Role of Chemokines, Neuronal Projections, and the Blood-Brain Barrier in the Enhancement of Cerebral EAE Following Focal Brain Damage

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Abstract. The role of focal brain damage as a trigger for autoimmune inflammation in multiple sclerosis (MS) is unclear. In this study we examine mechanisms by which experimental autoimmune encephalomyelitis (EAE) is enhanced by focal brain damage. EAE was produced in Lewis rats by footpad inoculation; focal brain damage, in the form of a cortical cryolesion (cryolesion-EAE), was induced 8 days post-inoculation (d.p.i.). The distribution of inflammation and chemokine production in cryolesion-EAE and EAE-only were compared. Inflammation in the brain, measured by immunocytochemistry for T lymphocytes (W3/13) and microglial activation (MHC class II -OX6), was significantly enhanced in cryolesion-EAE 11–15 d.p.i. (p < 0.01–0.05) but by 20–40 d.p.i., equated with EAE-only. Inflammation in cryolesion-EAE related to breakdown of the blood-brain barrier (BBB) at the site of the cryolesion and also to the corticospinal tracts and thalamus, reflecting the afferent and efferent neuronal connections with the cryolesioned cortex. Semiquantitative RT/PCR dot-blot hybridization assay showed a 6-fold increase in mRNA for specific chemokines in the brain in cryolesion-EAE at 9 d.p.i. (MCP-1) and 11 d.p.i. (MCP-1 and MCP-5) with no significant increase in RANTES, GRO-α, or MIP-1α. By 14 d.p.i., the levels of MCP-1 and MCP-5 mRNA equated with EAE-only animals. These results suggest that enhancement and location of autoimmune inflammation in the brain following focal cortical injury initially involve chemokines such as the macrophage chemoattractants MCP-1 and MCP-5, and the activities of afferent and efferent neuronal connections with the site of damage. By analogy, similar factors may modulate or reactivate autoimmune inflammation in MS.

Key Words: Autoimmune; Chemokines; Encephalomyelitis; Inflammation; Monocyte chemoattractant protein-1; Trauma.

INTRODUCTION

Pathological studies of brain and spinal cord in patients with multiple sclerosis (MS) suggest that the disease has an autoimmune pathogenesis, but the factors that initiate the inflammation in MS and result in its relapse are unknown (1). The role of trauma as an initiating factor in MS remains controversial (2). Nevertheless, studies using experimental autoimmune encephalomyelitis (EAE) as a model for the autoimmune aspects of MS show that focal brain damage enhances the severity of inflammation in the brain in EAE (3, 4).

In acute EAE, clinical signs of weakness and paralysis of the hind limbs are related to lymphocyte infiltration and microglial activation in the spinal cord; the cerebral hemispheres are relatively spared, at least in the initial stages of the disease. However, focal brain damage in the form of a cryolesion to one cerebral hemisphere 8 days post-inoculation (d.p.i.) for EAE results in a 6-fold enhancement of autoimmune inflammation, with lymphocyte infiltration and microglial activation, in the cerebral hemispheres (cryolesion-EAE) (4). The role played by cervical lymph nodes in such enhancement in cryolesion-EAE was investigated in the knowledge that tracers injected into the CSF drain to regional lymph nodes in the neck in the rat (5), and that antigens injected into the rat brain or CSF induce antibody production in cervical lymph nodes (6). Thus, when cervical lymphadenectomy was performed in cryolesion-EAE animals at the time of the focal brain injury, there was a 50% reduction in the enhancement of inflammation in the cerebral hemispheres (7). In further studies it was shown that adoptive transfer of lymphocytes from cryolesion-EAE animals induced a significant enhancement of cerebral EAE when compared with lymphocytes from EAE-only animals (8). The results of these experiments suggested that, firstly, the cervical lymph nodes are a significant source of T lymphocytes targeting the brain in EAE and, secondly, drainage of antigen from the brain to cervical lymph nodes may be involved in stimulating cervical lymph node lymphocytes to target the brain (9).

However, removal of the cervical lymph nodes results in only a 50% reduction in the level of enhancement of cerebral inflammation in cryolesion EAE (7). In the current studies, therefore, we tested the hypothesis that local...
factors within the brain are also involved in the enhancement of autoimmune inflammation in cryolesion EAE. We concentrated on 3 factors: the afferent and efferent neuronal connections with the site of the cryolesion; the integrity of the blood-brain barrier (BBB); and chemokines released from the site of the focal brain damage (10).

