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Journal of Neuroimmunology 89 (1998) 88–96

Journal of Neuroimmunology

Accumulation of passively transferred primed T cells independently of their antigen specificity following central nervous system trauma

David L. Hirschberg [a,1,2], Gila Moalem [a,b,1], Jun He [a,b], Felix Mor [b], Irun R. Cohen [b], Michal Schwartz [a,*]

[a] *Department of Neurobiology, The Weizmann Institute of Science, 76100 Rehovot, Israel*
[b] *Department of Immunology, The Weizmann Institute of Science, 76100 Rehovot, Israel*

Received 10 February 1998; revised 16 April 1998; accepted 16 April 1998

Abstract

The central nervous system (CNS) enjoys a unique relationship with the immune system. Under non-pathological conditions, T cells move through the CNS but do not accumulate there. CNS trauma has been shown to trigger a response to CNS self-antigens such as myelin basic protein (MBP). Here, we examined whether the injured CNS tissue undergoes changes that permit T cell accumulation. We found that injury to CNS white matter, such as the optic nerve, led to a transiently increased accumulation of T cells (between days 3 and 21). In Lewis rats with unilaterally injured optic nerves, systemic administration of passively transferred T cells recognizing either self-antigen (MBP) or non-self-antigen (ovalbumin) resulted in accumulation of the T cells in injured optic nerve, irrespective of their antigenic specificity. The effect of the T cells on the damaged nerve, the lack of selectivity in T cell accumulation and the mechanism underlying non-selective accumulation are discussed.

Keywords: T cell lines; CNS–immune interactions; Nerve injury; Multiple sclerosis; Experimental allergic encephalomyelitis

1. Introduction

The failure of mammalian central nervous system (CNS) tissue to repair itself has been attributed to its special relationship with the immune system. The CNS is considered to be an immunologically privileged site, in contrast to the peripheral nervous system (PNS). The presence of the blood–brain barrier (BBB) (Reese and Karnovsky, 1967) and the lack of lymphatic drainage (Barker and Billingham, 1977) have been suggested to account for immunological isolation of the CNS. Isolation of the CNS from a normal inflammatory response can also be explained by the presence of substances in the brain that downregulate macrophage function (Thanos et al., 1993; Hirschberg and Schwartz, 1995). This immune isolation might have arisen to protect the CNS from the large-scale damage that could result from an inflammatory response (Streilein, 1995).

Isolation from the immune system may not be the best way to describe immune function in the CNS. Previous studies have shown that leukocytes routinely cross the BBB into the CNS even if there is no observable pathology (Wekerle et al., 1986; Zeine and Owens, 1992; Wekerle, 1993b; Fabry et al., 1994). The T cells that do not cause disease also cross into the CNS, provided that they are activated (Wekerle et al., 1987; Owens et al., 1994). Of the lymphocytes that enter the CNS, it has been suggested that only those that are activated and that recognize CNS antigens can, under special conditions, cause a local autoimmune disease (Zeine and Owens, 1992).

Recently there has been growing interest in the T cell response to traumatic injury in the CNS. It has been demonstrated that CNS trauma, such as spinal injury, triggers a systemic response to self-epitopes such as myelin basic protein (MBP) (Popovich et al., 1996).

In this study, we examined whether damage in the CNS alters the accessibility to, and accumulation of, T cells. We

* Corresponding author. Tel.: +972 8 9342467; fax: +972 8 9344131; e-mail: bnschwartz@wiccmail.weizmann.ac.il

[1] The first two authors contributed equally to this manuscript.

[2] Current address: Department of Neurology and Neurological Sciences, Stanford University, Beckman Center B002, Palo Alto, CA 94305-5429; e-mail: hirschberg@stanford.edu

0165-5728/98/$19.00

found that endogenous T cells, as well as passively transferred T cells, accumulate in injured CNS tissue irrespective of the antigenic specificity of the injected activated T cells. The passively transferred T cells used in the present study did not accumulate either in injured PNS or in injured non-neural tissues such as muscle and skin.

