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Immunization With a Cannabinoid Receptor Type 1 Peptide Results in Experimental Allergic Meningocerebellitis in the Lewis Rat: A Model for Cell-Mediated Autoimmune Neuropathology

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Neuronal elements are increasingly suggested as primary targets of an autoimmune attack in certain neurological and neuropsychiatric diseases. Type 1 cannabinoid receptors (CB1) were selected as autoimmune targets because they are predominantly expressed on neuronal surfaces in brain and display strikingly high protein levels in striatum, hippocampus, and cerebellum. Female Lewis rats were immunized with N-terminally acetylated peptides (50 or 400 μg per rat) of the extracellular domains of the rat CB1 and killed at various time points. Subsequent evaluation using immunohistochemistry and in situ hybridization showed dense infiltration of immune cells exclusively within the cerebellum, peaking 12–16 days after immunization with the CB1 peptide containing amino acids 9–25. The infiltrates clustered in meninges and perivascular locations in molecular and granular cell layers and were also scattered throughout the CB1-rich neuropil. They consisted primarily of $CD4^+$ and $ED1^+$ cells, suggestive of cell-mediated autoimmune pathology. There were no inflammatory infiltrates elsewhere in the brain or spinal cord. The results show that neuronal elements, such as neuronal cell-surface receptors, may be recognized as antigenic targets in a cell-mediated autoimmune attack and, therefore, support the hypothesis of cell-mediated antineuronal autoimmune pathology in certain brain disorders.

Key words: T lymphocytes; autoimmunity; EAE/MS; MHC; neuroimmunology

Autoimmune processes directed against the CNS are implicated in the pathogenesis of a variety of neurological and mental diseases. A large body of evidence indicates that autoimmune attack directed against CNS white matter causes multiple sclerosis, a human condition affecting hundreds of thousands of people worldwide (Adams and Victor, 1993). In addition, there is increasing evidence that neurons per se may constitute the primary target of an autoimmune attack in certain brain disorders. Antineuronal autoimmunity is implicated in the pathogenesis of paraneoplastic degeneration of the cerebellum and limbic system (Dalmau et al., 1999), Rasmussen encephalitis (Rogers et al., 1994), Alzheimer's disease (Singh, 1997; Chorsky et al., 2001), and Sydenham's chorea (Husby et al., 1976; Kotby et al., 1998). Antineuronal autoimmunity has also been implicated in neuropsychiatric disorders, such as obsessive-compulsive disorder, Tourette syndrome, and Tic disorders (Kiessling et al., 1994; Swedo et al., 1994; Singer et al., 1998), particularly in the context of streptococcal infections (Swedo et al., 1997; Leonard and Swedo, 2001).

Antineuronal autoimmunity of the CNS is often suspected on the basis of antineuronal antibodies detected in serum or cerebrospinal fluid (CSF) of affected individuals (Graus et al., 1988; Kiessling et al., 1994; Rogers et al., 1994; Vega et al., 1994; Dalmau et al., 1999). However, in many instances, passive transfer of preparations containing antibodies failed to elicit the expected CNS disease, so a causal relationship between presence of antineuronal antibodies and brain disease has not been established (Sillevis Smitt et al., 1995; Tanaka et al., 1995a,b; Dalmau et al., 1999; Whitney and McNamara, 1999).

On the other hand, there is circumstantial evidence that T-cell-related mechanisms may play a major pathogenic role in some syndromes with suspected antineuronal autoimmune etiology. This evidence is based on the presence of T-cell infiltrates in affected brain regions (Graus et al., 1990; Dalmau et al., 1991; Hormigo et al., 1994; Jean

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et al., 1994), and the detection of early pleocytosis (Peterson et al., 1992), activated T cells (Albert et al., 2000), T-cell-associated neuronal death (Bien et al., 2002), and Th1 cytokines in the CSF of affected individuals (Mittleman et al., 1997). However, because neurons under normal circumstances lack expression of the antigen-presenting major histocompatibility complex (MHC) class I or class II antigens, the mechanism whereby antigen-specific T cells could recognize antigens expressed by neurons remains unknown. Therefore, it is unclear whether the cellular infiltrates present in affected brain regions of patients suffering from paraneoplastic CNS disease, for example, reflect a primary T-cell response against a neuronal antigen or a consequence of neuronal damage caused by other, as yet unknown mechanisms (Dalmau et al., 1999).

In susceptible animals, immunization with myelin- or glia-associated antigens in complete Freund's adjuvant (CFA) leading to experimental allergic encephalomyelitis (EAE) is a well-established model of $CD4^+$ T-cell-mediated autoimmune disease directed against CNS white matter (Cohen and Miller, 1994). EAE is widely applied to study aspects of multiple sclerosis. By contrast, animal models evaluating the role of antineuronal autoimmunity are less well established, frequently because of unknown target antigens and disease mechanisms (cellular vs. humoral). However, because knowledge of the underlying disease-inducing mechanism is crucial for disease management and the development of effective therapeutic strategies, the present study sought to test the principal hypothesis regarding whether it is possible to induce a cell-mediated autoimmune response against a neuronal antigen in vivo. To address this question, the Lewis rat model of EAE was adapted to a neuronal antigen.