The earliest intrathecal inflammatory event in EAE is probably the accumulation of activated T-cells; and subsequent recruitment of additional inflammatory cells into the CNS is a critical step in the development of the disease. As chemoattractant and activating factors for specific subsets of leukocytes, chemokines have emerged as likely candidates to mediate inflammatory cell recruitment into the CNS (11, 12). Chemokines are categorized into subfamilies based on structural and functional characteristics (13, 14). One subgroup of α-chemokines, which possess a glutamate-leucine-arginine (ELR) motif, such as interleukin-8 and GRO-α, is comprised of neutrophil chemoattractants. The β-chemokines, including the monocyte chemoattractant proteins (MCP)-1 through 5, macrophage inflammatory protein (MIP)-1α and MIP-1β, and regulated on activation normal T cell expressed and secreted (RANTES) attract T-cells, monocytes, basophils, and eosinophils.

Two general patterns of CNS chemokine expression in model neurological disorders have been described (11, 12, 15, 16). In immune-mediated inflammation, as seen in EAE, multiple α- and β-chemokines are expressed in the spinal cord and are closely related to the onset of clinical signs. These chemokines are produced immediately after the earliest signs of lymphocyte invasion of the CNS (17). Sources of chemokines include both infiltrating cells (RANTES, MIP-1α) and resident astrocytes (MCP-1, IP-10, GRO-α) (18). We have proposed that this burst of chemokine expression serves to amplify the recruitment of inflammatory cells after the entry of autoreactive T-cells into the CNS (19). In contrast to immune-mediated inflammation, direct injury to the CNS (as with a cryolesion) frequently evokes early expression principally of the monocyte chemoattractant, MCP-1 (10, 16, 20, 21). MCP-1 is expressed by astrocytes within hours of mechanical injury to the CNS, before leukocyte accumulation occurs, and is thought to mediate recruitment and activation of mononuclear phagocytes (21).

In the present study, we report the nature and distribution of inflammation in the brains and spinal cords of rats with cryolesion-EAE and with EAE-only across an extensive time course. Factors that influence inflammation are identified as afferent and efferent cortical neuronal projections, disruption of the BBB, and specific β-chemokine expression.

**MATERIALS AND METHODS**

**Animals**

Female Lewis rats (MHC type RT-1), 2–3 months old and weighing 170–200 g (Charles River Ltd, Kent, UK) were used for all experiments. Animals were housed at the Biomedical Research Unit, University of Southampton, in groups of up to 5 rats per cage, at room temperature 21°C–23°C; humidity 45%–65%; with water and food provided ad libitum. Animals were handled and weighed daily 1 wk before immunization and throughout the experiments. All animal procedures were carried out under the Home Office Animal Act (UK).

Acute EAE was induced by footpad immunization of 50 μl inoculum containing 1 mg/ml heat-killed Mycobacterium tuberculosis (Difco, Franklin Lakes, NJ) at a final concentration of 500 mg/ml guinea pig spinal cord homogenate in complete Freund’s adjuvant. Clinical signs were assessed daily on the following scale: 0 = normal, 1 = weight loss only, 2 = tail weakness, 3 = hind limb weakness, 4 = hind limb paralysis, 5 = moribund (4).

Cryolesions were induced 8 days after inoculation of antigen through the application, for 90 s, of a 5-mm-diameter metal rod cooled in liquid nitrogen to the exposed right side of the skull under full anesthesia as described previously (4, 7). The coordinates for the cryolesion were 1 mm rostral to the bregma, 3 mm to the right of the sagittal suture in the right frontal isocortical region. Thus the cryolesion damaged frontal cortical Fr1 and Fr2, which is part of the motor cortex and has extensive connections with other parts of the CNS, including the thalamus and spinal cord (22).

**Preparation of Animals**

In a total of 91 rats, EAE was induced in 77 by the inoculation of antigen (day zero); 42 rats then received a cryolesion at 8 d.p.i. of antigen (cryolesion-EAE); 35 rats with EAE received no cryolesion (EAE-only). Animals were killed by transcardiac perfusion with 10% buffered formalin for the immunocytochemical studies. Cryolesion-EAE and EAE-only animals were killed at 8, 9, 11, 13, 15, 20, 30, and 40 d.p.i. Twelve normal rats, which received a cryolesion only, were killed at 30 min, 1, 3, 5, 7, 12, and 22 days post-cryolesion. Two normal rats served as controls. Brains and spinal cords were removed and further fixed with 10% buffered formalin and embedded in paraffin wax.

For the chemokine RT/PCR study, cryolesion-EAE and EAE-only animals were killed at 9, 11, and 14 d.p.i.; the unfixed brains and spinal cords were removed, snap frozen in liquid nitrogen, and stored frozen.

