected with S100β-specific T cell lines is therefore associated with an enhanced permeability of the BBB sufficient to permit the 8-18C5 mAb to enter the CNS and initiate demyelination.

The Failure of S100β-specific T Cells to Induce Severe Clinical Disease Is Associated with a Decreased Activation of ED1$^+$ Macrophages in the CNS. To investigate the cellular/physiological basis responsible for the inability of S100β-specific T cell lines to induce severe neurological disease, we examined the

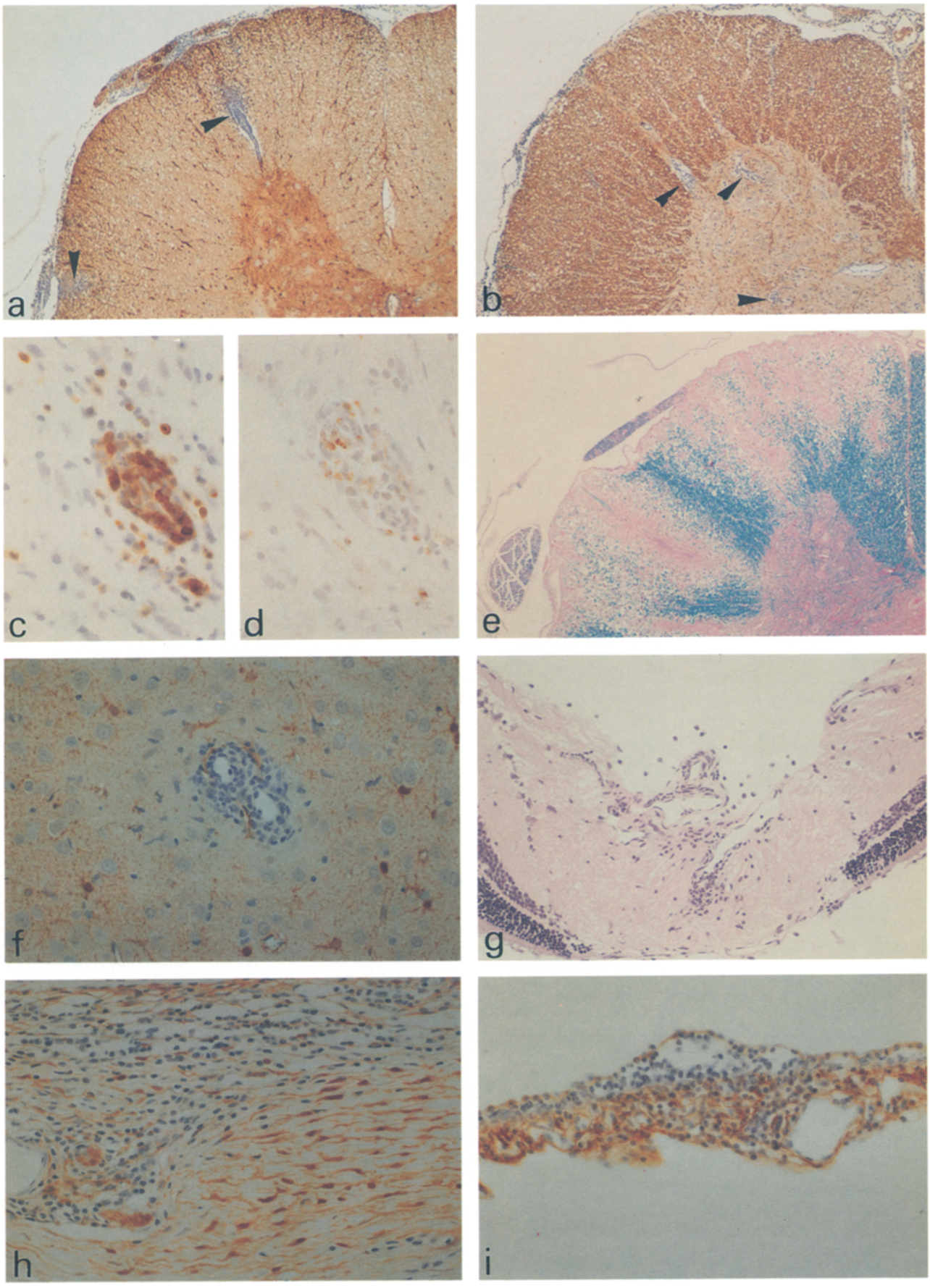
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Table 6. *Topographic Distribution of the Inflammatory Infiltrates in MBP- and S100β-specific T Cell–mediated Disease*