For the purpose of the present study, any neuronal antigen could have been chosen. The brain-type cannabinoid receptor (CB1) was selected for the following reasons: Cannabinoid receptors (CB) belong to the superfamily of G-protein-coupled receptors (Devane et al., 1988), which are frequently involved in autoimmunity of other tissues (Whitney and McNamara, 1999). Type 1 receptors (CB1) are found predominantly in brain tissue and to a lesser extent in peripheral organs, whereas type 2 receptors (CB2) are expressed almost exclusively in the periphery, particularly in B cells of the immune system (Galiegue et al., 1995; Felder and Glass, 1998). In the brain, CB1 are among the most abundant receptors (Herkenham et al., 1991). The vast majority of immunostaining for CB1 protein is on neuronal plasmalemma (mostly axon terminals) and some intracellular organelles in neurons (Katona et al., 2001), although one study indicated the possible presence of immunostained perivascular glial elements (Rodriguez et al., 2001). The high level of CB1 expression in basal ganglia and cerebellum indicates a role for cannabinoids in the control of movement and movement disorders (Felder and Glass, 1998; Sanudo-Pena et al., 1999). CB1 have also been implicated in short-term memory, modulation of mood and emotions, addictive behavior, nociception (Martin et al., 1998; Ledent et al., 1999), and tic disorders and Tourette syndrome (Muller-Vahl et al., 1997, 1998, 1999). The discrete spatial distribution of CB1 in the brain, with defined regions of high levels of CB1 expression (basal ganglia, hippocampus, and cerebellum) contrasting with regions of low expression (spinal cord and brainstem; Herkenham et al., 1990; Pettit et al., 1998; Egertova and Elphick, 2000), makes CB1 an excellent candidate for determining the specificity of a potential immune response against the antigen.

MATERIALS AND METHODS

Animals

Female Lewis rats, 6–8 weeks of age, were purchased from Charles River (Wilmington, MA). Animals were housed in pathogen-free, standard-facility conditions with lights on from 0600 to 1800 hr and ad libitum access to food and water. Animal procedures were approved by the NIMH Intramural Research Program Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Peptides

Synthetic peptides corresponding to amino acids (aa) 9–25, aa 22–38, aa 87–103, and aa 257–274 of the rat CB1 were synthesized commercially (Research Genetics, Huntsville, AL). Because previous studies have shown that acetylation of the N-terminal amino acid may enhance antigenicity (Rothbard et al., 1989; Zhao et al., 1994), the peptides were N-terminally acetylated. Peptides were identified by matrix-assisted laser desorption mass spectrometry, and purity was determined by reverse-phase high-performance liquid chromotography (HPLC) and reported as >95% by the supplier.

Antigen-Specific Proliferation Assay

N-acetylated CB1 peptides were dissolved in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml and emulsified with an equal volume of CFA containing 5 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI). Rats (n = 3/peptide) were injected with 0.1 ml of peptide-CFA emulsion intradermally into each hindlimb (200 μg of antigenic peptide/rat). Nine days later, the draining popliteal lymph nodes were removed, dissociated, and cultured in the presence of the immunizing peptide at different concentrations (0.1–20 μg/ml) for 2 days. Controls were cultured in the presence of concanavalin A (Con A; Sigma, St. Louis, MO) at 5 μg/ml. The medium was RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 1% heat-inactivated rat serum (Gibco BRL, Grand Island, NY), 5% NCTC-109 (BioWhittaker), 5×10^{-5} M 2-ME (Sigma), 2 mM glutamine (BioWhittaker), 10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml fungizone (BioWhittaker). Lymph node cells were cultured in triplicate in 0.1 ml of the medium at 4×10^6 cells/ml in a 96-well microtiter plate. At 48 hr, each well received 0.5 μCi [^{3}H]TdR in a volume of 50 μl. The cells were harvested after an additional 5 hr of culture using a Microbeta cell harvester (Wallac, Gaithersburg, MD). CPMs were determined by liquid scintillation counting as an indicator of cell proliferation.

Active Immunization for Disease Induction

For disease induction, rats were actively immunized with 400 μg of acetylated (ac) 9–25, ac 22–38, ac 87–103, and ac 257–274 (n = 3/peptide). Emulsion was injected subcutaneously at four sites, one at each of the flanks (0.1 ml/site). Thirteen days later, animals were killed by decapitation. Brains and spinal cords were snap frozen and stored at –70°C.

Severity and Reproducibility of Inflammatory Lesions Using ac 9–25

Additional animals were immunized with emulsion containing 50 μg of ac 9–25 (two injection sites, 0.1 ml/site, n = 9), 400 μg of ac 9–25 (four injection sites, 0.1 ml/site, n = 9), and PBS-CFA only (four injection sites, 0.1 ml/site, n = 2). Animals were killed 13 days later and processed as described above.

Time Course of Events Using ac 9–25

Additional animals were immunized with emulsion containing 400 μg of ac 9–25 (four injection sites, 0.1 ml/site) and killed 10, 12, 14, 16, 19, and 27 days following immunization (n = 3–4/time point). Animals immunized with CFA only were killed 13 days postimmunization (n = 4).

Histology

Cryostat-cut, 15-μm-thick, slide-mounted coronal (brain) and horizontal (spinal cord) sections were further processed for immunohistochemistry and in situ hybridization histochemistry. Sections were collected in a spaced series at levels concentrated in striatum/globus pallidus, hippocampus, substantia nigra, cerebellum (high CB1 density), and spinal cord (low CB1 density). Slides were stored at –35°C.

For immunohistochemistry, monoclonal antibodies used were mouse anti-rat CD45 (leukocyte common antigen; 1:500 dilution; Serotec, Oxford, England), ED1 (1:500; Serotec), CD4 (1:100; Serotec), CD8 (1:500; Serotec), CD54 (ICAM-1; 1:500; Serotec), CD80 (B7-1; 1:100; Pharmingen, San Diego, CA), CD86 (B7-2; 1:500; Pharmingen), and OX-6 (MHC class II; 1:500; Serotec). Slides were fixed in Histochoice (Amnesco, Solon, OH) for 12 min; washed in 1× PBS, pH 7.4; incubated in methyl alcohol containing 0.5% hydrogen peroxide for 20 min; washed in 1× PBS; and incubated in blocking reagent containing 1× PBS, 2% bovine serum albumin (BSA; Sigma), and 2% normal horse serum (Vector, Burlingame, CA) for 1 hr. Thereafter, slides were incubated with the respective primary antibodies overnight at 4°C. After additional washes in 1× PBS, slides were incubated with biotinylated secondary anti-mouse antibody (1:200; Vector) for 45 min, incubated with avidin-biotin (Vector) reagent for 30 min, then developed with 3,3′-diaminobenzidine in the presence of hydrogen peroxide (Vector). Alternatively, sections were quenched following incubation with the primary antibody, prior to application of the secondary antibody. Most sections were counterstained with hematoxylin.