Immunohistochemical staining was performed on 5-μm paraffin sections from 4 coronal levels of cerebrum, i.e. anterior to the cryolesion, through the cryolesion at the level of the septal nuclei; through the cryolesion at the level of the thalamus; and at the level of cerebellum and pons and from transverse sections of cervical, thoracic, and lumbar spinal cord. After dewaxing, endogenous peroxidase was quenched with 0.5% hydrogen peroxide in methanol for 10 min. Sections were pretreated in 1% trypsin, pH 7.8 (Sigma, St. Louis, MO) at 37°C for 15 min and incubated with primary antibodies in appropriate dilution in TBS for 30 min at room temperature. Mouse anti-rat monoclonal antibodies OX-6 for MHC class II antigens (1:100, Serotec, Oxford, UK), W3/13 (pan-T lymphocytes) (1:2,000, Serotec), ED-2 for perivascular cells (1:400, Serotec) were used. Polyclonal antibodies were used for GFAP (1:2,000, Neuropathology Laboratory, Southampton, UK) and...
serum albumin (1:3,500, Binding Site, UK). After washing with
TBS, sections were incubated with biotinylated rabbit anti-
mouse IgG or swine anti-rabbit IgG (1:200, Dako, Denmark)
for 30 min, washed, then reacted with avidin-biotin peroxidase
complex (Dako) for 30 min. Bound antibodies were visualized
with 3’, 3’-diaminobenzidine and sections counterstained with
hematoxylin. Immunocytochemistry was controlled by omitting
the primary antibodies.

Qualitative analysis of immunocytochemical preparations en-
tailed determining the anatomical distribution of perivascular
cuffs of lymphocytes and of MHC class II expression on mi-
croglial cells in immunocytochemical preparations. Photograph-
ic images of brain or spinal cord sections were generated with
a Mackintosh G3 computer attached to a slide scanner, using
the Adobe Photoshop 5.0.2 system, silver fast process.

Quantitative Analysis of Immunocytochemical
Preparations

The levels of MHC class II expression in histological sec-
tions were quantified using computerized image analysis of sec-
tions of brain and spinal cord stained immunocytochemically
using the antibody OX-6. Four levels of brain and 3 levels of
spinal cord were analyzed using a Zeiss videocamera linked to an
Apple Macintosh PC, with image 1.7 software (NIH, Bethes-
da, MD) and a light microscope. The results were expressed as
a percentage of the area of the section stained for MHC class
II expression. The number of EAE lesions was estimated by
using a light microscope with a × 10 objective for counting the
number of vessels which had at least 4 W3/13-positive lym-
phocytes in the perivascular space; the data were analyzed using
the two-tail Student t-tests assuming unequal
variances.

RNA Preparation

Brains and spinal cords of all animals were maintained at
−70°C until processing and analysis. For RNA extraction, tis-
sues were crushed to a fine powder with chilled mortar and
pestle (precooled with liquid nitrogen) and mixed with Trizol
(Life Technologies, Gaithersburg, MD) following the manufac-
turer’s instructions. After precipitation and resuspension in wa-
ter, the concentration and purity of the RNA were measured by
UV absorbance at 260 nm and ratio of absorbance at 260 nm/
280 nm with a DU 600 spectrophotometer (Beckman Instru-
tments, Fullerton, CA) and further assessed by electrophoresis
of 1 µg of each RNA sample on an ethidium bromide-contain-
ing agarose gel. Visualization of the 28S and 18S ribosomal
RNA bands on a UV transilluminator was used to confirm
quantity and integrity of the RNA samples.

Semi-quantitative RT/PCR dot-blot hybridization assays of rat
chemokine mRNA, including sequences of the primers, have been
described in detail previously (20). Briefly, first-strand
cDNA was synthesized by using 1 µg of total cellular RNA,
oligo-dT primers and Superscript II reverse transcriptase (Gib-
co, Gaithersburg, MD) according to the manufacturer’s instruc-
tions. The cDNA product was then amplified by PCR using
pairs of rat chemokine gene-specific primers and optimal PCR
conditions, yielding a linear relationship between cDNA input
and PCR product as previously described (23). PCR performed
without cDNA input provided negative controls. Amplification
of α-tubulin transcript confirmed intact RNA and absence of
DNA contamination in all samples. PCR products were dena-
tured and transferred to nylon membrane (Micron Separations
Inc., Westborough, MA) by vacuum blotter (Schleicher and
Schuell, Keene, NH) and then hybridized with radiolabeled
cDNA probes. Hybridization signal was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and
relative signal intensities expressed as arbitrary densitometric
units. We have previously documented close correlation be-
tween levels of chemokine mRNA determined by this method
and chemokine protein as determined by ELISA of tissue ho-
logenates (18).

The Kolmogorov-Smirnov test was used to assess distribu-
tion of the level of chemokines. Comparisons between groups
were made with the Mann–Whitney U-test, with p < 0.05 con-
sidered to indicate significant differences.

