EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN THE ABSENCE OF A CLASSICAL DELAYED-TYPE HYPERSENSITIVITY REACTION

Severe Paralytic Disease Correlates with the Presence of Interleukin 2 Receptor–positive Cells Infiltrating the Central Nervous System

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Experimental allergic encephalomyelitis (EAE),[1] is an autoimmune paralytic and inflammatory disease of the central nervous system (CNS) induced by the injection, in CFA, of heterologous myelin basic protein (MBP) or whole brain homogenate. It is one of the principal animal models for human CNS inflammatory and demyelinating disorders such as multiple sclerosis (MS); both diseases are characterized by considerable infiltration of leukocytes into the CNS, whose phenotypes have been extensively documented in the rat (1), mouse (2), guinea pig (3), and in brain tissue from MS patients (4).

While there is now little doubt that EAE after MBP injection has a cellular pathogenesis and that its induction is dependent on the presence of functioning T cells of the helper/inducer (CD4$^+$) lineage (5–8), it is not yet known how these cells induce disease. Moreover, the use of defined CD4$^+$ encephalitogenic T cell lines and clones in both mice (7) and rats (8), while greatly increasing our understanding of some aspects of this disease, in particular the means by which self antigen may be presented to such autoreactive cells, has not significantly furthered our understanding of the actual mechanisms of damage in the CNS. The accumulation of a considerable cellular infiltrate in the CNS of animals with EAE, whether induced with T cell lines (7, 8) or via conventional means after injection of MBP and adjuvant or transfer of cells from actively immunized animals (9, 10), has been taken as evidence that EAE may be the clinical manifestation of a delayed-type hypersensitivity (DTH) reaction in the CNS. All that can be said with confidence, however, is that appropriate MBP-specific CD4$^+$ cells, when injected into recipient rats or mice, are sufficient to induce both clinical signs and classic histological evidence of EAE (i.e., perivascular leukocyte infiltration reminiscent of a DTH reaction), but exactly which of the infiltrating

[1] *Abbreviations used in this paper:* CNS, central nervous system; DTH, delayed-type hypersensitivity; EAE, experimental allergic encephalomyelitis; L-CA, leukocyte-common antigen; MBP, myelin basic protein; MS, multiple sclerosis.

cells, whether acting alone or in collaboration with others, is essential for pathogenesis has not yet been established.

Herein, evidence is produced that severely weakens the link between EAE and DTH. As we now show in the rat, EAE may be mediated by a small number of CD4$^+$ IL-2-R$^+$ encephalitogenic cells that act in the virtual absence of other infiltrating leukocytes to induce clinical signs of EAE, possibly by producing vascular damage.

Materials and Methods

Animals. Specific pathogen–free male or female Lewis (RT1^l) rats, 8–12 wk old, were used in all experiments. Rats were obtained from the animal breeding unit of the MRC Cellular Immunology Unit, Oxford.

In Vitro Stimulation of EAE Effector Cells. MBP-reactive cells for adoptive transfer to recipient animals were from rats injected subcutaneously in the hind footpads with 50 μg MBP, prepared as described previously (5) emulsified in CFA. 12–15 d later, when the majority of animals displayed clinical signs of disease (limp tail or hind limb paresis), splenocytes were prepared from these animals and cultured in the presence of MBP for 3 d as previously described (11). Control (OVA-reactive) cells for transfer to recipient animals were generated essentially as described above except that the donor Lewis rats were injected with 100 μg OVA (Sigma Chemical Co., St. Louis, MO) in CFA. Spleens were removed 15 d later and splenocytes were cultured in the presence of 20 μg/ml OVA.

Passive Transfer of EAE. Recipient animals were either treated with cytotoxic drugs or gamma radiation (see below) or left untreated, before receiving between 4 and 6 × 10^7 viable, 3-d cultured splenocytes intravenously. The animals were subsequently observed for clinical signs of disease over the ensuing 2 wk and culled for histological examination of the CNS where appropriate.

Treatment of Recipient Animals. Removal of the majority of leukocytes in recipient rats was effected in two ways. Both protocols induced considerable leukopenia (peripheral blood leukocyte count equal to 3–6% of untreated rats) when measured 5–7 d after treatment.

Irradiation. Rats received 600 Rad on two occasions, 3 d before cell transfer and again, 4 h before receiving cultured cells (^{137}Cs, 88 rad/min; Gamma Cell 40).