The in situ hybridization procedures were performed as described previously for ribonucleotide (cRNA) probes (Stern et al., 2000). Tissue sections were fixed with 4% formaldehyde solution; acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0; dehydration with ethanol; and delipidated with chloroform. Antisense probes were transcribed using the Riboprobe System (Promega Biotech, Madison, WI) with α-^{35}S-UTP (New England Nuclear, Boston, MA) and T7, T3, or SP6 RNA polymerase. The applied rat cRNA probes consisted of interleukin (IL)-1β (a 498 bp-fragment acquired from Dr. Ronald Hart, Rutgers University), tumor necrosis factor (TNF)-α (gift from Dr. Karl Decker, Albert-Ludwigs Universität, Freiburg, Germany), IL-1α and IL-4 (Pharmingen), IL-12 p40 subunit [generated from PCR primers used by Tanuma et al. (2000) and subcloned into the pGEM T-easy vector], transforming growth factor (TGF)-β1 (gift of Joanna Jankowsky, California Institute of Technology), IL-10 (gift of Lily Feng, Baylor College of Medicine), and CB1 (gift of Mary Abood, California Pacific Medical Center, San Francisco, CA). TUNEL labeling of apoptotic cells was performed using the ApopTag Kit (Intergen, Purchase, NY) according to the manufacturer's specifications.

The radiolabeled probes were diluted in a riboprobe hybridization buffer and applied to brain sections (approximately 500,000 cpm/section). After overnight incubation at 55°C in a humidified chamber, slides were washed first in 20 μg/ml RNase solution and then for 1 hr each in 2× SSC (50°C) and 0.2× SSC (55°C and 60°C) solutions. The slides were then dehydrated, air dried, apposed to film (BioMax MR; Kodak, Rochester, NY) for 2–14 days, and developed. Selected slides were dipped in nuclear track emulsion (NTB-2; Kodak) and exposed for 1 month. Images were captured from a light box or a microscope (Leica DMR) using a digital camera (CoolSnap cf, Photometrics, Tucson, AZ) and IPLab Spectrum Software (Scanalytics, Fairfax, VA). All images were processed in Adobe PhotoShop.

Histological Analysis

Sections stained with hematoxylin and CD45 were evaluated for the presence of inflammatory infiltrates. Severity of inflammatory lesions was graded on the basis of CD45 immunoreactivity.

CB1 Receptor Binding

CB1 receptor binding was performed as described previously (Herkenham et al., 1990). Slides containing fresh-frozen brain sections were incubated for 2 hr at 37°C in 50 mM Tris, pH 7.4, + 5% BSA (Sigma) containing 10 nM of [^{3}H]CP-55,940 (specific activity 180 Ci/mmol; New England Nuclear). Control sections were incubated in the same buffer containing also 10 nM CP 55,244 (Pfizer, Groton, CT). Subsequently, slides were washed for 4 hr in 50 mM Tris, pH 7.5, + 1% BSA at 4°C. Sections were then fixed for 5 min at room temperature in 50 mM Tris, pH 7.4, + 0.5% formaldehyde. After sections were blow dried, slides were placed in X-ray cassettes, apposed to film (Hyperfilm-^{3}H; Amersham, Arlington Heights, IL) for 7 days, and developed.

Other Measures

Animals were observed for gross behavioral abnormalities and weighed on a daily basis. Serum was not collected for analysis for possible development of a humoral response, because the calculated optimal survival times were too brief for production of IgG antibodies to CB1.

TABLE I. Peptides Derived From Rat Brain Type Cannabinoid Receptor (CB1) Are Positioned in Ways That Align Glutamic Acid (E) or Aspartic Acid (D) in the Peptide at Position 9 of the MHC Class II (RT1.BL) Molecule in the Lewis Rat (see Wauben et al., 1997)*

	Position
Peptide	1 3 7 8 9
9–25	AD**T**T**F**RTIT**TD**LLYVGS
22–38	YVG**S**NDI**OYE**DIKFDMA
22–38	YV**G**SNDIQYEDIKGDMA
22–38	YVGSNDIQ**Y**EDI**KGD**MA
87–103	LS**S**FKE**NE**NIQCGENF
87–103	LSSFKE**N**EENIQC**GE**NF
257–274	NCKK**L**QSVC**SD**FPLIDE
257–274	NCKKLQSVC**S**DFPL**IDE**
257–274	NCKKLQSV**C**SDFPLID**E**

*Positions 1, 3, 7, 8, and 9 of the MHC class II molecule contribute to peptide binding. Amino acids shown in boldface are those that are predicted to enhance the binding of the peptide to the MHC class II packet.

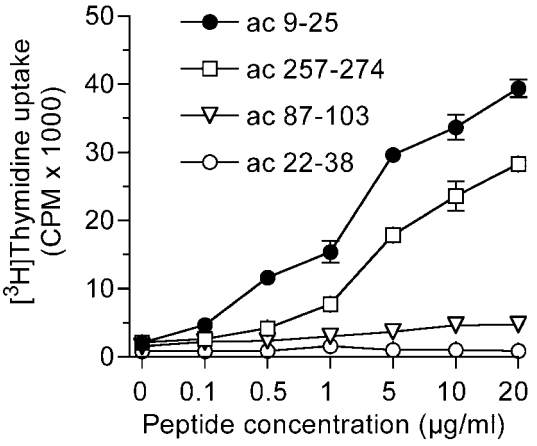

Fig. 1. T-cell proliferation, measured by [^{3}H]thymidine uptake, in response to presentation of selected type 1 cannabinoid receptor (CB1) peptide sequences to cultured lymph node cells harvested from rats previously immunized with the same peptides. Data are mean ± SEM of triplicate determinations.