RESULTS

Clinical Signs

In the EAE animals, clinical signs were related to spi-
nal cord pathology with the progressive development of
tail and hind limb weakness as outlined in the “Materials
and Methods” section. No clinical signs related to the
cerebral cryolesion were detected in either the EAE rats or
the cryolesion-only animals. The progression of clin-
ical signs in the cryolesion-EAE animals was similar to
that in the EAE-only animals except that the mean score
for clinical signs in the cryolesion-EAE animals at 9
d.p.i. was greater than in the EAE-only animals (p =
<0.05). Thereafter, there was no statistical difference be-
tween the 2 groups of animals.

Breakdown of the Blood-Brain Barrier

Immunocytochemical staining for serum albumin was
used to assess the breakdown of the BBB associated with
cryolesions and EAE lesions. At 30 min after a cryole-
sion in normal animals, there was intense staining for
serum albumin in the region of the cryolesion and ex-
tending into the white matter. By 24 h, the serum albumin
staining involved most of the cerebral cortex of the ip-
silateral hemisphere but did not extend into the deep gray
matter. By 3 days after the cryolesion, albumin staining
extended from the cortex surrounding the lesion into the
ipsilateral corpus callosum, caudate-putamen, internal
capsule and fornix, and to a lesser extent, the contralateral
cerebral cortex. Diffuse staining for albumin persisted 7
days after the cryolesion but was less intense and no ob-
vious albumin staining associated with the cryolesion re-
maind after 12 days.

Increased permeability of the BBB to serum proteins
accompanying the onset of EAE resulted in serum albu-
min staining as patches around perivascular lesions from
9 to 20 d.p.i. in both cryolesion-EAE and EAE-only. By
30 d.p.i., only very weak albumin staining was seen de-
spite the widespread involvement of the brain by EAE
lesions. In animals exhibiting clinical relapse at 30 d.p.i.,
however, there was strong perivascular serum albumin staining around perivascular lymphocyte cuffs.

Inflammatory Reactions to the Cryolesion Alone

In the 12 animals that received a cryolesion only, edema and a small number of neutrophil polymorphonuclear leukocytes were present at the site of the cryolesion 1–3 days post-cryolesion. Few, if any, lymphocytes were detected. MHC class II expression was present on ED2-positive perivascular cells, which were widely distributed throughout the cerebral hemispheres at 30 min post-cryolesion. MHC class II expression on microglia was seen 1 day later. By 3 days post-cryolesion, perivascular cells, microglia, and macrophages expressed MHC class II antigen and this persisted at the site of the cryolesion until 7 days on perivascular cells and microglia and until 12 days on macrophages. From day 1–12, inflammation was restricted to a thin rim of tissue around the cryolesion except for the widespread response by perivascular cells.

Distribution of Inflammation in EAE and Cryolesion EAE

Histology of the EAE lesions in both the cryolesion-EAE and EAE-only animals revealed lymphocyte cuffing of vessels and activation of perivascular microglia. The lymphocyte cuffs were clearly identified by the lymphocyte marker W3/13 in immunostained sections. Microglial activation was illustrated by MHC class II antigen expression using the OX-6 antibody. Widespread reactivity of perivascular cells (ED2) and astrocytes (GFAP) was seen throughout the cerebral hemispheres in cryolesion-EAE from 11 d.p.i. The histological characteristics of these lesions at 15 d.p.i. have been previously documented (4, 7)

Days 11–15 Post-inoculation

In cryolesion-EAE animals, concentration of MHC class II expression around the cryolesion (Fig. 1a) was observed at 11 d.p.i. (3 days after the cryolesion) extending into the corpus callosum and into the ipsilateral caudate putamen. The septal nuclei were heavily involved as were the hypothalamus and optic chiasm.

Staining was less intense and less widespread in the brains of EAE-only animals; a predilection for the septal nuclei, hypothalamus, and optic chiasm was seen at 11 d.p.i. (Fig. 1b) and there was also some staining in the corpus callosum.

By day 15 post-inoculation, cryolesion-EAE animals showed much more intense staining for MHC class II expression in the brain (Fig. 1c). Staining extended from the cryolesion into the ipsilateral caudate putamen, corpus callosum, and fornix. The thalamus and hypothalamus were heavily stained and focal staining for MHC class II expression was seen in the contralateral caudate putamen. Patchy subpial expression of MHC class II was observed, particularly in the hemisphere ipsilateral to the cryolesion but there was also some staining contralateral to the cryolesion.

In EAE-only animals at 15 d.p.i. (Fig. 1d), MHC class II expression was mainly concentrated in the septal nuclei, hypothalamus, optic chiasm with some expression of MHC class II antigen in the corpus callosum, and in the subpial region inferiorly in the hemispheres.