2. Materials and methods

2.1. Animals

Female Lewis rats were supplied by Harlan Olac (Bicester, UK), matched for age (8–12 weeks) and housed four to a cage in a light- and temperature-controlled room.

2.2. Proteins used for T cell stimulation

Myelin basic protein (MBP) was prepared from guinea pig spinal cord as previously described (Ben-Nun and Cohen, 1982). Chick ovalbumin (OVA) was purchased from Sigma (Israel). Heat-inactivated *Mycobacterium tuberculosis* H37RA (*M. tuberculosis*) and incomplete Freund's adjuvant (IFA) were purchased from Difco laboratories (Detroit, MI, USA).

2.3. Media

Proliferation medium contained the following: Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Israel) supplemented with 2 mM L-glutamine (L-Glu, Sigma, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma), penicillin (100 IU/ml; Biological Industries), streptomycin (100 μg/ml; Biological Industries), sodium pyruvate (1 mM; Biological Industries), non-essential amino acids (1 ml/100 ml; Biological Industries) and autologous rat serum 1% (v/v) (Mor et al., 1990). Propagation medium contained DMEM, 2-ME, L-Glu, sodium pyruvate, non-essential amino acids and antibiotics in the same concentration as above and also 10% fetal calf serum (FCS), and 10% T cell growth factor (TCGF) obtained from the supernatant of concanavalin A-stimulated spleen cells (Mor et al., 1990).

2.4. Establishment of T cell lines with active EAE induction

The MBP or OVA were dissolved in PBS (1 mg/ml) and emulsified with an equal volume of IFA supplemented with 4 mg/ml *M. tuberculosis*. Rats were immunized subcutaneously in the hind footpads with 0.1 ml of the emulsion. At day 9 (1–3 days before clinical onset of disease), animals were euthanized and draining lymph nodes were surgically removed and dissociated under sterile conditions. The cells were washed and placed in proliferation medium with irradiated thymocytes (2000 rds) and 10 μg/ml either of MBP or OVA for 3 days. Cells were then washed and placed in propagation medium for 5 to 10 days and were then restimulated with irradiated thymocytes and peptides in proliferation medium. The T cell lines were expanded by stimulation and propagation and tested for specificity in an antigen-specific T cell proliferation assay. Lines were expanded and stocks were frozen in liquid nitrogen. For experiments, the cells were thawed and stimulated once before being used.

2.5. Passive transfer of labelled and non-labelled T cell lines

The T cell lines were activated by restimulation in vitro with their own antigen (10 μg/ml) in proliferation medium, as above. After incubation for 48–72 h at 37°C, 90% relative humidity and 7.5% CO_2, the cells were washed and viable cells were isolated on Percoll, washed and suspended in 10^7 μM Hoechst 33342 stain (Molecular Probes, USA) for 10 min at 37°C. The cells were washed twice with 50 ml volumes of phosphate-buffered saline (PBS) and then resuspended at 10×10^6 cells/ml on ice until used. Animals were injected intraperitoneal (i.p.) with 1 ml of the T cell suspension. For transfer of non-labelled T cells, T cell lines were activated as above, washed and suspended in PBS. Animals were injected with 10×10^6 cells/ml i.p. and control rats were injected with 1 ml PBS, i.p.

2.6. Crush injury of rat optic nerve

Crush injuries were performed as previously described (Hirschberg et al., 1994). Briefly, rats were deeply anaesthetized by i.p. injection of xylazine (10 mg/kg; Rompun) and ketamine (50 mg/kg; Vetalar). Under a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. A moderate crush injury was inflicted on the optic nerve, 2 mm from the eye, using calibrated cross-action forceps (Duvdevani et al., 1990). The contralateral nerve was left undisturbed and was used as a control.

