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Lymphocyte Recruitment Following Spinal Cord Injury in Mice is Altered by Prior Viral Exposure

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Abstract

The inflammatory response induced by mechanical lesion of the spinal cord is known to include the recruitment of neutrophils and macrophages, while the involvement of lymphocytes has been largely ignored. We have studied the pattern of lymphocyte recruitment following partial transection of the mouse spinal cord. Using immunohistochemical techniques, all three types of lymphocytes (CD4-positive T-cells, CD8-positive T-cells and B-cells) were found in the vicinity of the lesion site within hours and persisted for up to 7 days. There was a predominance of B-lymphocytes during the first 3 days. A second, late phase of cell infiltration, dominated by CD8-positive T-lymphocytes, occurred in mice that had been raised in a conventional breeding unit and had acquired antibody titres to a common murine virus (mouse hepatitis virus). In contrast, mice kept in specific pathogen-free facilities did not show this late-phase response. These findings suggest a possible role for lymphocytes in secondary tissue loss, local demyelination, scar formation, cytokine-mediated inflammatory responses or trophic processes. They also provide evidence that a virus infection can significantly enhance the reaction of T-cells to a spinal cord lesion.

Introduction

The cellular response to central nervous system (CNS) trauma is known to be dominated by phagocytic cells, notably blood-borne neutrophils, monocytes/macrophages and microglia (Means and Anderson, 1983; Giulian *et al.*, 1989; Perry *et al.*, 1993; Clark *et al.*, 1994; Kreutzberg, 1995). In rats, neutrophil granulocytes invade spinal cord lesions in large numbers during the first 2 days (Dusart and Schwab, 1994; Bartholdi and Schwab, 1995), and macrophages dominate the inflammatory response from day 4 onward. So far, the presence and possible role of lymphocytes following CNS trauma has not been extensively investigated. In a rat model of middle cerebral artery occlusion, recruitment of a small number of lymphocytes to the vicinity of the lesion has been shown (Schroeter *et al.*, 1994; Jander *et al.*, 1995). The contribution of these cells to the development of the lesion is not known, but the fact that lymphocytes represented only a small part of the cellular response may explain the lack of attention paid to these cells after CNS trauma. In contrast, in experimental allergic encephalomyelitis (EAE) T-cells are known to mediate inflammatory reactions which result in breakdown of the blood–brain barrier, myelin damage and oligodendrocyte and axon destruction (Wekerle, 1993). Treatment of rats with a T-cell receptor-specific monoclonal antibody can suppress the development of EAE-associated neurological symptoms (Imrich *et al.*, 1995).

Mechanical trauma to the CNS results not only in a primary lesion, but frequently develops into a more widespread area of cell damage, resulting in exacerbation of the lesion (Sloan *et al.*, 1992; Dusart and Schwab, 1994). This secondary tissue loss has been attributed to a number of factors, such as ischaemia resulting from disrupted and blocked blood vessels, glutamate excitotoxicity, intracellular Ca^{2+} accumulation, and free radical damage (Tator and Fehlings, 1991; for review see Schwab and Bartholdi, 1996). The roles played by the various types of inflammatory cells are not clear at present. Damaging effects as well as trophic roles have been suggested in addition to phagocytosis (Coffey *et al.*, 1990; Blight, 1992; Logan and Berry, 1993). At later time points scars and cavities are formed. Evidence for myelin loss in originally intact fibre tracts exists in animal spinal cord injury models and in spinal cords of paraplegic patients (Bunge *et al.*, 1993; for review see Waxman, 1992).

To investigate whether cells of the immune system also react to spinal cord injury, we studied the appearance of T- and B-lymphocytes over a period extending from 10 min to 5 weeks after a mechanical spinal cord lesion in the adult mouse. Since pathogens commonly found in conventional breeding units (CU) might provoke an additional immune response that could complicate the interpretation of results, mice obtained from a specific pathogen-free (SPF) unit were also examined. We provide evidence for selective recruitment of lymphocytes at certain intervals following

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partial spinal cord transection. The magnitude and duration of this recruitment is influenced by prior exposure of the mice to viruses, e.g. mouse hepatitis virus (MHV).

Materials and methods

Experimental animals

C57Bl6J mice (total $n = 83$) were used throughout the experiments with the exception of the studies in rats, which were carried out in Lewis Hannover rats. The majority of animals were lesioned between 5 and 7 weeks of age; both sexes were used at each time point. One group of mice and all rats were bred and housed in a conventional, open animal unit (CU; at our Institute). A second group of mice was obtained from an SPF breeding facility (Institute of Laboratory Animal Science, University of Zürich, Switzerland). All animals were kept under controlled conditions of light and temperature, with food and water available *ad libitum*. Microbial and viral testing was performed at the Institute of Laboratory Animal Science, University of Zürich, Switzerland.