RESULTS

In the Lewis rat, most autoimmune-associated CD4$^+$ T cell responses are MHC class II RT1.BL restricted. The extracellular domains of the rat CB1 receptor, based on the extended MHC class II-peptide binding motif for the Lewis rat (Wauben et al., 1997), were scanned for potential RT1.BL molecule binding peptide sequences. Table I shows the four peptide sequences that were ultimately chosen for immunization.

Antigen-Specific Proliferation of Lymph Node Cells

In the presence of the immunizing antigen, lymph node cells from animals immunized with peptides ac 9–25 and ac 257–274 displayed a strong, dose-dependent, antigen-specific proliferation (Fig. 1).

Specificity of the Inflammatory Response

On day 13 postimmunization (p.i.), animals immunized with peptides ac 22–38, ac 87–103, and ac 257–274 showed no evidence of an inflammatory response at any level examined. By contrast, animals immunized with ac 9–25 showed an inflammatory response that was strictly confined to the cerebellum (Fig. 2a,e,f). There was no apparent inflammation at any other level examined, i.e., striatum/globus pallidus (Fig. 2c), hippocampus, substantia nigra, or spinal cord (Fig. 2d). Likewise, there was no inflammatory infiltration observed in animals immunized with CFA only (Figs. 2b, 3b). In animals immunized with ac 9–25, CD45-immunoreactive (-IR) cells appeared in the meninges, in perivascular/parenchymal infiltrates, and scattered throughout the adjacent molecular and granular cell layers of the cerebellum (Figs. 2a, 3a). The inflammatory response was further confirmed by strong upregulation and expression of ICAM-1 (Figs. 2f, 3g) and MHC class II antigens (Figs. 2e, 3h), which closely matched the spatial distribution of CD45 immunoreactivity. In some animals (with significant infiltration), cerebellar white matter tracts subjacent to the affected gray matter layers also showed perivascular cuffing, scattered infiltrating cells, and apparently reactive microglia. However, more distant cerebellar white matter tracts were practically devoid of infiltrating cells, as were the cerebellar nuclei and the brainstem (Fig. 2). Overall, the spatial distribution of the inflammatory infiltrates in the cerebellar gray matter matched the location of dense CB1 binding (Fig. 2g), strongly arguing for specificity of the obtained immune response.

Characterization of Inflammatory Infiltrates by Immunohistochemistry

Infiltrating cells displayed monocytic and lymphocytic morphology, based on hematoxylin staining, whereas neutrophils were hardly detected. In animals immunized with ac 9–25, CD45-IR cells were numerous (Fig. 3a). By contrast, in animals immunized with CFA only, rare cells in the cerebellar meninges were CD45$^+$ (Fig. 3b). The inflammatory response was dominated by ED1$^+$ (Fig. 3d) and CD4$^+$ cells (Fig. 3e), frequently observed clustered in perivascular/parenchymal infiltrates, whereas CD8$^+$ cells were less prominent and more scattered throughout the parenchyma (Fig. 3f).

ICAM-1 immunoreactivity was selectively upregulated in the cerebellar meninges and more diffusely throughout the parenchyma, the parenchymal pattern resembling capillary staining (Fig. 3g). In addition, there was strong cellular staining of MHC class II protein, visualized by OX-6 immunoreactivity, in the meninges and parenchyma (Fig. 3h). The cell types expressing OX-6 could not be determined, but their locations suggested that they were meningeal or perivascular cells. There was no clear evidence of neuronal staining of MHC class II in the granule cell layer. The costimulatory molecules B7-1 and