2.7. Sectioning of nerves

At specified time points, rats were euthanized by over-anaesthesia with ether and their optic nerves were surgically removed, immersed in Tissue-Tek (Miles, USA), and frozen in liquid nitrogen cooled in isopentane (BDH, UK). The nerves were then transferred to dry ice and stored at −70°C until sectioning. Longitudinal cryostat nerve sections (20 μm thick) were picked up onto gelatin-coated

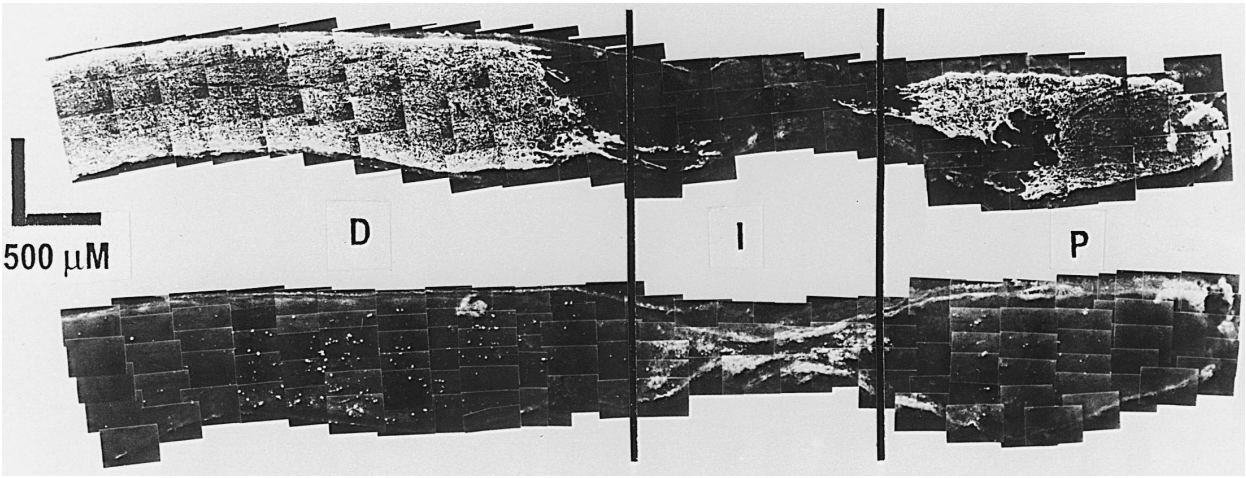

Fig. 1. Passively transferred T_{MBP} cells selectively accumulate in injured optic nerve. The figure is a montage of epifluorescence micrographs of an optic nerve section excised 14 days after a controlled crush injury. At the time of the crush, animals were injected i.p. with 10×10^6 primed T_{MBP} cells prelabelled with Hoechst stain. One section was stained with GFAP to visualize the injury site (lack of GFAP-positive astrocytes) and photographed using filters that detect rhodamine, and the other was photographed using filters that detect Hoechst stain to visualize the labelled cells (which appear as white dots). The largest concentration of cells was in the injury site and just distal to the injury (P: proximal; I: site of injury; D: distal).

glass slides (four sections per slide) and frozen at −20°C until viewed or prepared for fluorescence staining.

2.8. Data analysis of T cells in nerve sections

Nerves excised at various time periods after injury were prepared and sectioned. Hoechst-labelled nuclei or immunostained cells in each section were counted using the fluorescence microscope. For each time point, four to six sections were counted and the numbers were averaged.

2.9. Immunolabelling of nerve sections

Sections were thawed and fixed in ethanol for 10 min at room temperature, washed twice with double-distilled water (ddH_2O), and incubated for 3 min in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20; Sigma, USA). They were then incubated overnight at 4°C with a mouse monoclonal antibody directed against rat macrophages (ED1; 1:400; Serotec, UK) and antibody against rat glial fibrillary acidic protein (GFAP; 1:100; BioMakor), all diluted in PBS containing 3% FCS. Staining of T cells was accomplished by incubating nerve sections for 1 h at room temperature with a mouse monoclonal antibody directed against rat T cell receptor (TCR) (1:100; Hunig et al., 1989), in PBS containing 3% FCS and 2% BSA. After three washes with PBS containing 0.05% Tween-20, the sections were incubated with goat anti-mouse $F(ab')_2$ conjugated to either fluorescein isothiocyanate (FITC; BioMakor) or tetramethyl rhodamine iso-