Day 20 Post-inoculation

In the cryolesion-EAE animals at 20 d.p.i., MHC class II expression was less centered on the site of the cryolesion and was more evenly spread throughout the hemispheres (Fig. 1e). There was, however, predominance of MHC class II expression in the caudate putamen ipsilateral to the cryolesion, although staining was also seen in the contralateral caudate putamen and cortex.

MHC class II expression in EAE-only animals (Fig. 1f) was much more prominent at 20 d.p.i. than at 15 d.p.i. and showed no significant difference from the cryolesion-EAE animals. There was extensive expression of MHC Class II antigen bilaterally in the caudate putamina, septal nuclei, hypothalamus, and optic chiasm.

Days 30 and 40 Post-inoculation

In both cryolesion-EAE and EAE-only animals, MHC class II expression had declined by day 40. At this time, however, there was a clear pattern of MHC class II expression reflecting the afferent and efferent neuronal projections of the region of the cortical cryolesion. Figure 2 shows MHC class II expression in the cortico-spinal tracts and in the mediodorsal and ventrolateral thalamus of the cryolesion-EAE animals, reflecting the afferent and efferent connections with the region of frontal cortex damaged by the cryolesion. In the same animal illustrated in Figure 2, MHC Class II expression is also prominent in the corticospinal tract in the spinal cord (Fig. 3). W3/13 immunocytochemistry for T lymphocytes did not reveal a predilection for lymphocyte cuffs at 40 d.p.i. in the cortico-spinal tract or in the thalamus. Except in the 4 animals that showed clinical relapse, the number of lymphocytes in the brain and spinal cord at 40 d.p.i. was substantially reduced, compared with animals at 15–20 d.p.i.

Control Animals

No lymphocytes stained by W3/13 immunocytochemistry were observed in the 2 control normal rats and neither was there any detectable MHC class II expression in paraffin sections of normal rat brain or spinal cord.

Quantitative Analysis of Inflammation in Cryolesion-EAE and EAE-only Animals

The intensity of MHC class II expression was quantified in whole coronal sections from 4 levels of cerebral
Fig. 1. The pattern of inflammation in cryolesion-EAE (left) and EAE-only (right) illustrated by immunocytochemistry for MHC class II expression at different time points: (a) Cryolesion-EAE at 11 d.p.i.; (b) EAE-only at 11 d.p.i.; (c) Cryolesion-EAE at 15 d.p.i.; (d) EAE-only at 15 d.p.i.; (e) Cryolesion-EAE at 20 d.p.i.; (f) EAE-only at 20 d.p.i. The area of inflammation extends from the superior surface of the cerebrum on the right (a, c, e), into the ipsilateral hemisphere at 11 d.p.i. (a), and then to the contralateral hemisphere by 15 d.p.i. (c). By 20 d.p.i., animals with cryolesion-EAE and EAE-only exhibit equivalent degrees of inflammation in the cerebral hemispheres. Immunocytochemistry using OX6 for MHC class II antigen.

hemispheres and in whole transverse sections from 3 levels of spinal cord stained with OX-6 from 11 d.p.i. to 40 d.p.i. in cryolesion-EAE and in EAE-only animals. Analysis with a 2-tail Student t-test showed a significant increase of MHC class II expression in the brain sections of cryolesion-EAE animals at 11d.p.i. (p < 0.01), 13 d.p.i. (p < 0.05), and 15 d.p.i. (p < 0.05) compared with the brains of EAE-only animals. However the differences at days 20, 30, and 40 d.p.i. were not significant (p > 0.05) when cryolesion-EAE was compared with EAE-only (Fig. 4a).

MHC class II expression in the spinal cord (Fig. 4b) showed a similar trend to that in the cerebral hemispheres and was higher in cryolesion-EAE than in EAE-only
animals at 11 to 20 d.p.i.; however, this increase was not significant (p > 0.05).

The number of EAE lesions was counted in sections stained with the W3/13 antibody from 4 levels of cerebral hemispheres and 3 levels of the spinal cord (Fig. 5a, b). Statistical analysis showed a significant increase in the number of EAE lesions in the cerebral hemispheres of cryolesion-EAE compared with EAE-only at days 11 (p < 0.05), 13 (p < 0.05), and 15 (p < 0.05) d.p.i. (Fig. 5a). At days 20, 30, and 40 the numbers of EAE lesions in cryolesion-EAE and EAE-only animals showed no significant difference (p > 0.05). There was no statistical difference between the number of EAE lesions in the spinal cords of cryolesion-EAE and EAE-only animals at any time point (Fig. 5b).