Tissue	T cell line specificity	
	MBP	S100β
Cortex*	0.004 ± 0.002	0.746 ± 0.088
White matter*	0.107 ± 0.053	0.310 ± 0.050
Diencephalon*	0.209 ± 0.055	0.965 ± 0.200
Mesencephalon*	0.499 ± 0.109	1.500 ± 0.064
Cerebellum	0.571 ± 0.187	0.235 ± 0.043
Medulla	1.711 ± 0.380	1.355 ± 0.228
Spinal Cord	2.040	2.990

Lewis rats were injected intravenously with activated MBP-specific (CP1; $n = 7$), or S100β-specific (LS1; $n = 6$) T line cells and perfused 6 d later as described in the text. The number of inflammatory infiltrates/mm² determined in multiple tissue sections. The distribution of the lesions is strikingly different in the two models, particularly with respect to the cortex, white matter, diencephalon, and mesenencephalon that are intensively involved in the S100β paradigm, but only exhibit moderate inflammation following the transfer of MBP-specific T cells (* significance at 98% in analysis of variance).

composition and distribution of the infiltrating inflammatory cells in the CNS. This data was compared with that obtained from studies on MBP-specific T cell–mediated EAE.

Even after hematotoxylin–eosin staining it was apparent that in the S100β-specific T cell induced disease model, the perivascular lesions were very large as compared with those seen in MBP-specific T cell–induced EAE. Large numbers of cells were tightly packed into clearly delineated perivascular cuffs of inflammatory cells. Quantitative immunohistochemical analysis revealed that the absolute number of W3/13$^+$ T cells present in the CNS on day 6 was at least 10 × greater in both the perivascular and parenchymal compartments of animals infected with the S100β-specific T cell lines, than in MBP-induced EAE (Table 7).

However, in contrast to the numbers of T cells found within the CNS, the expression of ED1 as a marker for lysosomal activation in macrophages was lower in rats with S100β-induced CNS inflammation. ED1$^+$ cells are the major component of the classical EAE lesion (Table 7), where they account for up to 80% of the infiltrating inflammatory cells, both in the perivascular space and parenchyma. This is not the case in S100β-specific T cell mediated CNS inflammation. Although the absolute number of ED1$^+$ cells present in the CNS is approximately equal in both MBP- and S100β-specific paradigms, the proportion of this cell type entering the parenchyma is low (Table 7). Moreover, the intensity of staining with the ED1 mAb appeared weaker in the S100β paradigm, suggesting that these cells are poorly activated in this model. To ensure that this observation was not a staining artifact, macrophage infiltration was also evaluated using additional markers for rat macrophage populations on fresh frozen sections. The macrophage-specific mAbs ED3 and TRPM3 also demonstrated that in S100β-mediated EAP, the number of activated macrophages present in the perivascular cuffs and parenchymal infiltrates was lower than that seen in MBP-mediated EAE. In contrast, the mAb ED2, which stains a population of resident perivascular cells in the CNS, was found to identify a similar number of cells in both MBP- and S100β-mediated diseases. Similarly, no difference in the phenotype of ramified microglia in the parenchyma was found between the two experimental models.