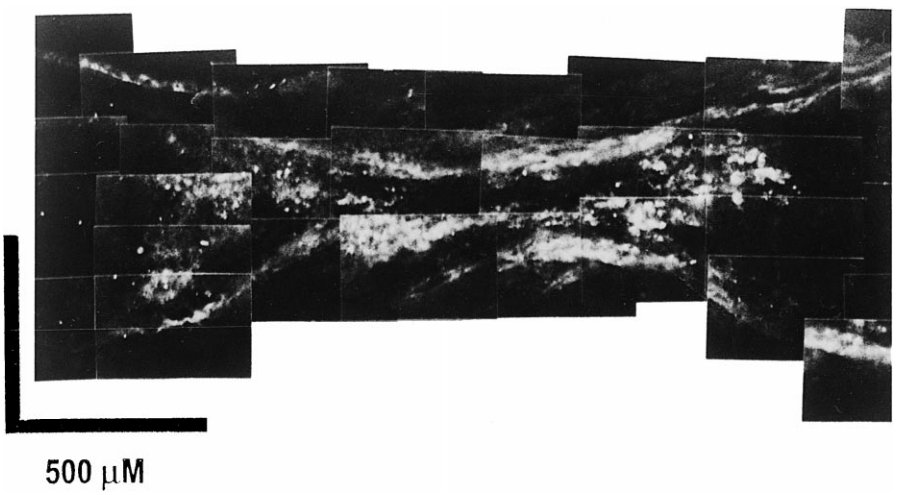

Fig. 2. Higher magnification of the optic nerve injury site shown in Fig. 1, showing the large concentrations of injected cells localized at the site of injury.

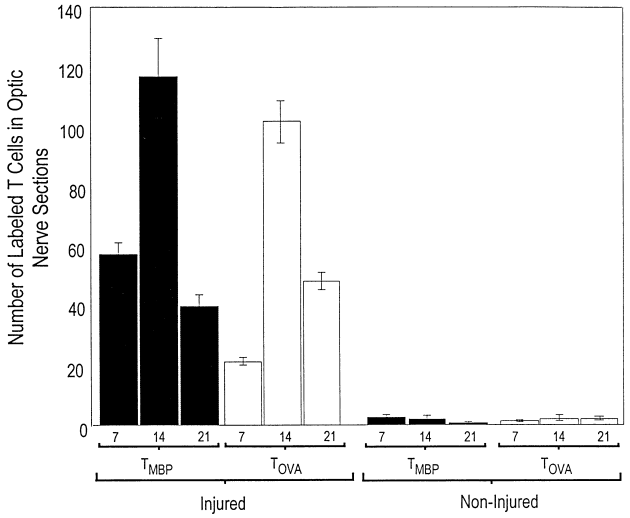

Fig. 3. Accumulation of primed T cells in the site of injury is not dependent on the antigen that primed them. Hoechst-prelabelled T_{MBP} and T_{OVA} cells were injected into groups of animals at the time of optic nerve crush. Ipsilateral (injured) and contralateral (non-injured) nerves were removed and prepared for microscopy 3, 7, 14 or 21 days later. The bar graph shows the number of labelled cells counted in nerve sections ($n = 5$).

thiocyanate (TRITC; BioMakor) at a dilution of 1:100 and 1:50, respectively, for 1 h at room temperature. They were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane (Sigma), to inhibit quenching of fluorescence. The sections were viewed with a Zeiss Universal fluorescence microscope using filters that detect TRITC, FITC and Hoechst stains (Blaugrund et al., 1992a,b).

2.10. Analysis of the BBB status

Crush injury of the optic nerve was performed. The contralateral nerve was left undisturbed and was used as a control in the experiments. Immediately after the injury or 3, 7 and 14 days later, the rats were injected intravenously (i.v.) with 3 ml/kg of 2% Evans blue dye (EBD) in PBS. The solution was allowed to circulate for 30 min and then the rats were euthanized and the nerves were removed. Frozen cryostat nerve sections were collected (20 μm thick) and analyzed microscopically for EBD fluorescence.