Chemokine mRNA Expression in Cryolesion-EAE and EAE-only

**EAE-only:** Chemokine expression in the CNS was present at basal levels at the early preclinical time point (9 d.p.i.) in brains and spinal cords of rats immunized to develop EAE (20). There were no significant differences between 9 d.p.i. and 11 d.p.i. for chemokine mRNA levels in either brain or spinal cord of EAE rats (Tables 1, 2), and these levels were typical of those observed in control rat tissues (20). Therefore, for statistical analyses,
Fig. 4. Quantitation of inflammation in cryolesion-EAE (light bars) and EAE-only (dark bars) by the extent of MHC class II expression in cerebral hemispheres (a) and spinal cord (b). Abbreviations: CLEAE = cryolesion-EAE; EAE = EAE-only. Significance values: ** = p < 0.01; * = p < 0.05.

The induced accumulation of chemokine mRNA was compared with 9 d.p.i. EAE levels. At the onset of clinical signs of EAE, MCP-1 and MCP-5 levels rose markedly and significantly, with means at 14 d.p.i. increased from 5- to 15-fold over 9 d.p.i. in both brain and spinal cord (Tables 1, 2; Figs. 6, 7). Expression of RANTES and MIP-1α mRNA was modest in both brain and spinal cord at all time points examined, with a tendency for MIP-1α to increase at the onset of EAE (Tables 1, 2). GRO-α mRNA expression was analyzed only in brain and did not change significantly during the time course of EAE (Table 1). These results were consistent with predominant, selective expression of chemokines that act towards monocytes and T-cells, tightly linked to clinical
Fig. 5. Quantitation of inflammation in cryolesion-EAE (light bars) and EAE-only (dark bars) by the number of lesions (i.e., perivascular lymphocyte cuffs) in cerebral hemispheres (a) and spinal cord (b). Abbreviations: CLEAE = cryolesion-EAE; EAE = EAE-only. Significance values: ** = p < 0.01; * = p < 0.05.

onset of disease, as previously reported by several investigators (reviewed in [19]).

Cryolesion-EAE: A cryolesion at 8 d.p.i. resulted in selective, massive, and significant increase of MCP-1 expression in the brains but not in the spinal cords at 9 d.p.i. (1 day after cryolesion; Tables 1, 2; Figs. 6, 7). MCP-1 expression levels in brain were sustained at 11 d.p.i. (3 days after the cryolesion). At 14 d.p.i., levels of MCP-1 mRNA expression in brain were not significantly different from the elevated levels in the brains of rats with EAE-only (Table 1; Fig. 6). MCP-1 mRNA was present at basal levels in the spinal cord at 9 d.p.i. in cryolesion-EAE animals, but was significantly elevated by 11 d.p.i. (Table 2; Fig. 7), increasing still further with
the onset of clinical signs of EAE by 14 d.p.i. Increased expression of MCP-5 was not detected in cryolesion-EAE brains until 11 d.p.i., when levels were significantly increased as compared with EAE-only (Table 1; Fig. 6). The disparate regulation of MCP-1 as compared with MCP-5 was striking, as MCP-5 is highly related to MCP-1 and is considered to be a biologically relevant ligand for the MCP-1 receptor, CCR2 (24). By 14 d.p.i., both EAE-only and cryolesion-EAE brains contained elevated levels of MCP-5. MCP-1 and MCP-5 rose in concert in the spinal cords of EAE-only rats, at 14 d.p.i. (Fig. 7). In cryolesion-EAE rats, the spinal cord MCP-1 and MCP-5 response appeared blunted, although significant increases over controls levels were present at 14 d.p.i. (Fig. 7).

MIP-1α was not highly expressed in the CNS of animals with EAE-only at any time point, although expression modestly increased in the cryolesion-EAE brains 9, 11, and 14 d.p.i., with a slight increase detected in spinal cords at 14 d.p.i. RANTES and GRO-α expression did not change significantly during these experiments (Tables 1, 2).

**DISCUSSION**

The present study has shown that the enhancement of cerebral EAE by a focal cryolesion at 8 d.p.i. is centered on the period 11–15 d.p.i. By 20 d.p.i., the level of inflammation in the brains of cryolesion-EAE animals equates with that of EAE-only. In the early stages (11–20 d.p.i.), the distribution of MHC class II expression on microglia in the cryolesion-EAE animals relates to the cryolesion and corresponds to the pattern of spread of serum protein leaking from breakdown of the BBB at the cryolesion itself. Semiquantitative RT/PCR-blot hybridization assays showed that both MCP-1 and MCP-5 mRNA expression is increased in the brain in cryolesion-EAE 1 and 3 days after the cryolesion for MCP-1 and 3 days after the cryolesion for MCP-5 when compared with EAE-only, but by 5 days post-cryolesion, levels of both these chemokines have fallen to that of EAE-only animals. These findings suggest that MCP-1 and MCP-5, related to the cryolesion, play a key role in the induction of the inflammatory response in cryolesion-EAE as their expression peaks in the period before the maximum degree of inflammation is seen within the brain.