Spinal cord lesions

Mice were anaesthetized with intraperitoneal (i.p.) Hypnorm (Janssen Laboratories; 0.25 mg in 250 µl per 20 g body weight) and Dormicum (Roche; 0.3 mg in 125 µl per 20 g, i.p.). The skin overlying the vertebral column was then incised, the muscles were detached from the vertebrae and a partial laminectomy was performed at thoracic vertebra 7 or 8. After opening the dura, the spinal cord was lesioned using fine iridectomy scissors so as to produce a partial transection including both dorsal funiculi and the lateral hemicord on the right side. Durafilm (Codman & Shurtleff, Randolph, MA) was used to replace the dura and a small piece of Gelfoam (Upjohn Company, Kalamazoo, MI) was inserted before closure of the muscles. The skin was re-apposed with wound-clips and all animals were warmed on a heating plate until completely awake. In all cases recovery was uneventful, except to note the anticipated transient paralysis of the right hindlimb during the first 2 postoperative days.

Tissue preparation

At 10 min, 3, 6, 12 h, 1, 2, 4 days, and 1, 2, 3 and 5 weeks mice were decapitated and the spinal cord was immediately dissected, embedded in Tissue Tek (Miles Laboratories, West Haven, CT) and frozen at –40°C. Sagittal sections were cut at 20 µm on a cryostat and mounted on Superfrost$^+$ slides (Fisher Scientific, Pittsburgh, PA). Series of adjacent sections were processed for immunohistochemical staining.

Antibodies for lymphocyte detection

Primary antibodies

Rat monoclonal antibodies directed against mouse CD4 (T-helper lymphocytes), CD8 (cytotoxic/suppressor T-lymphocytes) and CD45 (B220 on B lymphocytes) were used. These antibodies were kindly supplied by the laboratories of Dr R. M. Zinkernagel and Dr B. Odermatt, University of Zürich) and were diluted as follows: CD4, 1:6000; CD8, 1:10 000; B220, 1:50.

Secondary antibodies

Goat anti-rat, alkaline phosphatase-conjugated (Tago, Burlingame, CA), diluted 1:75, and donkey anti-goat, alkaline phosphatase-conjugated (Jackson Immunoresearch Laboratories, Westgrove, PA), diluted 1:75, were used.

Antibodies for macrophage/microglia detection

An antibody against F4/80, which detects an EGF–TM7 molecule on the surface of activated macrophages and microglia (McKnight *et al.*, 1996), was used at a dilution of 1:1000 (Lawson *et al.*, 1990). Neutrophils were recognized by their characteristic nuclear morphology in cresyl violet-stained sections (Dusart and Schwab, 1990).

Immunohistochemistry

Sections were fixed in acetone at room temperature for 10 min and air-dried. To minimize detachment of tissue sections, slides were stored overnight in sealed containers at –20°C and acetone fixation was repeated. After air-drying, primary antibodies were applied for 1 h and then rinsed with Tris-buffered saline, pH 7.4. Milk powder was added as a blocking agent to the goat anti-rat alkaline phosphatase-coupled secondary antibody, which was left on the sections for 40 min. Rinsing was repeated and a donkey anti-goat alkaline phosphatase-labelled antibody was applied for 30 min. After another rinsing, the sections were processed for the alkaline phosphatase reaction with Naphthol Phosphate (Sigma, N-2250) and New Fuchsin (Sigma, N-0638) in Tris-buffered saline (pH 9.0) for 15 min, again rinsed in Tris-buffered saline, pH 7.4, counterstained with Mayer's haemalaun solution for 2 min, rinsed, and then mounted in undiluted Kaiser's glycerol–gelatine (Merck 1.09242), which had previously been incubated at 60°C. Control sections were processed in the same manner but with the primary antibody omitted.

Analysis and quantification

Serial sections were viewed under a Zeiss Axiophot microscope and specifically labelled cells were counted. Cells were classified into two groups according to their location: (i) cells within the parenchyma (completed diapedesis); (ii) cells adhering to blood vessels in the luminal as well as the abluminal space (it was often not possible at the light microscopic level to reliably determine whether a single cell was associated with the luminal or abluminal side of an endothelial cell).

All the labelled cells over a 10 mm length of the spinal cord (5 mm rostral, 5 mm caudal to the lesion site) were counted on every fifth section of a complete series of sagittal sections. An estimate of the total number of cells was extrapolated for the whole thickness of the spinal cord. A minimum of five animals was analysed for each time point for the CU mice. The statistical significance of the results was evaluated by the unpaired Student's *t*-test.

Results

Immunohistochemical analysis of unlesioned control mice

Unlesioned control mice (6–8 weeks old) of both CU and SPF origin had no detectable lymphocytes in the parenchyma or adhering to blood vessel walls of the spinal cord. In the vicinity of the dorsal root entry zones, however, the meningeal vessels contained significant numbers of B-cells.

Time course of lymphocyte infiltration into the lesioned mouse spinal cord

Early phase following lesion: days 0–7

The earliest time point examined was 10 min after partial transection of the spinal cord. The lesion could be identified by the disruption of spinal cord tissue (both white and grey matter) and a variable degree of haemorrhage at the lesion site. Erythrocytes and leukocytes, including some lymphocytes, were present: the distribution of T- and

B-lymphocytes was identical to that found in peripheral blood of the same mouse strain (Table 1). No leukocyte margination was apparent at this time point. Changes in these values at later time points can, therefore, be interpreted as resulting from specific adhesion and active cell infiltration.