3. Results

3.1. Accumulation of passively transferred T cells in injured CNS nerves

We used the optic nerve as a model of CNS white matter. In order to find out whether axonal injury in the CNS is accompanied by any change in the tissue susceptibility to T cell accumulation, we compared the accumulation of passively transferred T cells in injured and non-injured optic nerves. We first used activated T cell lines that recognize MBP, a CNS antigen (Ben-Nun and Cohen, 1982). These MBP-specific T cells (T_{MBP}) are known to cause experimental allergic encephalomyelitis (EAE) (Ben-Nun and Cohen, 1982). The T_{MBP} cells were stimulated with MBP for 2–3 days, labelled with Hoechst stain, and then injected i.p. into rats within 5 min after the optic nerve was subjected to crush injury (Duvdevani et al., 1990; Yoles et al., 1997). The nerves were excised, cryosectioned and analyzed microscopically for the presence of labelled T cells 3, 7, 14, or 21 days later. The T_{MBP} cells were detected in the injured optic nerves on day 3 and their number increased until they reached a peak on day 14. Fig. 1 shows the accumulated labelled T cells in an

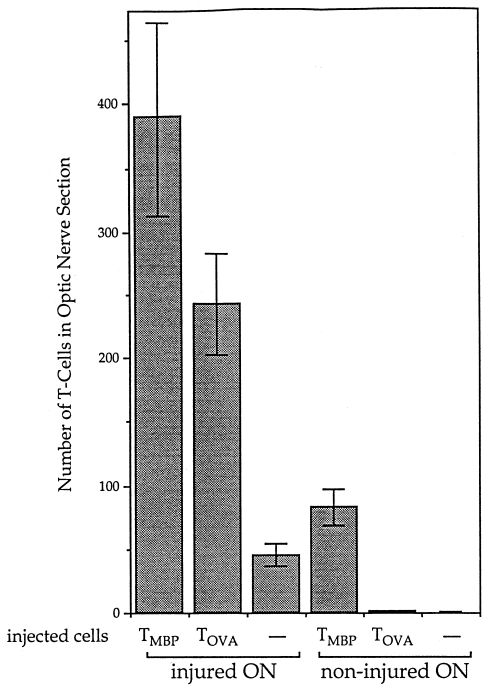

Fig. 4. Accumulation of passively transferred and endogenous T cells in injured optic nerve (ON). The T_{MBP}, T_{OVA} cells or PBS were injected i.p. into adult Lewis rats immediately after unilateral crush injury of the optic nerve. Seven days after injury, both optic nerves were excised, cryosectioned and immunostained with monoclonal antibody to TCR. Note the striking increase in T cell accumulation in injured nerves of rats injected with either anti-MBP or anti-OVA T cells. The bar graph shows the mean number of total T cells $\pm$ SE counted in three sections of each nerve. Each group contained three to four rats. Statistical analysis (ANOVA) revealed significant differences in T cell numbers in (a) injured optic nerve of rats injected with anti-MBP T cells vs. injured optic nerve of rats injected with PBS ($p < 0.001$); (b) injured optic nerve of rats injected with anti-OVA T cells vs. injured optic nerve of rats injected with PBS ($p < 0.05$); (c) injured optic nerve vs. uninjured optic nerve of rats injected with anti-MBP T cells ($p < 0.001$); (d) injured optic nerve vs. uninjured optic nerve of rats injected with anti-OVA T cells ($p < 0.01$); and (e) injured optic nerve vs. uninjured optic nerve of rats injected with PBS ($p < 0.05$). No significant differences in T cell numbers were observed in injured optic nerve of rats injected with anti-MBP T cells vs. injured optic nerve of rats injected with anti-OVA T cells ($p > 0.05$).

injured optic nerve 14 days after injury. Large clusters of T_{MBP} cells were observed at the injury site; individual cells were seen proximal and distal to it (Fig. 2). No T cells were seen in the non-injured contralateral optic nerves.