In the later stages of cryolesion-EAE, there was marked activation of microglia in a distribution that corresponded to the afferent and efferent neuronal pathways damaged by the cortical cryolesion. This was particularly well illustrated in the 40 d.p.i. animals with upregulation of MHC class II expression in the thalamus and in the
Fig. 6. MCP-1 and MCP-5 mRNA accumulation in the brains of rats with cryolesion-EAE and EAE-only. Graphic representation of mean MCP-1 and MCP-5 mRNA levels (Table 1) in the brains of rats 9–14 d.p.i. Rats receiving a cryolesion at 8 d.p.i. demonstrated a selective increase in MCP-1 (open circles) but not MCP-5 (asterisks) at 9 d.p.i., 1 day after the cryolesion. MCP-5 levels were elevated by 11 d.p.i., 3 days after cryolesion. In EAE-only animals, brain levels of both MCP-1 (closed circles) and MCP-5 (diamonds) rose moderately and significantly at 14 d.p.i., coincident with the onset of EAE.

Fig. 7. MCP-1 and MCP-5 mRNA accumulation in the spinal cords of rats with cryolesion-EAE and EAE-only in the spinal cords of rats 9–14 d.p.i. Graphic representation of mean MCP-1 and MCP-5 mRNA levels in Table 2. Note marked difference in the time course of chemokine expression in brain (Fig. 6) as compared with spinal cord shown here. Rats receiving cryolesion at 8 d.p.i. demonstrated a modest increase in MCP-5 (asterisks) but not MCP-1 (open circles) at 9 d.p.i., 1 day after cryolesion. Spinal cord levels of MCP-1 (open circles) and MCP-5 (asterisks) levels rose continuously but modestly after cryolesion. In EAE-only, spinal cord levels of both MCP-1 (closed circles) and MCP-5 (diamonds) rose abruptly and significantly at 14 d.p.i., coincident with onset of EAE. This increase in chemokine expression was more striking than that observed in brains of EAE-only rats (Fig. 6), reflecting the predominant site of inflammation in the spinal cord.
corticospinal tracts. The cortex damaged by the cryolesion was frontal cortex Fr1 and Fr2 (22). Fr1–3 receive afferent connections from the ventrolateral thalamic nucleus and Fr2 from the mediodorsal thalamic nucleus. Efferent connections of these areas of frontal cortex terminate in the parietal cortex and, via the internal capsule and the corticospinal tract, fibers descend to the spinal cord.

Given the potential for chemokines to determine the cellular composition and activation of leukocyte subsets in the CNS, the present results suggest that a sequential pattern of chemokine expression plays a role in the cryolesion enhancement of EAE. We examined the expression patterns of 5 chemokine mRNAs, the β-chemokines MCP-1, MCP-5, MIP-1α, and RANTES and the α-chemokine GRO-α, during the time course of an initial attack of EAE, with or without enhancement by a cryolesion. The β-chemokines we studied are chemoattractants towards monocytes/macrophages and T-cells. GRO-α is a highly selective neutrophil chemoattractant. Chemokine message levels were analyzed by a sensitive and specific RT/PCR dot-blot hybridization assay, previously validated in models of CNS trauma in rats (20). Increased expression of MCP-1 in the brain occurred 1 day after cryolesion. At this early time point after the injury, MCP-1 induction was quite selective, as other β-chemokines including MCP-5 were not similarly increased. Cryolesion-induced MCP-1 expression was also spatially restricted to the brain, as the spinal cord did not demonstrate altered chemokine expression at this time point. By 3 days postcryolesion, both MCP-5 and MCP-1 were elevated in brain and, to a modest extent, in spinal cord. In contrast, chemokine message levels in EAE-only animals remained at basal levels in both brain and spinal cord until the onset of EAE at 14 d.p.i. These results indicate the sequential pattern of chemokine expression that may operate in the cryolesion enhancement of EAE. Furthermore, the results underline the remarkably restricted pattern of focal MCP-1 expression shortly after physical trauma to the CNS.

As a chemoattractant for monocytes in vivo, MCP-1 is likely to play an important role in the CNS response to injury and in enhancing the evolving inflammation of EAE (25). A cryolesion is accompanied by local breakdown of the BBB (3), by macrophage infiltration, and by microglial and astrocyte activation. The early onset and relative transience of cryolesion-induced MCP-1 expression, shown in the present study, is consistent with the seemingly narrow window during which a cryolesion augments EAE. Previously, we observed that a cryolesion at 3 d.p.i. did not result in enhanced EAE, suggesting important interactions between lymphocytes, which are first detected in the CNS at 5–6 d.p.i., and the acute effects of a cryolesion (4).