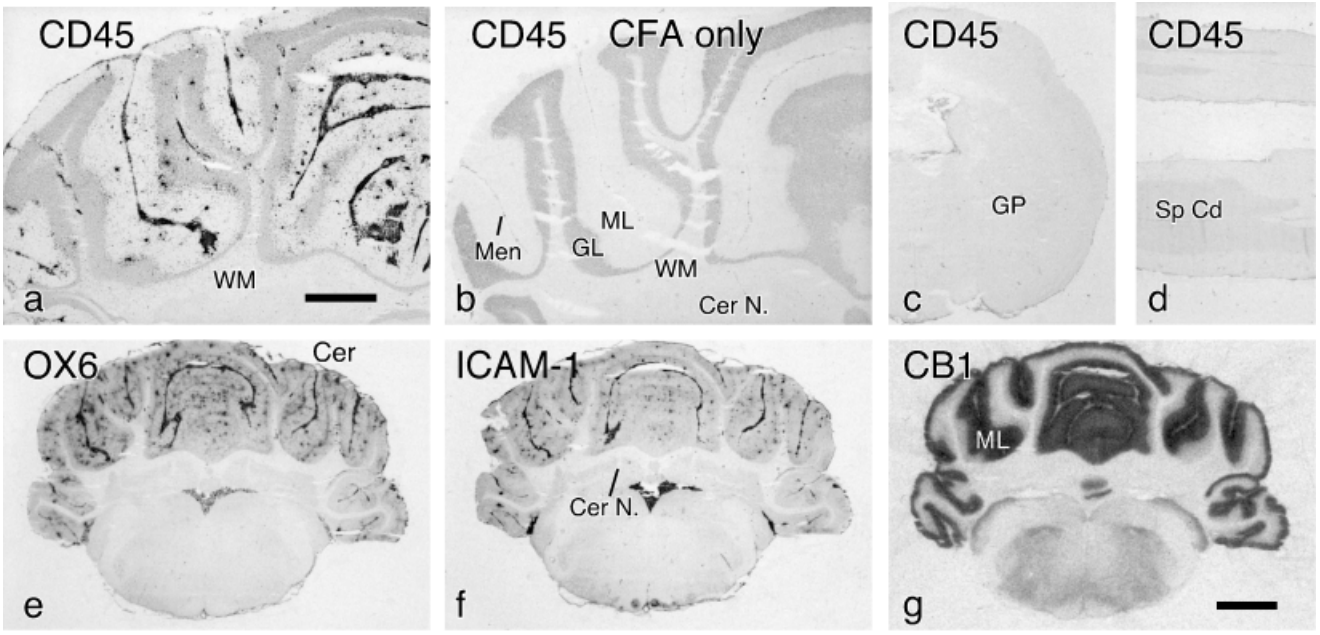

Fig. 2. Spatially discrete immune response in the brain 13 days after s.c. immunization with ac 9–25 in CFA (**a,c–f**) contrasts with no response to CFA only (**b**). Leukocyte (CD45 IR) infiltration into the meninges (Men) and granule cell layer (GL) and molecular layer (ML) of the cerebellum (Cer) is shown in a. There was no infiltration in the deeper white matter (WM) or deep cerebellar nuclei (Cer N.). Likewise, there was no immune response elsewhere in the brain, including the CB1-rich globus pallidus (GP; c), or spinal cord (Sp Cd; d). The $CD45^+$ areas of cerebellum showed identical patterns of OX6 (e) and ICAM-1 (f) immunostaining of MHC class II and intercellular adhesion molecules, respectively. A film autoradiograph shows dense cannabinoid receptor (CB1) binding of [^{3}H]CP-55,940 (**g**) in the cerebellar molecular layer in a near-adjacent section. Scale bars = 1 mm in a (for a,b); 2 mm in g (for c–g).

B7-2 were both present in cells within the meninges (Fig. 3i,j). Within the parenchyma, B7-1 immunoreactivity was light (Fig. 3i), whereas B7-2 immunoreactivity was more pronounced in the infiltrates (Fig. 3j).

Characterization of Inflammatory Infiltrates by In Situ Hybridization Histochemistry

Induced mRNA expression of the examined cytokines was restricted to the locations of infiltrates (Fig. 4). Whereas IL-1α mRNA was induced predominantly within perivascular/parenchymal infiltrates (Fig. 4a), IL-1β mRNA showed not only strong perivascular/parenchymal but also strong meningeal expression (Fig. 4b,c). In contrast to the strong expression of IL-1α and IL-1β mRNA, expression of TNF-α mRNA was less frequently observed, predominantly in scattered cells, occasionally associated with clustering infiltrates (Fig. 4f). Expression of IL-12 p40 mRNA was rarely detected, and in distinct cells only, in close association with parenchymal infiltrates (Fig. 4e). Among the counterinflammatory cytokines, there was strong meningeal and perivascular/parenchymal expression of TGF-β1 mRNA (Fig. 4d), whereas IL-4 and IL-10 mRNA expression was not observed (data not shown). Animals immunized with CFA only did not display significant mRNA expression of any of the probes examined (data not shown).

Severity and Reproducibility of the Inflammatory Lesions

To assess severity, reproducibility, and dose dependence of the inflammatory response, female Lewis rats were immunized with 400 μg or 50 μg of ac 9–25 or CFA only and evaluated 13 days p.i. Quantification of the inflammatory response, based on CD45 immunoreactivity, revealed variation in the severity of the inflammatory response (Table II). Among nine animals immunized with 400 μg of ac 9–25, all showed some infiltration, and four animals showed severe infiltration (severity score = 3). Animals immunized with 50 μg of ac 9–25 were affected less frequently, though equally severely. The spatial distribution was identical in all animals affected.

Time Course of Events Using 400 μg ac 9–25

To assess the temporal elaboration of the inflammatory response, immunized animals were evaluated at 10, 12, 14, 16, 19, and 27 days p.i. At day 10, only a few $CD45^+$ cells were detected within cerebellar meninges and parenchymal blood vessels (Fig. 5a). At day 12, cuffing of the meninges became evident, whereas perivascular/parenchymal infiltrates were still largely absent (Fig. 5b). At days 14 and 16, meningeal infiltration was accompanied by perivascular/parenchymal infiltration (Fig. 5c,d), whereas, from day 19 onward, perivascular infiltration was no longer evident (data