The finding that T_{MBP} cells accumulated in the injured optic nerve, but not in the non-injured nerve, suggested that the injured nerve might either provide signals that attract and bind the cells or have lost the signal that allows repulsion/elimination of these cells. Are these signals dependent on the recognition of MBP? To determine whether optic nerve injury allows non-discriminative accumulation of activated T cells or only accumulation of T cells specific to a CNS antigen, the experiments were repeated using a T cell line sensitized to OVA, an antigen not present in the rat CNS. The T cells sensitized to OVA (T_{OVA}) were also found to accumulate in the injured but not in the non-injured optic nerve, and their pattern of accumulation in the injured nerve was similar to that of the T_{MBP} cells. Examination of the labelled cells in longitudinal sections of optic nerve excised 3, 7, 14 and 21 days after injury revealed that T_{MBP} cells were detectable 2 days earlier than T_{OVA} cells, but no significant differences were observed in the total numbers of T_{MBP} and T_{OVA} cells that accumulated (Fig. 3). Assessment of the two cell lines by a T cell proliferation assay using thymidine uptake experiments confirmed the absence of antigenic cross-reactivity. These results suggested that antigen specificity does not play a role in the homing and accumulation of T cells in the injured optic nerve. Resting T cells did not accumulate in the injured optic nerve. No labelled T cells were found in crushed sciatic nerve or in muscle or skin tissue that was examined at the same time points post-injury (data not shown), indicating that accumulation of activated T cells with no antigenic specificity appears to be unique to injured CNS.

3.2. Accumulation of endogenous and passively transferred T cells

To exclude the possibility that the accumulation of activated labelled T cells in injured CNS and the apparent lack of antigenic selectivity might have been caused by the accumulation of phagocytic cells that had phagocytized the labelled T cells, we repeated the experiment, but instead of counting the Hoechst-labelled cells, we assessed the number of T cells using an antibody that recognize T cells only. Seven days after injury, a significant number of T cells was detected at the site of injury using the anti-TCR antibody. No TCR-immunoreactive cells were detected in the non-injured optic nerves of PBS-injected rats (Fig. 4). We also examined accumulation of T cells in the non-injured nerves following injection of T cells specific to either MBP or OVA. In non-injured nerve, injection of T_{MBP} cells into rats resulted in accumulation of relatively small numbers of T cells, whereas no T cells were detected following injection of T_{OVA} cells. Thus, there were differences in the extent of T cell accumulation in the non-injured nerves following injection of non-CNS-relevant T cells (T_{OVA}) and CNS-specific T cells (T_{MBP}) into rats (Fig. 4). In contrast, in injured nerves of rats injected with T cells specific to either MBP or OVA, large numbers of T cells were found in the site of the injury with no significant differences between the two T cell lines. Fig. 5 shows an example of accumulated T cells in cryosections of a

Fig. 5. Photomicrographs of injured rat optic nerve stained for T cell receptor and GFAP. The T_{MBP} cells (10×10^6) were injected i.p. at the time of the injury. Seven days later, the nerves were excised, cryosectioned and processed for immunocytochemistry. Note that the density of T cells is highest at the site of the lesion, as delineated by the GFAP staining.

Fig. 6. Kinetics of BBB resealing as a function of the time after injury. Unilateral crush injury was inflicted on the optic nerve and EBD was injected i.v. immediately or 3, 7 or 14 days after the injury (D0, D3, D7, D14, respectively). At each time point, the nerves were excised 30 min after the dye injection, cryosectioned and analyzed. The photomicrographs show that on day 14, the nerve was almost back to normal (N).

nerve 7 days after lesion and T cell injection. The site of injury was delineated by GFAP immunoreactivity. These results confirmed that CNS white matter lesion is followed by accumulation of activated T cells, and that selectivity is indeed lost (i.e., no antigenic discrimination after injury). In all cases, however, accumulation was transient.