The observation that cryolesion-induced MCP-1 expression was initially present in the brain but not in spinal cord indicates that this chemokine was directly upregulated by local tissue stimuli rather than by systemic factors. Following a cryolesion, widespread tissue changes, such as microglial MHC Class II antigen expression, occur in the absence of BBB breakdown and probably contribute to the enhancement of histological EAE. The results of the present study showing a close association between microglial activation and the afferent and efferent neuronal projections suggest that axonal damage and surrounding glial reactions may lower the threshold for inflammation to develop in neuronal projection pathways. Similar conclusions can be drawn from the work of Konno et al (3). They reported microglial MHC Class II upregulation at distal sites of Wallerian degeneration in the visual pathways, including the contralateral optic tract and superior colliculus in adoptively-transferred EAE after unilateral enucleation. (3). Our present findings of delayed increase in spinal cord chemokine production after a cryolesion are consistent with the proposal that Wallerian degeneration distal to a focal lesion may signal chemokine expression, as previously observed in the cortex contralateral to a cryolesion injury (10).

We and others have reported selective and early increase in MCP-1 expression in several models following mechanical or ischemic insult to either CNS or PNS (9, 21, 26–29). This pattern is distinct from immune-mediated inflammatory conditions such as EAE, in which multiple chemokines are expressed (18, 19, 30–32). Although it was unexpected, the indispensable role of MCP-1 in certain forms of inflammation has been firmly established by analysis of gene-targeted MCP-1-null mice (33–35). There are 5 monocyte chemoattractant proteins (MCPs), which exhibit remarkable functional similarity in vitro. One interpretation of our results is, therefore, that differential expression may help to define the roles of the various MCPs in specific pathological and physiological processes (36). We have not excluded the possible expression of other members of the family in the brains of cryolesioned rats, as MCPs have been reported to be coexpressed in several examples of neural inflammation including MS (37–39).

The results of the present study have shed new light upon the sequence of events in the pathogenesis of cerebral EAE. A cryolesion enhances cerebral EAE in the early phases of the disease, between 11 and 15 d.p.i., at the time of maximum pathology and clinical effect in the spinal cord and it is not until 20 d.p.i. that inflammation in the brain in EAE-only equates with cryolesion-EAE. One question, therefore, is why is there delay in the inflammatory response in the brain in EAE-only animals?

Results from previous studies (4, 7, 8) and from the present experiments suggest that a combination of factors is involved in the enhancement of EAE by a cryolesion.
that could explain the delay in inflammation in the brain in EAE-only animals. Removal of the cervical lymph nodes significantly reduces cerebral inflammation in cryolesion-EAE (7), which indicates that focal injury to the brain may stimulate cervical lymph node T-cells to target the brain, possibly in response to the drainage of antigens from the site of injury to the cervical lymph nodes. This concept is supported by studies showing restriction of integrin expression by lymphocytes in the CNS (40), which suggests some degree of selective targeting of the CNS by certain subsets of lymphocytes (9). The present study emphasizes that the local release of chemokines and stimulation of neuronal projections also play a significant role in the enhancement of EAE by a cryolesion.

How then may these factors operate in EAE-only? It seems probable that drainage of antigen from the injection site in the footpad to popliteal and lumbar lymph nodes accounts for the early targeting of the spinal cord in EAE. The lumbar nodes are the regional lymph nodes for the spinal cord (5) and thus possibly the main source of T lymphocytes targeting the cord. With regard to the onset of cerebral inflammation in EAE-only, adoptive transfer experiments have shown that activated T lymphocytes may enter the CNS from the circulation regardless of specificity (41). Thus initial entry of T-cells into the brain in EAE may be nonspecific, and more specific mechanisms may only engage at a later stage. Tissue damage in the early stages of cerebral EAE resulting from nonspecific entry of T-cells may trigger the local release of chemokines, stimulation of neuronal connections, and the drainage of antigens to cervical lymph nodes, all of which, as outlined above, may combine to promote more specific T-cell targeting of the cerebral hemispheres.

By analogy with EAE, focal brain damage in patients with MS may initiate similar sequences of events. In some patients, the entry of nonspecific activated T lymphocytes may initiate the lesion. But, in others, a small area of ischemia, damage by virus infection or mechanical trauma, in a susceptible individual, may determine the site and timing of an autoimmune lesion in the CNS by a similar mechanism to cryolesion-EAE. Although in cryolesion-EAE, upregulation of MCP-1 may proceed in part through signals generated by injured neurons, in MS, neuronal injury is probably a secondary phenomenon with axonal damage resulting from inflammation and demyelination (42–44). It is possible that such axonal damage leads to enhancement of CNS inflammation and disease chronicity through glial activation and MCP-1 expression. Further studies of the role of chemokines in inflammatory and degenerative diseases should facilitate the development of effective therapeutic agents, for MS.

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