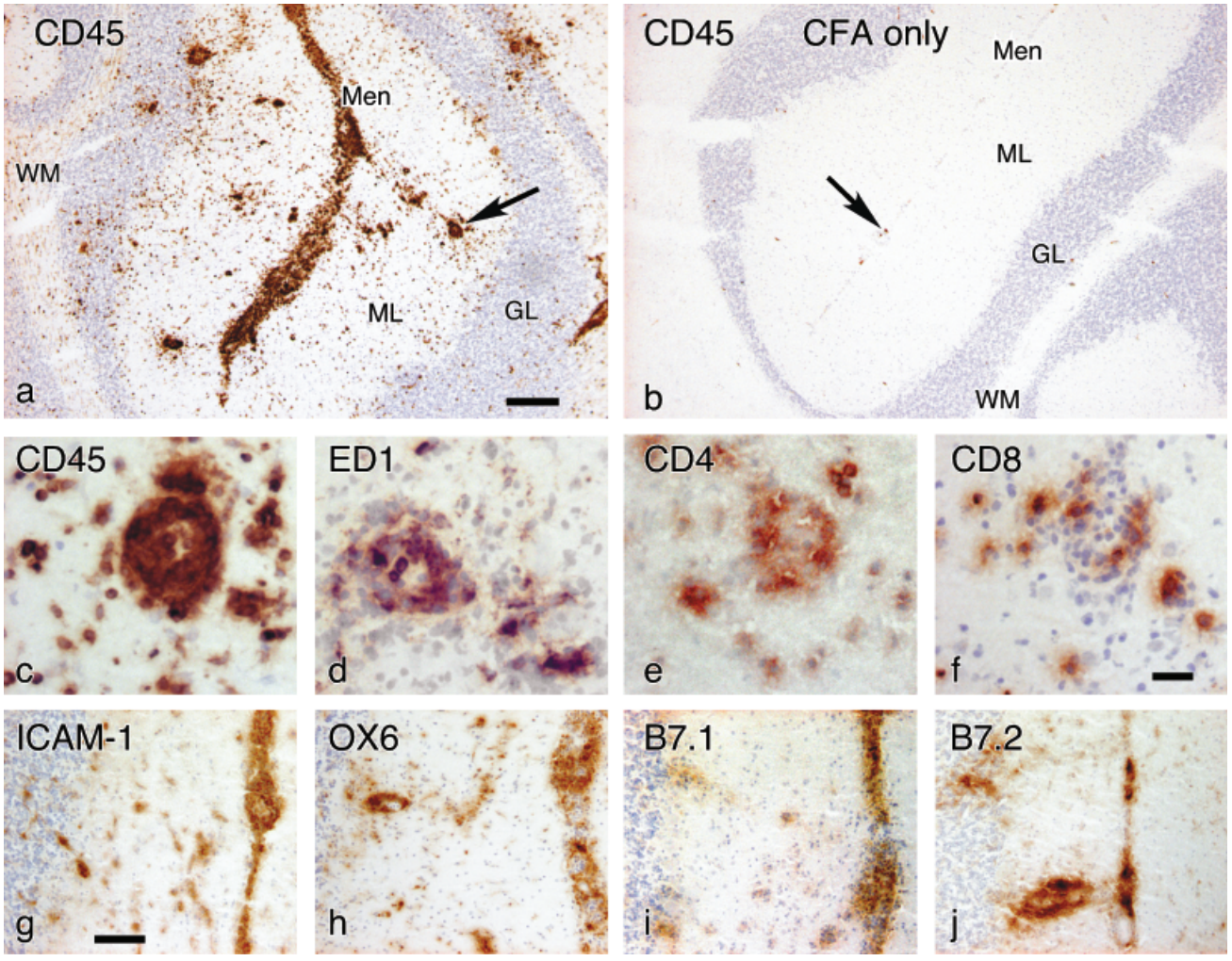

Fig. 3. Brightfield photomicrographs of coronal brain sections from animals evaluated 13 days after immunization with 400 μg of ac 9–25 (**a,c–j**) or CFA only (**b**) showing immunostaining for various immune markers and lightly counterstaining. Sections in a–c are stained with CD45; sections in c–j are stained with ED1 (d), CD4 (e), CD8 (f), ICAM-1 (g), OX-6 (h), B7-1 (i), or B7-2 (j). Photomicrographs in c–f are taken at the perivascular infiltrate marked by the arrow in a, whereas photomicrographs in g–h are taken at other, comparable locations within the cerebellar molecular layer. For abbreviations see legend to Figure 2. Scale bars = 200 μm in a (for a,b); 20 μm in f (for c–f); 100 μm in j (for g–j).

not shown). There was no evidence of infiltration in any other brain area outside the cerebellum at any time point. Animals immunized with CFA did not show signs of inflammation.

Behavioral Evaluation

No pathological behavior was observed in any animals at any time point. Some animals showed substantial weight loss during the first 2 days p.i., which did not persist. Starting at about day 12 p.i., some animals developed swelling of their joints. However, neither early weight loss nor later development of joint swelling was specific for peptide-immunized animals. Interestingly, however, animals immunized with ac 9–25 displaying severe joint inflammation tended to show less severe inflammatory lesions in the cerebellum.

TUNEL Staining for Apoptosis

TUNEL-positive cells were found almost exclusively in the same regions of the cerebellum as the CD45-IR cells (Fig. 6). The positive cells were identified as lymphocytes and monocytes on the basis of shape, size, and location. However, cellular definition was not good at high magnification (Fig. 6b, inset). There was no clear evidence of neuronal apoptosis, notably in the granule cell layer, where infiltrates were commingled with densely packed small neurons, although the possibility cannot be ruled out. In addition, in the hematoxylin-stained sections, no degenerating (pyknotic) neurons were apparent adjacent to infiltrates.

Cannabinoid Receptor Binding and CB1 mRNA Expression

The pattern of protein binding and CB1 mRNA expression were the same in the Lewis rat brain as has been

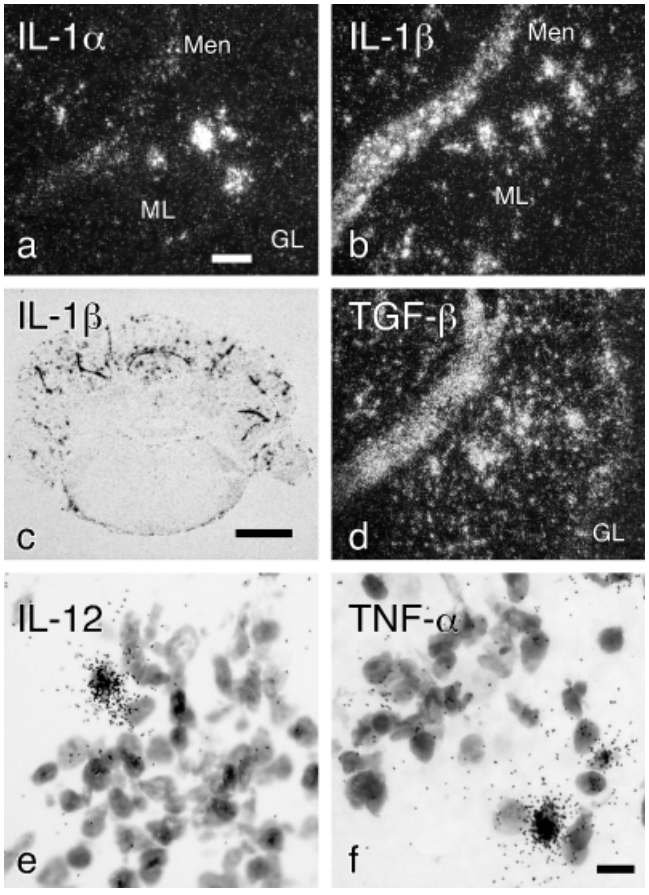

Fig. 4. Representative film autoradiograph (**c**) and darkfield (**a,b,d**) and brightfield (**e,f**) photomicrographs of rat brain sections taken from animals 13 days after immunization with ac 9–25 showing patterns of cytokine gene induction in the cerebellum. Tissue was hybridized with IL-1α (a), IL-1β (b,c), TGF-β1 (d), IL-12 p40 (e), or TNF-α (f) cRNAs. For abbreviations see legend to Figure 2. Scale bars = 100 μm in a (for a,b,d); 2 mm in c; 10 μm in f (for e,f).