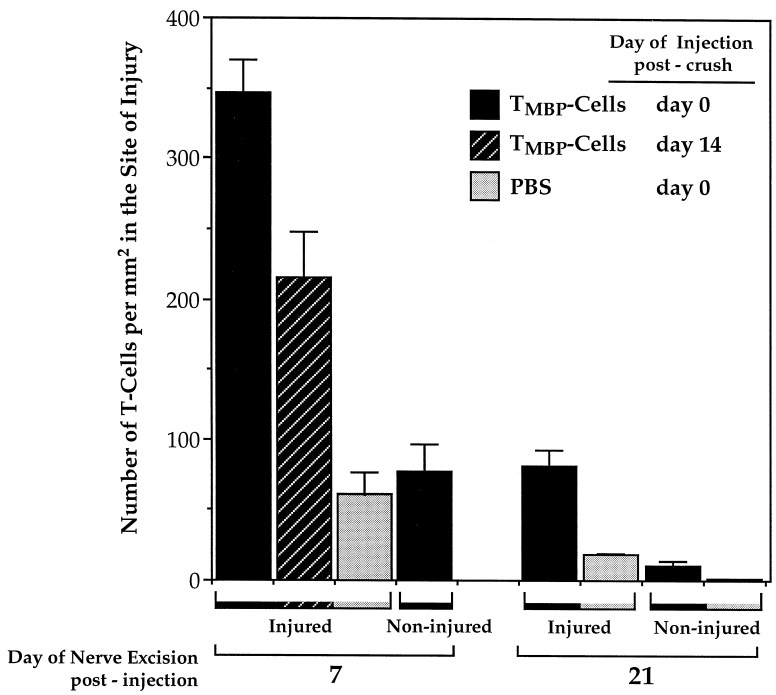

Fig. 7. Relationship between BBB breakdown and T cell accumulation. The bar graph shows the mean number of T cells ± SE counted in three sections of each nerve. Each group contained three rats. Statistical analysis revealed a significant increase in the number of accumulated T cells following optic nerve injury, relative to non-injured nerve, when T cells were injected immediately after the injury or 2 weeks after injury, both analyzed a week later ($p < 0.001$; ANOVA). No significant difference was observed between the two injured groups (i.e., the group injected immediately after injury and the group injected 2 weeks after injury) when each was analyzed 1 week after T cell injection.

3.3. Accumulation of T cells is not directly related to the breakdown of the blood–brain barrier

The accumulation of T cells can be explained either by a general increase in the permeability of the nerve to T cells (e.g., due to breakdown of the BBB) and/or by the changes in the characteristics of the nerve as a result of the injury. To examine whether the accumulation of T cells reflected a breakdown of the BBB, we examined the kinetics of BBB resealing after crush injury of the optic nerve. Examination of the penetration of intravenously injected EBD showed that the BBB was almost completely resealed within 2 weeks after injury (Fig. 6). We therefore carried out an experiment to compare T cell accumulation in rats injected with T_{MBP} cells immediately after injury with that of rats injected with T_{MBP} cells 2 weeks later, when significant recovery of the BBB is detected. The T cell accumulation, analyzed a week after the T cell injections, was found to be similar in both cases. Accumulation of T cells injected immediately after injury and analyzed 3 weeks later was lower than when the T cells were injected 2 weeks after injury and analyzed a week later (Fig. 7). It thus appears that the accumulation of T cells was not a reflection of BBB permeability but more likely of changes in the characteristics of the nerve as a result of the injury (Fig. 7).

4. Discussion

This study demonstrates that the injured CNS, unlike the non-injured CNS, appears to enjoy a special relationship with activated T cells, characterized by an absence of specificity restrictions with regard to accumulation of T cells to injured tissue. It has already been established that antigen-stimulated T cells patrol the intact CNS but do not accumulate there under non-pathological conditions (Wekerle, 1993a). Our findings suggest that injury to the CNS causes striking changes both in the extent and in the antigenic selectivity of the accumulated T cells. In non-injured nerves, T cell levels were low and the numbers of T cells following T_{MBP} (self-antigenic) cell injection were significantly higher than the numbers of T cells following T_{OVA} (non-self-antigenic) cell injection. However, after injury, selectivity was apparently lost and the extent of accumulation increased. It seems reasonable to assume that cytokines, growth factors and surface adhesion molecules undergo changes (up- or down-regulation) in CNS tissue after injury, resulting in accumulation of T cells. We have yet to determine whether the accumulating T cells represent a specific T cell subpopulation.