Three hours after the lesion, the margination of lymphocytes within dorsal and ventral blood vessels both rostral and caudal to the lesion was prominent in CU mice (Fig. 2), with particularly high numbers of B-cells (Fig. 2c). The number of lymphocytes was also increased in the area of the lesion and surrounding tissue in CU animals (Fig. 1), where B-cells reached peak levels (Fig. 1c, Fig. 2a). The number of lymphocytes in blood vessels was lower in SPF mice than in mice from conventional units (CU) (Fig. 2c, d). Marginated neutrophils were seen in the vessels in CU and SPF mice (data not shown).

Six hours after the lesion, fewer B-cells were seen in the parenchyma of CU animals than at 3 h (Fig. 2a). In SPF animals B-cell infiltration peaked at 6 h (Fig. 2c). Neutrophil granulocytes were observed in the parenchyma in CU and SPF mice (data not shown).

Twelve and 24 h after the lesion, B-cells remained the predominant population of lymphocytes (Fig. 2). Neutrophil numbers reached their maxima at these time points. These cells were found predominantly in the lesion site. They outnumbered the lymphocytes by a factor of ~10 (Table 2).

Four days after the lesion, the neutrophils had almost disappeared, and the presence of some pyknotic figures at day 2 suggests that their elimination could occur at least in part by apoptosis. In contrast, all three classes of lymphocytes (B-cells, $CD4^+$ and $CD8^+$ T-cells) were still present in appreciable numbers (Fig. 2, Fig. 3).

One week after the lesion, there was a decline in T- and B-cell numbers, and only very few lymphocytes were observed in the tissue (Fig. 2a, b), most of them being $CD4^+$ T-cells located in the immediate vicinity of the lesion site. Margination in blood vessels was minimal for all three subpopulations (Fig. 2c, d). Large numbers of $F4/80^+$ macrophages/microglia were present in the area of the lesion from day 4 onward and persisted in degenerating fibre tracts throughout the time of these experiments.

Late phase following lesion: 2–5 weeks

Marked differences in the presence of lymphocytes were observed at these late time points when comparing mice raised in CU with SPF mice. In SPF mice, the number of lymphocytes remained low (Figs 2b and 4c). In marked contrast, CU animals showed a strong increase in the number of infiltrating $CD8^+$ T-cells 2 weeks after the lesion (Fig. 2a). Clusters of cells appeared throughout the rostrocaudal portion of the region examined (Fig. 4b), but were most frequent close to the lesion (Fig. 4a). Although nerve fibre pathways are known to undergo Wallerian degeneration as a consequence of the initial lesion, $CD8^+$ T-cells were not primarily found in these regions; infiltrates appeared scattered across areas of both grey and white matter. Although less numerous, $CD4^+$ T-cells were found in the same clusters together with the $CD8^+$ T-cells. The ratio of $CD4^+$ T-cells to $CD8^+$ T-cells was ~1:6. The number of B-cell in the parenchyma remained low, but an increase in marginating B-cells was observed. (Fig. 2a, c).

The number of CD8+ T-cells present 3 weeks after the lesion remained highly elevated in CU animals, and further increased to almost 2-fold by 5 weeks (Fig. 2a).

Since there was a dramatic difference in the level of $CD8^+$ T-cells between lesioned animals from CU and SPF environments, the two colonies were submitted to microbial and viral screening. The animals from the CU were found to have elevated titres of antibodies against mouse hepatitis virus (MHV). No evidence of exposure to other common viral or bacterial pathogens was found.