TABLE II. Summary of Histologic Evaluation of Rats Immunized With Peptide ac 9–25

Peptide conc.	Inflammation[a]	Severity score
400 μg/rat	9/9[b]	2.2 ± 0.8
50 μg/rat	3/9	2.2 ± 1.4
CFA only	0/2	0

[a]Presence of $CD45^+$ cells in cerebellar meninges/parenchyma, graded as follows: 0, no inflammation; 1, meningeal infiltration only; 2, meningeal infiltration and rare, scattered, small foci of parenchymal inflammation; 3, meningeal and multiple foci of parenchymal infiltration.
[b]Number of animals showing inflammatory lesions/number of animals immunized.

reported previously for the Sprague Dawley rat (Herkenham et al., 1991). No inflammation-induced changes were seen in the cerebellum or elsewhere in levels of binding or mRNA expression (data not shown).

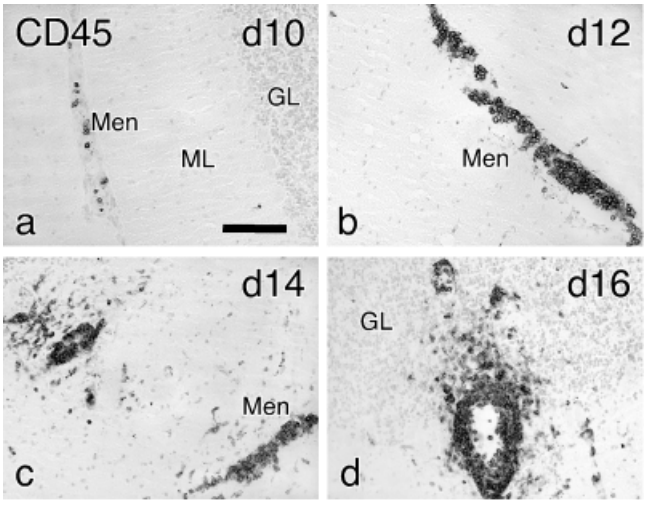

Fig. 5. Photomicrographs showing CD45-immunostained cerebellum sections taken from animals immunized with ac 9–25 and evaluated at different time points: 10 days (**a**), 12 days (**b**), 14 days (**c**), or 16 days (**d**). For abbreviations see legend to Figure 2. Scale bar = 100 μm.

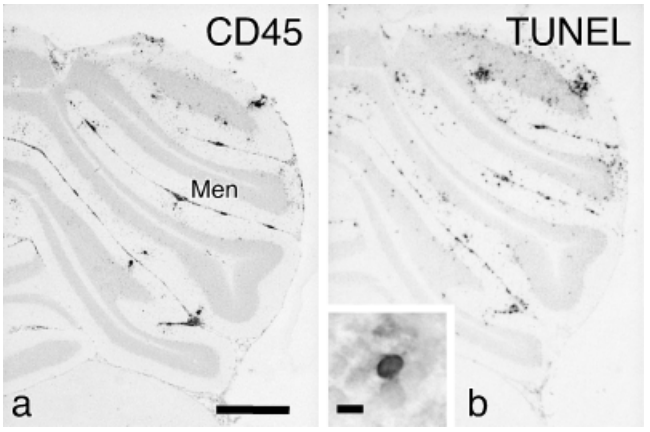

Fig. 6. Photographs of nearly adjacent sections stained either for leukocyte common antigen (CD45; **a**) or apoptosis (ApopTag stain for TUNEL-positive cells; **b**) show that putative apoptotic cells were highly restricted to those locations where infiltrating leukocytes were found. Men, meninges. **Inset** shows a single TUNEL-positive cell at the border of the granule cell layer. Scale bars = 1 mm in a (for a,b); 5 μm in inset.

DISCUSSION

The present study was undertaken to evaluate whether neuronal antigens can become targets of a primary cell-mediated immune response. Two (ac 9–25 and ac 257–274) of the four rat CB1 receptor-derived N-terminally acetylated peptides elicited antigen-specific lymphocyte proliferation in vitro for cells taken from female Lewis rats injected in an EAE-like immunization paradigm. In addition, active immunization of rats with the peptide ac 9–25 resulted in a spatially restricted encephalitogenic immune response within a highly CB1-

enriched area of the brain. The inflammatory infiltrates were primarily lymphocytic and monocytic, affected the cerebellum only, and were therefore characterized as experimental allergic meningocerebellitis.

The Lewis rat strain is particularly prone to experimental autoimmune diseases in which self-reactive $CD4^+$ T cells are considered crucial in disease propagation (Cohen and Miller, 1994; Wekerle et al., 1994). In the Lewis rat, most $CD4^+$ T-cell responses associated with autoimmune disease are MHC class II RT1.BL restricted (Chou et al., 1989; Offner et al., 1989; Kojima et al., 1994; Wegmann et al., 1994; Zhao et al., 1994). Previous studies have defined peptide-MHC binding motifs for rat MHC class II RT1.BL molecules (Reizis et al., 1996; Wauben et al., 1997) that were used successfully in designing self-reactive peptides that induced $CD4^+$ T-cell-mediated autoimmune responses (Wegmann et al., 1994; Zhao et al., 1994). The present study demonstrates that the same strategy can be applied to determine self-reactive epitopes on neuronal proteins. Recognition of neuronal antigens by self-reactive $CD4^+$ T cells per se does not seem surprising, because antigen recognition depends not on identity or function of the respective peptide but rather on its amino acid sequence, which may or may not allow binding to the MHC class II RT1.BL molecule. However, in light of the much debated question of whether neurons may or may not qualify as targets for a primary cell-mediated response, this study provides the first experimental evidence that CNS neuronal antigens may indeed become targets of a primary cell-mediated attack in vivo and elicit an encephalitogenic response within the brain. The data, therefore, are highly supportive of the increasing number of publications suggesting an important role for primary T-cell-mediated autoimmune mechanisms in the pathogenesis of antineuronal autoimmune disease of the CNS (Graus et al., 1990; Jean et al., 1994; Mittleman et al., 1997; Benyahia et al., 1999; Albert et al., 2000; Bien et al., 2002). Importantly, antineuronal autoimmunity may not only play a major role in human diseases with alleged neuronal targets, e.g., paraneoplastic diseases of the CNS, but also may be involved in the pathogenesis of multiple sclerosis, insofar as recent reports have shown axonal injury (Ferguson et al., 1997; Trapp et al., 1998) and the presence of cellular and humoral reactivity against neuronal proteins in multiple sclerosis patients (Acarin et al., 1996; Sadatipour et al., 1998; Polak et al., 2001).