Astrocyte Interactions with S100β-specific T Cell Lines In Vitro. The cytotoxicity of several pathogenic CD4$^+$ S100β-specific T cell lines was assessed in vitro using syngeneic astrocytes as targets in a chromium release assay. The Lewis rat MBP-specific T cell line Z85 (40) and PPD-specific T cell line LP-PPD1 (37) were used as positive and negative controls for T cell mediated cytotoxicity. In agreement with previous studies the Z85 T cell line effectively lysed IFN-induced, Ia$^+$ astrocytes in the presence of its specific antigen, MBP (40). In striking contrast, the S1008-specific T cell lines LS1, S1-D2 and SP-D3, which induce an inflammatory response in the CNS in vivo, are completely unable to lyse Ia$^+$ astrocytes in vitro in the presence of S100β (Table 8). Thus, unlike classic encephalitogenic MBP-specific T cells (40), pathogenic CD4$^+$ S100β-specific T cell lines are not cytotoxic. Moreover, although the astrocytes used in these studies

Figure 3. Inflammatory lesions in the rat brain after the adoptive transfer of S100β- or MBP-specific T cell lines. (*a*) Perivascular inflammatory infiltrates are present in the meninges and spinal cord tissue (*arrows*) 6 d after the adoptive transfer of 10^7 S100β-specific T line cells. S100β-specific immunocytochemistry (*brown*) reveals that S100β is confined to astrocytes in the white and grey matter. Immunocytochemistry for S100β, nuclear staining with hematoxylin; ×70. (*b*) Inflammatory infiltrates in the spinal cord (*arrows*) 6 d after the adoptive transfer of 5×10^6 MBP-specific T line cells. MBP-specific immunocytochemistry (*brown*) reveals that MBP is present in the myelin sheaths of both the white and grey matter. Note however the distribution of MBP between white and grey matter is the reverse of that seen for S100β in *a*. Immunocytochemistry for MBP, nuclear staining with hematoxylin; ×70. (*c*) After the adoptive transfer of S100β-specific T cells the majority of the cells in the inflammatory infiltrates are T cells. Immunocytochemistry with W3/13; ×360. (*d*) Adjacent serial section to that in *c* stained with ED1 to identify macrophages in the lesion, note only very few ED1$^+$ cells are present in the infiltrate; ×360. (*e*) Co-transfer of S100β-specific T cells followed 5 d later by an intravenous injection of monoclonal MOG-specific antibody. Injection of the antibody induces the formation of extensive confluent plaques of demyelination in the spinal cord white matter. Klüver myelin stain; ×70. (*f*) Inflammatory infiltrate in the frontal cortex induced by the adoptive transfer of S100β-specific T cells. S100β immunoreactivity (*brown*) is confined to astrocytes. Immunocytochemistry for S100β, nuclear staining with hematoxylin; ×250. (*g*) The adoptive transfer of S100β-specific T cells induces a perivenous inflammatory infiltrate in the retina (periphlebitis retinae); hematoxylin–eosin; ×175. (*h*) Extensive inflammation in the uvea and sclera of the eye induced by the adoptive transfer of S100β-specific T cells. Immunocytochemistry for S100β reveals the presence of S100β in many cells throughout the tissue. Immunocytochemistry for S100β, nuclear staining with hematoxylin; ×250. (*i*) Extensive inflammation in the iris induced by the adoptive transfer of S100β-specific T cells, iris stroma cells are immunoreactive for S100β. Immunocytochemistry for S100β, nuclear staining with hematoxylin; ×250.

Table 7. *Quantitation of Macrophage and T Cell Infiltration into the CNS*

T cell specificity	Perivascular			Parenchym		
	ED1	W3/13	Ratio*	ED1	W3/13	Ratio
MBP	289 (31)	38 (41)	7.6	803 (52)	129 (4)	6.2
S100β	700 (126)	1,158 (6)	0.6	415 (40)	1,173 (3)	0.4

W3/13+ and ED1+ cells were quantitated in multiple spinal cord sections as described in the text.

were S100β+ we were unable to demonstrate the presentation of autologous S100β by astrocytes to syngeneic T cell lines in either proliferation or cytotoxicity assays.

Discussion

This study demonstrates that in the Lewis rat, autoimmune T cells specific for an astrocyte-derived autoantigen, S100β, adoptively transfer an unusual type of inflammatory CNS disease, which can be best described as EAP. Clinical disease and histopathology in this new model of tissue specific autoimmunity differ dramatically from that seen in "classical" MBP-induced EAE, and in some respects may resemble more closely the human disease MS.