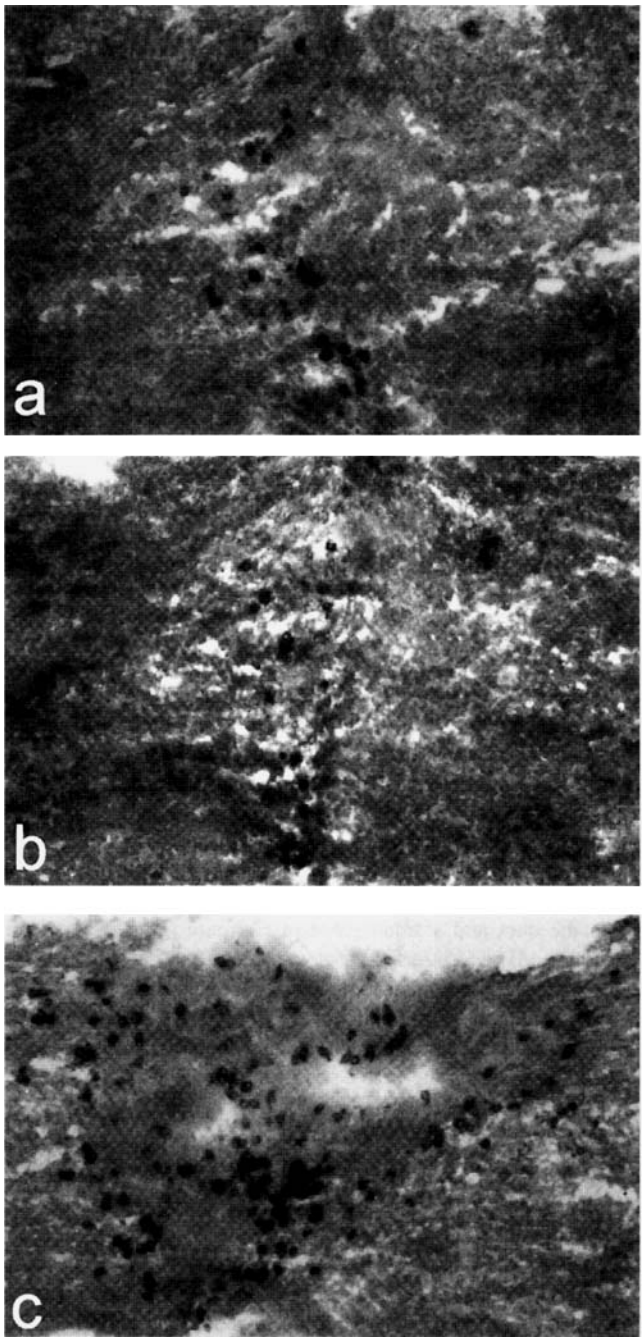

FIG. 1. Parasagittal, consecutive sections of a CU mouse spinal cord showing the lesion site 3 h after a mechanical lesion. (a) T-helper lymphocytes in the lesion site revealed by an antibody recognizing the CD4 receptor epitope. (b) Cytotoxic/suppressor T-lymphocytes demonstrated using CD8 receptor immunoreactivity. (c) B-lymphocytes stained using the monoclonal antibody B220. Although all major lymphocyte subpopulations can be demonstrated 3 h after the lesion, B-lymphocytes dominate the picture at this early time point in both CU and SPF mice. Magnification, 700×; section thickness, 20 μm.

In order to test the influence on lymphocyte recruitment of a prolonged interval between viral infection and experimental injury, two older CU animals (3 and 4 months of age, both asymptomatic and raised in the conventional unit of our institute) were analysed 2 weeks after spinal cord lesions. In these animals, the number of