Cytotoxic Drugs. The regimen of drugs for the removal of leukocytes and platelets in the rat was a slight modification of that described elsewhere (12, 13). 3 d before receiving cultured cells, animals were administered 20 mg/kg busulfan (Sigma Chemical Co.). The drug was first dissolved in DMSO then diluted to 10% in olive oil and 1 ml was injected intraperitoneally. 24 h later, animals received 12 mg/kg chlorambucil (Sigma Chemical Co.). This drug was resuspended in olive oil and 0.5 ml of the suspension was also injected intraperitoneally.

Assessment of Clinical Signs of EAE. Signs of EAE were assigned an arbitrary scale of severity as follows: −, no signs; 1, limp tail; 2, hind limb paresis; 3 and 4, unilateral and bilateral hind limb paralysis, respectively; 5, bilateral hind limb paralysis with incontinence and/or more severe signs.

Histological Examination of Recipient Rats. Paraffin or cryostat sections were prepared from the spinal cord, brain stem, cerebrum (all coronal sections), and cerebellum (para-sagittal sections) of recipient animals. Paraffin sections (7 μm) were stained with H and E, while cryostat sections (5 μm) were stained for cell surface antigens using a variety of mAbs (see below) in combination with the immunoperoxidase method (14) using a peroxidase-conjugated rabbit anti–mouse Ig as second antibody (Dako Corp., Santa Barbara, CA). Where staining was weak, enhancement of the brown diaminobenzidine substrate product was effected via a silver intensification procedure as described in detail elsewhere (15). All cryostat sections were lightly counterstained with hematoxylin.

Monoclonal Antibodies. mAbs used in these investigations were as follows: MRC OX-1 and MRC OX-30, noncompetitive antibodies that recognize the leukocyte-common anti-

gen (L-CA, reference 16); MRC OX-6, which recognizes a nonpolymorphic determinant on rat Ia, MHC class II antigen (17); MRC OX-8, which recognizes cytotoxic/suppressor T cells and NK cells (CD8, reference 18); MRC OX-19, which recognizes T cells and thymocytes (CD5, reference 18); MRC OX-21, which recognizes human C3b inactivator but not rat cells (19); MRC OX-39, which recognizes the IL-2-R (see Results); MRC OX-42, which recognizes macrophages and microglia via the iC3b receptor (20); MRC OX-52, which recognizes all T cells and thymocytes but is noncompetitive with MRC OX-19 (not CD5, reference 21); and W3/25, which recognizes T helper/inducer cells and macrophages (CD4, references 18 and 22).

Leukocytes were detected with a mixture of the mAbs MRC OX-1 and MRC OX-30 while T cells were stained with a combination of mAbs, MRC OX-19 and MRC OX-52. Tissue culture supernatants diluted one-third were used throughout.

Delayed-type Hypersensitivity. DTH responses in irradiated and nonirradiated animals receiving MBP or OVA reactive cells were tested as follows: 2 d after receiving in vitro–stimulated splenocytes, each animal was injected subcutaneously in one ear with 20 μg MBP in 20 μl PBS and, in the contralateral ear, with 40 μg OVA in 20 μl PBS (i.e., equimolar concentrations of antigen). 24 h later, the thickness of each ear was measured in triplicate using a Quicktester Micrometer gauge (H. C. Kroplin, Federal Republic of Germany) and a mean score for each ear was determined. Measurements were performed by an operator unfamiliar with the immunological status of the animals. After ear measurement, some animals were culled and ears were removed for histological examination. All remaining animals were assessed for clinical signs of EAE over the following 7–10 d.

Results

Severe Clinical Signs of EAE in Rats with a Minimal Leukocyte Infiltrate in the CNS. Our recent investigations in this laboratory designed to establish the host contribution to passive EAE noted that severe paralytic disease could be induced in recipient animals that had received lethal doses of irradiation before cell transfer. This finding was investigated further by an analysis of the cells infiltrating the CNS in both irradiated and nonirradiated recipients, using mAbs that recognize a range of cell- and tissue-specific antigens (see Materials and Methods). Of particular note here is MRC OX-39, which recognizes the rat Il-2-R. The specificity of this recently developed mAb has been defined by its ability to bind to activated, but not resting T cells and by its inhibition of binding of radiolabeled IL-2 to such activated cells. The antibody precipitates, from rat T blasts, a protein of 45–50 kD, similar in molecular mass to that of mouse (23) and human (24) IL-2-R (Jefferies, W. A., D. J. Paterson, P. Cortese, J. R. Green, M. Puklavec, and A. F. Williams, manuscript in preparation).