In their basic characteristics, autoaggressive S100β- and MBP-specific T cell lines are indistinguishable; both express the $CD4^+CD8^-$ membrane phenotype and use the TCR-α/β; their antigen specific responses are MHC class II ($RT1.B^1$) restricted, and, in addition, both MBP- and S100β-specific T cell lines are Th1-like, synthesizing IL-2 and IFN-γ upon activation. For practical reasons, the S100β-specific T cell lines were selected for reactivity against bovine S100β protein. Epitope mapping using synthetic peptides representing rat S100β sequences proved that most of these T cell lines are truly self-reactive. This was most clearly seen in the T cell lines LS1 and LS2 derived from the bulk lymph node cell cultures, in which the proliferative response to the COOH-terminal rat S100β peptide, amino acids 76–91, was virtually equivalent to that obtained with the native bovine protein. This epitope appears to be immunodominant in the bulk-derived S100β-specific T cell lines and its pathogenicity was confirmed using a T cell line generated using a synthetic rat $S100\beta_{76-91}$ peptide. In contrast to the MBP-specific T cell response, recognition of this immunodominant S100β epitope was not however found to be associated with preferential usage of the TCR $V_\beta 8.2$ gene.

Adoptive transfer experiments established that this S100β-specific T cell response is highly pathogenic. However the disease induced by S100β-specific T cell lines differs radically from that produced by MBP-specific T cells. Despite the intense inflammatory response in the CNS, clinical signs of neurological dysfunction were minimal. Moreover, the histo-

Table 8. *Cytotoxicity of S100β- or D3 Peptide-specific T Cell Lines*

Line	A	Effector/target (E/T) ratio				
		10:1	3:1	1:1	0.3:1	0:1
LS1	+	0	0	0	0	0
	−	0	0	0	0	0
S1-D2	+	0	0	0	0	0
	−	0	0	0	0	0
LS-D3	+	0	0	0	0	0
	−	0	0	0	0	0
Z85*	+	31.7 ± 3.5	17.6 ± 1.6	8.7 ± 2.5	0	0
	−	0	0	0	0	0
LP-PPD1	+	0	0	0	0	0
	−	0	0	0	0	0

Astrocytes were seeded as target cells in 96-well flat-bottom plates at a density of 10^4 cells/well. After a 48-h incubation with IFN- (100 U/ml) to induce Ia expression, the cells were labeled with ^{51}Cr (1 μCi/10^4 cells), and freshly activated T lymphoblasts added in different E/T ratios in the presence or absence of the appropriate antigen ([bovine S100β, guinea pig MBP, PPD] at 10 μg/ml). Lysis was determined using triplicate samples after a 12-h incubation. The results are given as the mean specific lysis ± SD.

pathology of S100β-specific T cell–mediated EAE differs from the MBP-mediated model with respect to the cellular composition and tissue distribution of the inflammatory infiltrates.

The inflammatory infiltrates in the CNS of rats injected with S100β-specific T cells were composed predominantly of T cells and contained approximately 10 times the number of infiltrating T cells seen in rats injected with an equivalent number of encephalitogenic MBP-specific T cells. In contrast, activated macrophages that are the dominant cell population in MBP-induced EAE lesions (43, 44), accounted for only a minority of the infiltrating inflammatory cells in the S100β paradigm.

The distribution of lesions in S100β-induced disease correlated roughly with the pattern of S100β expression within the CNS and adjacent tissues. S100β is present in astrocytes (in both the grey and white matter) in the CNS, in Schwann cells in the PNS and in astrocytes and Müller cells in the eye (45). Thus inflammatory infiltrates were noted throughout the CNS, including cortex, telencephalon, optic nerve, and cerebellum, as well as in the PNS and eye. This is in contrast to MBP-induced EAE in which the target autoantigen is only expressed by myelinating oligodendrocytes and Schwann cells and is not expressed in the uvea or retina as these tissues are not myelinated. Uveitis is therefore not seen in the MBP-induced model of EAE. MBP and S100β are, however, both expressed at high concentrations along the whole length of the spinal cord, as well as in the brain. It is therefore striking that whereas in MBP-induced EAE the lesions tend to be concentrated in the caudal segments of the spinal cord (46), in S100β-induced disease lesions are found throughout the CNS. The adoptive transfer of S100β-specific T line cells therefore induce an EAP that is clearly distinguishable from the classical model of MBP-induced encephalomyelitis. This observation indicates that as yet unspecified factors modulate the ability of local APCs within the brain to process and present specific autoantigens within different regions of the CNS.