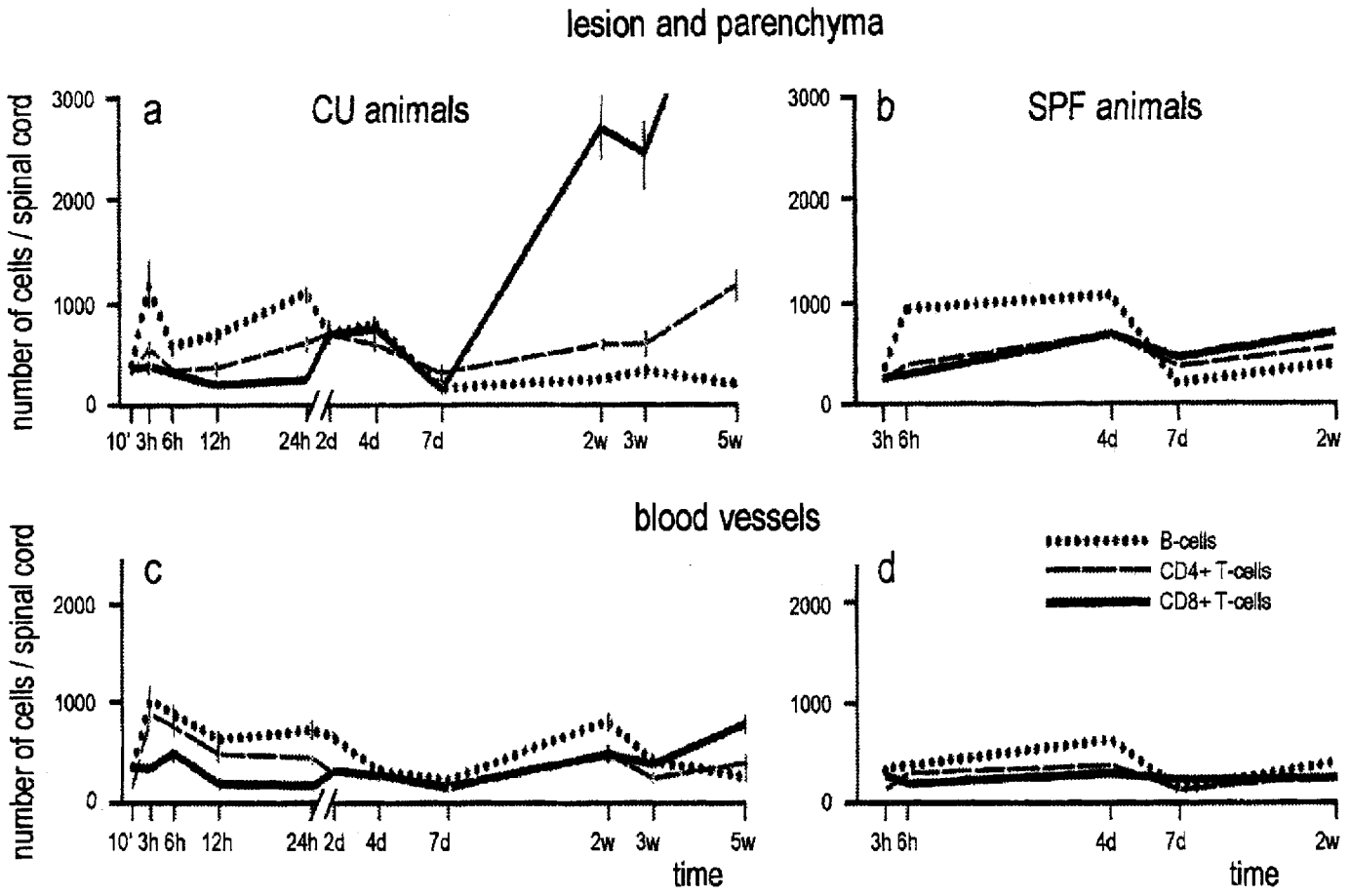

FIG. 2. Lymphocyte numbers as a function of time after spinal cord lesion. (a) CU animals: lesion and parenchyma. Note the large increase in numbers of $CD8^+$ T-lymphocytes at time points later than 2 weeks. (b) CU animals: marginating lymphocytes within and around blood vessels. (c) SPF animals: lesions and parenchyma. No late rise in $CD8^+$ cell count is seen. (d) SPF animals: lymphocytes associated with blood vessels.

TABLE 1. B- and T-lymphocyte distribution in peripheral blood of unlesioned CU mice and in the spinal cord of lesioned CU mice

		B-cells	T-cells
Peripheral blood (two animals, 200 cells each)		42.0%	58.0%
Spinal cord tissue	10 min after lesion	39.9%	60.1%
	3 h	87.3%	12.7%
	6 h	46.5%	53.5%
	12 h	54.8%	45.2%
	1 day	60.0%	40.0%
	2 days	42.8%	57.2%
	4 days	38.9%	61.1%
	1 week	25.7%	74.3%
	2 weeks	17.4%	82.6%
	3 weeks	11.4%	88.6%
	5 weeks	3.4%	96.6%

Values for the distribution of B- and T-lymphocytes in spinal cord tissue were obtained from the results in Figure 1.

TABLE 2. Neutrophil and lymphocyte recruitment into the lesion and surrounding tissue 24 h after spinal cord injury

Neutrophils (n = 5)	2694 ± 213
Lymphocytes (n = 4)	267 ± 19

Number of cells (mean ± SEM) in three 20 μm parasagittal sections including the centre of the lesion and 5 mm of rostral and caudal spinal cord. The data were obtained from CU animals.

cells was similar and the distribution of lymphocyte subpopulations exhibited the same pattern as seen at the younger ages (6–8 weeks) described above, where the time between viral exposure and lesion was only a few weeks.

Lymphocyte infiltration following spinal cord lesions in the rat

To compare the results obtained in mice with another species, similar lesions were performed in Lewis rats from the same CU facility. Lymphocytes, as identified by cresyl violet staining of spinal cord sections, were found adhering to blood vessels and in the proximate parenchyma as early as 3–6 h after the lesion (Fig. 5) and with distribution patterns similar to those observed in CU mice at later time points (results not shown).

Discussion

Partial transection lesions of the spinal cord in the mouse result in a leukocytic infiltrate which also includes the rapid recruitment of $CD4^+$, $CD8^+$ and B-lymphocytes to the cord parenchyma. Gradual