It is not known how the CB1 antigen in the CNS is initially recognized. There is no evidence of neurons expressing MHC class II antigens, so the most likely initial event is that CB1 antigen is presented by CNS antigen-presenting cells, such as microglia, perivascular macrophages, or dendritic cells (Hickey and Kimura, 1988; Thomas and Lipsky, 1996; Dittel et al., 1999; Gonzalez-Scarano and Baltuch, 1999; Aloisi et al., 2000; Serafini et al., 2000). Furthermore, it is argued that, because immunization was performed in healthy, naïve animals in which gross neuronal damage was not observed, the cellular infiltrates in the cerebellum of animals immunized with ac 9–25 represent a primary cellular response against the antigen rather than a secondary event.

The histopathological features observed in the cerebellum of affected animals—namely, meningeal and perivascular/parenchymal infiltration by T-lymphocytic and monocytic cells in conjunction with up-regulation of MHC class II, costimulatory molecule (B7-1 and B7-2) and adhesion molecule (ICAM-1) expression and in-situ-induced expression of pro- and counterinflammatory cytokine mRNAs—are nearly identical to the pathological features observed in myelin basic protein (MBP)-induced EAE (Archelos et al., 1993; Racke et al., 1995; Benveniste, 1997; Laman et al., 1998). However, the selective targeting of the molecular and granular cell layers of the cerebellum without any inflammatory infiltration in the spinal cord or brainstem is clearly different from the targeting in MBP-induced EAE in the Lewis rat, in which the spinal cord is the most severely affected region (Berger et al., 1997). Although myelin oligodendrocyte glycoprotein (MOG) has also been shown to target the cerebellum preferentially (Berger et al., 1997), ac 9–25 leads to preferential targeting of the gray matter (molecular and granular layers) as opposed to cerebellar white matter in MOG-induced EAE. Previous studies have shown for a number of myelin- and glial-associated antigens that antigen specificity of autoreactive T cells determines the spatial distribution of inflammatory infiltration in the brain (Kojima et al., 1994; Berger et al., 1997). The selective presence of inflammatory infiltration in CB1-enriched regions of the cerebellum in conjunction with the absence of inflammatory infiltration in regions with low CB1 expression (brain stem or spinal cord; Herkenham et al., 1991) strongly argues for the specificity of the immune response.

The reasons for the selective targeting of the cerebellum despite dense CB1 receptor distribution in other brain regions, such as basal ganglia or hippocampus, remain unclear. It may be of importance that the cerebellum is the only site where CB1-enriched regions are in direct and extensive contact with the meningeal surface and the subarachnoid space (SAS). Earlier studies, performed in MBP-induced EAE, have suggested the SAS as an important site for precursor T-cell proliferation and effector T-cell selection in the Lewis rat (Shin et al., 1995). This finding was further expanded by showing that effector T cells preferentially migrate into the subpial parenchyma from the SAS (Shin and Matsumoto, 2001) and similarly for dendritic cells in preclinical stages of mouse proteolipid protein-induced EAE (Serafini et al., 2000). The severe inflammatory infiltration of cerebellar meninges preceding perivascular and parenchymal infiltration (Fig. 5) underscores the importance of the SAS in propagation of the inflammatory response. Meningeal inflammation was restricted to the cerebellar meninges, indicating that, whereas entry of inflammatory cells into the SAS may occur at random, local accumulation (homing) appears to be more complex, depending on local antigen-specific factors.

The lack of clinical disease induction in conjunction with the absence of overt neuronal degeneration does not argue against specificity or encephalitogenicity of peptide ac 9–25, because severe subclinical inflammation of the CNS has also been observed in MOG-induced EAE (Linington et al., 1993) and experimental allergic myocarditis (Wegmann et al., 1994). In addition, lack of sustained demyelination despite severe inflammation is also a common feature in MBP-induced EAE in the Lewis rat (Martin and McFarland, 1995). In analogy to MBP-induced EAE (Prud'homme and Piccirillo, 2000), it is possible that the strong TGF-β1 expression in ac-9–25-immunized rats contributes to rapid down-regulation of the proinflammatory response and widespread apoptosis of infiltrating cells, preventing the establishment of a sustained proinflammatory milieu with secretion of cytotoxic substances or generation of $CD8^+$ cytotoxic T cells. Cytokines, for example, could not only exert direct neurotoxicity (Ma and Zhu, 2000; Stoll et al., 2000; Nicholas et al., 2001) but also induce neuronal MHC class I expression (Neumann et al., 1997), rendering neurons susceptible to $CD8^+$ T-cell-mediated neuronal damage. Future studies in TGF-β1 or other cytokine-gene knockout animals could address some of these questions.

CONCLUSIONS

The present study demonstrates for the first time in an experimental setting that neuronal antigens may become primary targets for a cell-mediated autoimmune attack, lending experimental support to an increasing body of literature suggesting an important, possibly primary role for T-cell-related mechanisms in the pathogenesis of antineuronal autoimmunity. The results further demonstrate that the Lewis rat EAE model can be adapted to evaluate encephalitogenicity of the potential antineuronal target antigens in vivo.

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