The ability of S100β-specific T cells to induce an intense inflammatory response throughout the CNS is in marked contrast to their inability to induce severe neurological dysfunction. Considering the number and the localization of the inflammatory infiltrates, one would expect the animals to develop severe, if not lethal disease. There are at least two observations that could account for this discrepancy, and these are by no means mutually exclusive.

First, it should be remembered that most, if not all, encephalitogenic MBP-specific T cells exhibit antigen specific, class II MHC–restricted cytotoxicity in several different species (40, 47, 48). It was postulated that target cell lysis, as measured in vitro, reflected the encephalitogenic potential of MBP-specific T cells in vivo. However, in contrast to MBP-specific T cells, none of the S100β-specific T cell lines tested were cytotoxic.

Second, it is well established that macrophages are critically involved in mediating clinical disease in MBP-mediated EAE. Depletion of the macrophage population has been shown to migrate clinical EAE (49, 50) despite the continued presence of inflammatory infiltrates in the CNS (50). Interestingly, in EAP there is a reduced migration of activated ($ED1^+$) macrophages from the perivascular space into the CNS parenchyma. This could account in part for the mild clinical course of EAP and can be best explained by a deficit in the production of cytokines known to recruit individual mononuclear blood cells into areas of inflammation. Although cytokine assays for IL-2, TNF-α/β and IFN-γ did not reveal gross differences between MBP and S100β-specific T cells (both behaved like Th1 cells), there could be dissimilarities in the release of other chemotactic cytokines such as IL-8, MIP-1, CP-1, MCP-1, etc., in vivo. These factors are under investigation at present, although at present we cannot yet establish any direct link between the absence of S100β-specific T cell–mediated cytotoxicity and the composition of the inflammatory infiltrates.

This new model of autoimmune-mediated CNS inflammation has direct relevance for our understanding of the immunopathogenesis of MS and other putative autoimmune inflammatory diseases of the nervous system. Although the human retina and uvea are not myelinated (51), these tissues are involved in some patients with MS (51, 52, reviewed in 26), periphlebitis retinae occurring in 10–40% of patients (51, 26) and in another series of cases inflammation of the uvea was seen in 5–27% of the patients studied (53–55). Moreover, pathological abnormalities in the retinal endothelium of patients with acute isolated optic neuritis are also a significant additional risk factor for MS (56). The absence of myelin in these tissues is difficult to reconcile with the concept that MS is exclusively caused by a myelin-specific autoimmune response, particularly as inflammation of these tissues was not seen in MBP-specific T cell–mediated EAE. Our results raise the possibility the primary autoantigen in some cases of MS may not be solely derived from myelin, but that other cellular compartments in the CNS may also provide appropriate autoantigenic targets. This notion that a nonmyelin autoantigen could have a role in a demyelinating inflammatory disease may at first seem bizarre. However, nuclear magnetic imaging studies in MS and EAE also indicate that the primary event in disease pathogenesis is an increased permeability of the BBB (57, 58) and that this is triggered by a T cell–mediated response in the CNS (59). We have now demonstrated that this inflammatory response need not necessarily be myelin specific, although an additional myelin-specific effector mechanism, such as MOG-specific autoantibody response is required to produce a demyelinating white matter pathology similar to that seen in MS. The immunopathogenesis of the MS lesion, at least in some cases could be a consequence of multiple autoimmune responses to a variety of CNS autoantigens.

Finally we predict that many additional myelin and nonmyelin brain determinants will be identified as potential encephalitogens. This concept is crucial for the future development of novel therapeutic strategies for MS, since once established disease may be driven by a polyclonal T cell–mediated autoimmune response against different CNS antigens. Such responses may also play an important role in the pathogenesis of other inflammatory brain diseases, including the inflammatory response that is often associated with neoplasia.

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Address correspondence to Dr. Christopher Linington, Department of Neuroimmunology, Max-Planck Institute for Psychiatry, 82152 Martinsried, Germany.

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