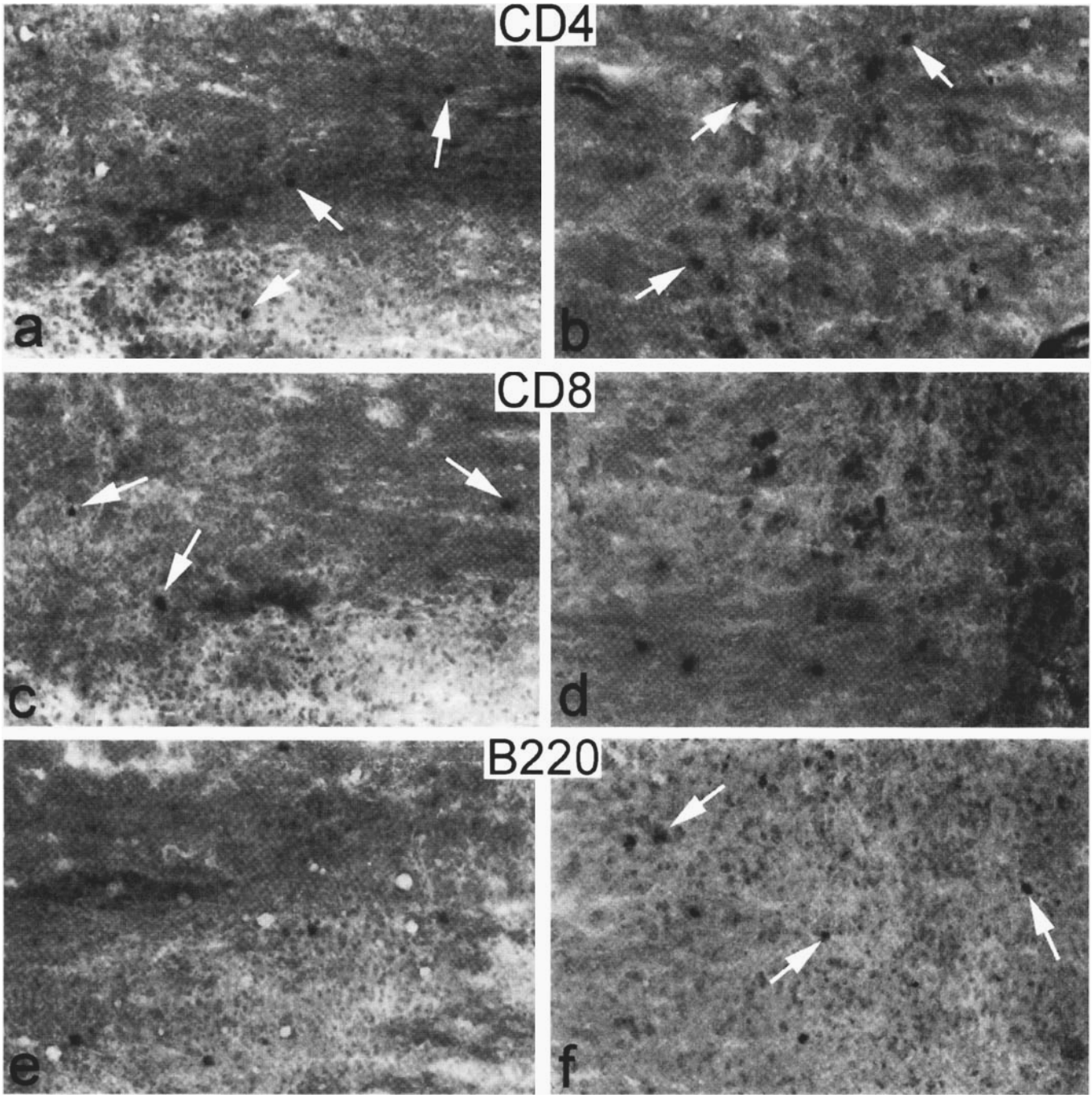

FIG. 3. Parasagittal sections through mouse spinal cords 4 days after lesion. Comparison of CU (a, c, e) and SPF (b, d, f) animals for the three subtypes of lymphocytes. Magnification, 700×; section thickness, 20 μm.

resolution of the lymphocyte infiltrate occurs within the first week. In mice raised in a conventional non-sterile animal unit (CU), but not in SPF mice, a second increase of predominantly $CD8^+$ T-lymphocytes throughout the spinal cord was observed at 2–5 weeks.

Leukocyte recruitment during the *early phase* resembles that seen following a lesion in non-nervous tissue such as skin; it is dominated by myelomonocytic cells. In addition to the well documented monocyte/macrophage/microglia and the neutrophil contribution, immunocytochemistry has allowed us to see that lymphocytes are also part of the population of inflammatory cells invading the lesion site. Interestingly, they are among the earliest cells to be recruited: B- and T-lymphocytes were seen within 3 h at the site of injury and were adhering to surrounding vessels. The response during the first day was dominated by B-lymphocytes, and the proportion of these cells was much larger than in the blood. Thus, selective recruitment of these cells appears to occur. The specific recruitment of leukocytes requires not only the expression of the adhesion molecules on the endothelium (for review see Springer, 1990), but also the presence of the appropriate chemokines to attract the cells to the site of injury (Schall *et al.*, 1993; Roth *et al.*, 1995; Berman *et al.*, 1996). Recent studies have shown that in acute neuronal degeneration and following challenge with inflammatory agents, CNS blood vessel endothelium expresses the