The T cell accumulation was measured by two independent techniques, one that measures only exogenously injected T cells, making use of their prelabelling, and one that involves detection by T cell-specific antibody, and therefore measures both endogenous and exogenous T cells. While the data observed by the two techniques are complementary, the absolute numbers and distribution are not identical. More T cells were detected by the TCR immunostaining technique than by prelabelling with Hoechst, and the TCR staining distribution was less restricted than the Hoechst prelabelled T cells to the injury site. These differences in the detected numbers of T cells and their distribution suggest that the injected T cells caused recruitment of additional T cells from the periphery to increase the local immune response. These endogenously recruited T cells would be detected only by the immunostaining technique.

The T cell accumulation in injured nerves was more pronounced than in non-injured nerves following injection of activated T cells, suggesting that a major part of the increased accumulation of the T cells was due to injury-induced changes in the nerve. That increased T cell accumulation was observed even when activated T cells were injected after the BBB had almost recovered (at least with respect to EBD permeability, an accepted marker for BBB permeability) further suggests that the increase is not merely due to injury-induced breakdown of the BBB. However, selective local post-injury changes in the BBB permeability to T cells cannot yet be excluded. The fact that T cell accumulation was transient may be related to immune privilege mechanisms in the CNS such as immunosuppression, involving inhibition of T cell adhesion in vitro (Hirschberg and Schwartz, 1995) and of T cell elimination by apoptosis. In fact, apoptosis of T cells was demonstrated in other immune-privileged sites such as the testis (although not yet in the brain), where any invading T cells are killed by apoptosis mediated by Fas and Fas ligand expressed by the privileged site (Griffith et al., 1995).

A major question emerges as to whether the accumulation of T cells has a negative effect such as induction of autoimmune disease (Mor et al., 1990; Steinman, 1992) or represents a physiological beneficial response. If the former, then the increase in transient accumulation of T cells at the injury site could be a default resulting from a local and transient loss of mechanisms of inhibition and/or exclusion of the primed T cells, presumably active in the intact and healthy CNS. If the latter, this would suggest that the system also undergoes physiological changes to allow accumulation of T cells which might contribute to the post-injury homeostasis of the nerve tissue. Such a beneficial effect may operate via production of neurotrophic factors by the accumulated T cells (Ehrhard et al., 1993). It is interesting to note that with respect to regeneration, T cells might be beneficial for removal of oligodendrocytes and hence of myelin-associated growth inhibitors (Schwab and Caroni, 1988). This is supported by the fact that oligodendrocytes were recently shown to be highly susceptible to attack by T cells (Scolding et al., 1990). It was further shown that CD4 + cells can induce lysis of oligodendrocytes (Antel et al., 1994), and that

anti-T cell monoclonal antibodies directed against CD4 or CD8 can promote remyelination (Rodriguez and Lindsley, 1992). Moreover, the accumulated T cells might be a source of IL-2 which, when dimerized, is cytotoxic to oligodendrocytes (Eitan et al., 1994; Eitan and Schwartz, 1993).

The accumulation of T cells irrespective of antigenic selectivity at the lesion site may be related to the unusual macrophage response to injury in CNS tissue as compared to PNS tissue. Following axonal injury in the PNS, macrophages promptly invade the injured site and the area distal to it, whereas macrophage invasion into the injured CNS is slow and for the most part confined to the site of injury (Perry et al., 1987; Brown et al., 1991; Hirschberg et al., 1994; Schwartz et al., 1994).

The accumulation of T cells following injury, in the absence of exogenous administration of activated T cells, might be a reflection not only of the changes in the dialogue with the T cells, but also of a systemic immune response against self-components exposed by the injury. This would seem to be in line with the reported injury-induced stimulation of a systemic response to MBP and hence the presence of MBP-primed T cells in animals with spinal cord lesions (Popovich et al., 1996). This, however, would not explain the homing of T cells specific for non-self antigens.

Acknowledgements

This study was supported in part by the Alan T. Brown Foundation of Nerve Paralysis. M. Schwartz is the incumbent of the Maurice and Ilse Katz Professorial Chair in Neuroimmunology. D. Hirschberg is supported by a postdoctoral fellowship from the National Multiple Sclerosis Society.

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