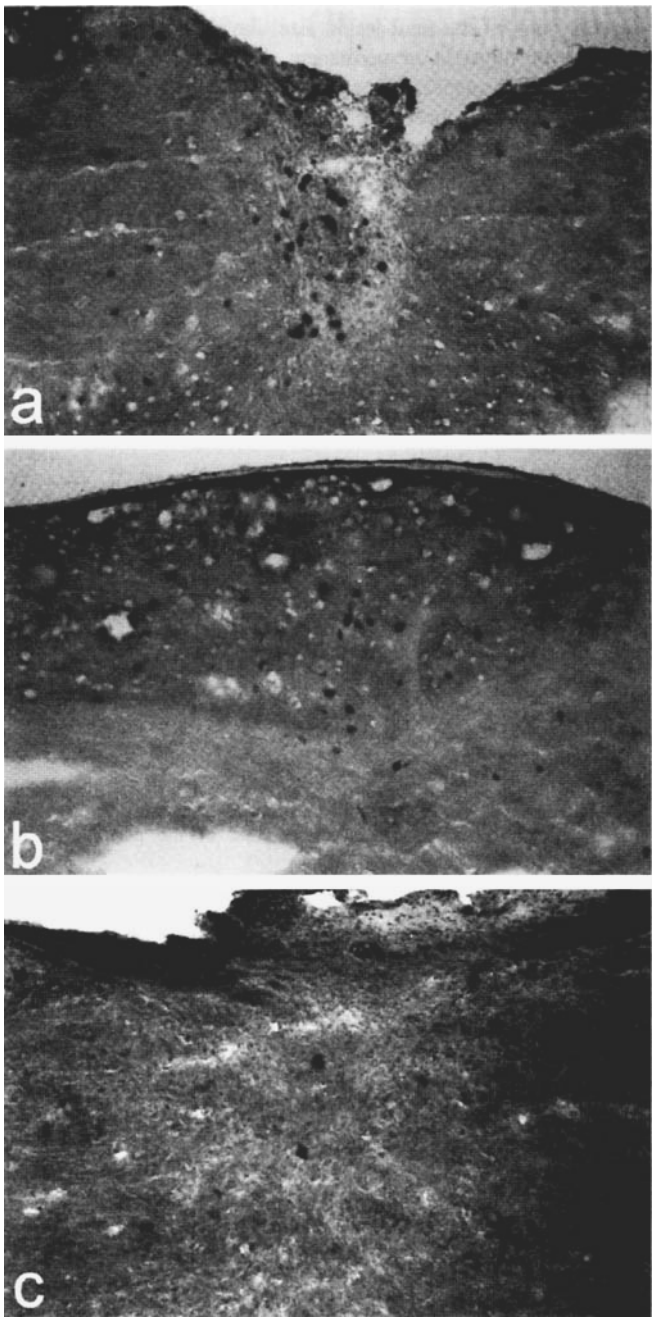

FIG. 4. Photomicrographs of parasagittal sections of mouse spinal cords 2 weeks after a lesion, showing $CD8^+$ cytotoxic T-cells. Cytotoxic lymphocytes in CU animals in the lesion site (a) and in clusters 3.6 mm rostral to the lesion (b). (c) Very few cytotoxic T-cells are found at the lesion site in SPF mice, and the widespread clustering throughout the parenchyma, seen in CU animals, is absent. Magnification, 700×; section thickness, 20 μm.

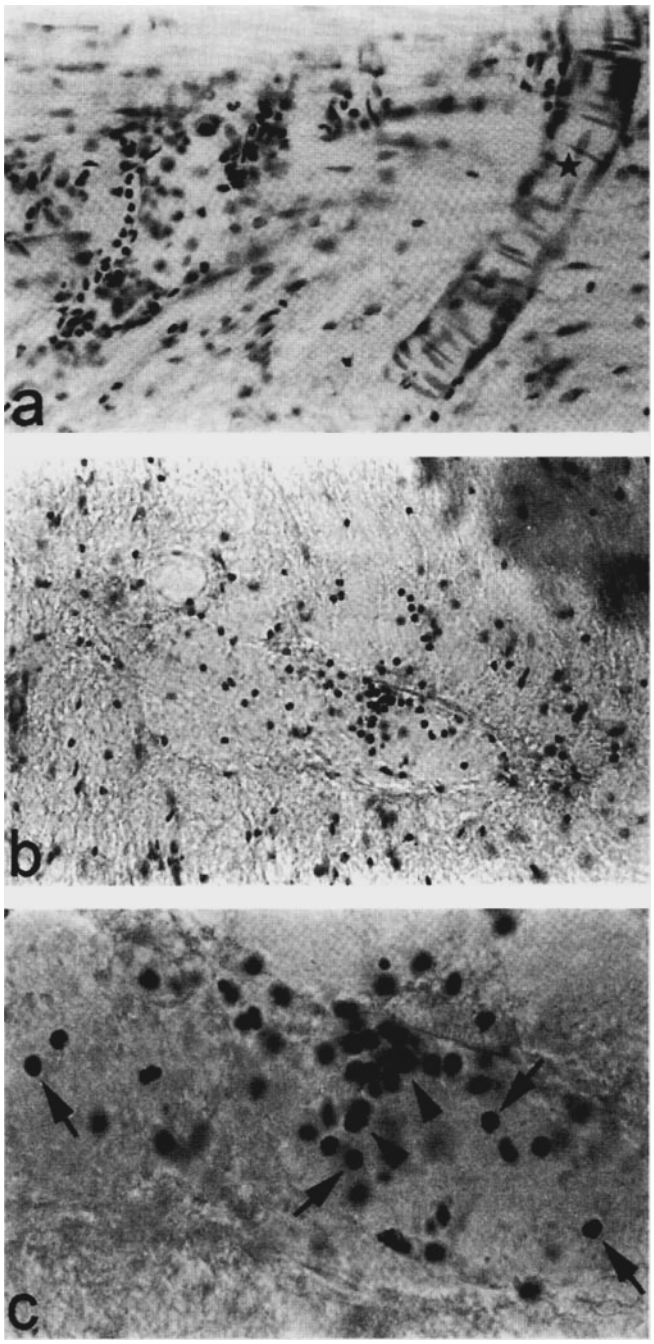

FIG. 5. Rat spinal cord 3 h after lesion. Photomicrographs of parasagittal sections, cresyl violet staining. (a) Ventromedial blood vessels 1 mm rostral to the lesion, illustrating recruitment of leukocytes predominantly in venules (note the staining of the smooth muscle cells of the arteriole (star). (b) Extravasation of mononuclear cells from a blood vessel close to the lesion. (c) Higher magnification, large mononuclear cells, probably monocytes, (arrowheads), and small mononuclear cells, probably lymphocytes (arrows), are seen adhering to the vascular endothelium and in the parenchyma. Magnification, 700× in a and b; 200× in c.

adhesion molecules necessary for leukocyte adhesion and diapedesis (Bell and Perry, 1995), and that macrophage chemotactic protein 1 is expressed in ischaemic brain tissue (Kim *et al.*, 1995). The synthesis of cytokines within the CNS parenchyma has not been well studied after traumatic injury. Up-regulation of interleukin-1 (IL-1)-like activity 3 days after brain injury has been shown (Nieto-Sampedro and Berman, 1987), but only recently has the expression of message for the proinflammatory cytokines IL-1, tumour necrosis factor and macrophage inflammatory protein-1 been detected at very early times after spinal cord lesions in the mouse (D. Bartholdi, submitted for publication).

T-lymphocytes also appeared in the lesion during the first week. During the first 24 h, the proportion of $CD8^+$ to $CD4^+$ cells in the tissue was comparable to their proportion in blood (twice as many $CD4^+$ cells as $CD8^+$ cells). Thus, the initial presence of these T-

cells might be largely caused by the injury-induced haemorrhage. At days 2 and 4, however, their relative proportions had changed and more $CD8^+$ cells were found in the tissue, again probably reflecting specific accumulation or recruitment of cells, possibly mediated by selective chemokine expression.

The pathophysiological role of the B- and T- lymphocytes in traumatic lesion sites is not known and it is only by their selective experimental depletion that this could be properly investigated in this model. Activated T-cells are known to secrete molecules such as interferon-γ, which may further activate the mononuclear phagocytes. T-cell-derived factors could also act at the level of glial cells (Eddleston and Mucke, 1993) or neurons (Erkman *et al.*, 1988; Neumann *et al.*, 1995). In addition to a possible influence on the recruitment and actions of inflammatory cell types, T-lymphocytes could therefore influence scar formation or the synthesis of trophic factors (Lindholm *et al.*, 1988).

In terms of numbers, lymphocytes represented only ~10% of neutrophils recruited after 24 h and an even smaller percentage of the macrophage number at later time points. However, they seemed to be amongst the first cells recruited and, therefore, could maintain a key position in the cascade of cell recruitment.

A *late-phase* T-lymphocyte response was observed exclusively in mice which had been raised in the conventional, non-sterile unit (CU). In these animals the lesion of the spinal cord led to an accumulation of T-cells at 2–5 weeks in the parenchyma several millimetres rostral and caudal of the lesion site. The clusters of lymphocytes consisted of $CD8^+$ T-cells and $CD4^+$ T-cells at a ratio of 6:1, which strongly supports a specific infiltration process. This late-phase lymphocyte response was first observed 2 weeks after lesioning and persisted up to 5 weeks, the latest time point investigated. The method of tissue preparation employed did not allow us to quantify the dimension of the secondary lesion and to analyse possible differences between SPF and CU animals.

Since it is well known that $CD8^+$ lymphocytes are involved in the elimination of virus-infected cells, we examined the SPF and the CU animals for evidence of infection with different viruses. The serum of CU animals was found to contain antibodies against MHV, a coronavirus which can lead to subclinical infection at 3–4 weeks of age. Being highly infectious, this virus is commonly found in conventional breeding facilities and often escapes detection (Homberger and Thomann, 1994). In rats, some strains of coronavirus have been shown to invade the CNS and cause transient demyelination (Nagashima *et al.*, 1979; Wedge *et al.*, 1984).

It is important to appreciate that the late-phase recruitment of T-cells in the cord required a combination of both a spinal cord lesion and prior exposure to virus: neither alone was sufficient.

Two hypotheses can be formulated for the late-phase recruitment of T-lymphocytes. (i) Viruses, e.g. MHV virus, could be sequestered in a latent form in the CNS parenchyma (Adami *et al.*, 1995) and be activated by the lesion. Activated T-cells patrolling the CNS could detect virus-infected cells and react to them (Hickey *et al.*, 1991; Wekerle *et al.*, 1991). (ii) Viral immune determinants could be shared by molecules of the CNS (Steinman, 1995; Wucherpfennig and Strominger, 1995). Exposure of these CNS peptides by the CNS lesion to cells of the immune system could specifically activate these cells which, in turn, detect and react to these determinants within the CNS (Lorentzen *et al.*, 1995). In either case, it is of interest to consider that in humans the reaction of the immune system following an injury of the CNS tissue could also be influenced by prior or ongoing subclinical or latent virus infections.

The contribution of lymphocytes to the processes of secondary injury following mechanical lesions of the CNS needs to be elucidated. A careful study of the final lesion size, the scar formation and the occurrence of transient or permanent demyelination at later time points in immune suppressed or CD4- or CD8-knockout mice could help to answer these questions.

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Abbreviations

CNS	central nervous system
CU	(animals raised in) conventional breeding unit
MHV	mouse hepatitis virus
SPF	specific pathogen-free

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