Lewis rats were injected with either MBP or OVA in adjuvant and their splenocytes were subsequently stimulated in vitro with the same antigen before transfer to either normal or irradiated recipient animals (Table I). 5–7 d after cell transfer when recipient animals exhibited signs of disease (or on day 7 in the case of recipients of OVA-reactive cells that never displayed any clinical signs), the animals were culled, brain and spinal cords were removed, and cryostat sections were assessed for infiltrating cells. In these studies, examination of spinal cord material extending from the cervical to cauda equina regions did not reveal any significant differences in the degree of cellular infiltration, and hence the data reported in Table I are those obtained only from material recovered from the thoracic cord.

It is clear from Table I that regardless of the status of the recipient (irradiated

TABLE I

Analysis of Infiltrating Cells in the Spinal Cord of Rats with Passive EAE

Group	Treatment of recipient	Animal	Disease stage when culled for histology	Class II	L-CA	Pan T	CD4	CD8	IL-2-R	MRC OX-42
A	Irradiated plus MBP-activated cells	1	5	5	10	ND	5	1	ND	18
		2	5	3	8	7	10	2	4	13
		3	5	3	11	8	6	1	7	7
		4	5	4	7	7	4	1	6	11
		5	5	5	10	5	7	0	6	24
		6	4	3	11	7	4	2	5	6
B	No irradiation plus MBP-activated cells	1	4	200	190	ND	79	19	ND	104
		2	4	194	216	ND	120	33	ND	134
		3	2	107	81	47	76	31	7	80
		4	5	106	78	34	51	14	7	85
		5	2	115	86	37	66	12	6	88
		6	4	135	122	51	73	19	6	86
C	Irradiated plus OVA-activated cells	1	—	0	2	1	1	0	0	8
		2	—	0	1	0	3	0	0	7
D	No irradiation plus OVA-activated cells	1	—	1	1	0	2	0	0	13
		2	—	0	2	0	1	0	1	10
E	Irradiated only	1	—	0	2	1	0	0	ND	5
		2	—	0	1	0	1	0	ND	7
		3	—	0	1	0	0	0	0	6
		4	—	0	0	0	3	0	0	10
F	No treatment	1	—	2	1	ND	1	0	ND	12
		2	—	1	0	ND	4	0	ND	10
		3	—	0	2	0	2	0	0	13

Cells/0.13 mm² in the spinal cord positive for: (column header spanning Class II, L-CA, Pan T, CD4, CD8, IL-2-R, MRC OX-42)

Figures shown are the number of nucleated cells recognized by mAbs against these cell markers in 10 random fields under × 800 magnification (a total area of 0.13 mm²). Animals in groups A and B received MBP-reactive splenocytes while those in groups C and D received OVA-stimulated splenocytes. Animals in groups E and F did not receive cells. Sections stained with the control mAb MRC OX-21 were uniformly negative (data not shown).

or not), clinical signs were induced when the animals received MBP-reactive cells. The similarity between the two groups ends here, however, as there is a clear disparity between the number of leukocytes detected in the spinal cords of these animals (see also Fig. 1). As expected, spinal cords of nonirradiated recipients were infiltrated by large numbers of leukocytes, many of which were class II MHC antigen Ia+. Both CD4+ and CD8+ cells were also present, although the majority of the CD4+ cells had the appearance of macrophages rather than lymphocytes, a finding supported by the observation that fewer cells were recognized by T cell–specific (pan T) mAbs than were detected by anti-CD4 mAbs alone (see Table I). MRC OX-42 antibody which, like Mac 1 in the mouse (25), recognizes macrophages and microglia, also labeled a considerable number of cells in the spinal cords of these animals. The increase above control values (groups D and F) was not as great as with other markers, however, as this antibody labeled microglia that were of course present in normal (non-EAE) animals. Infiltrating leukocytes, many of which were perivascular, were mainly clustered in discrete areas in the white matter (see Fig. 1, a and b).

In stark contrast to this typical picture of EAE, irradiated recipients (group A) contained markedly reduced numbers of infiltrating leukocytes and, as Fig. 1 c shows, there were no inflammatory foci. The few positively staining cells were
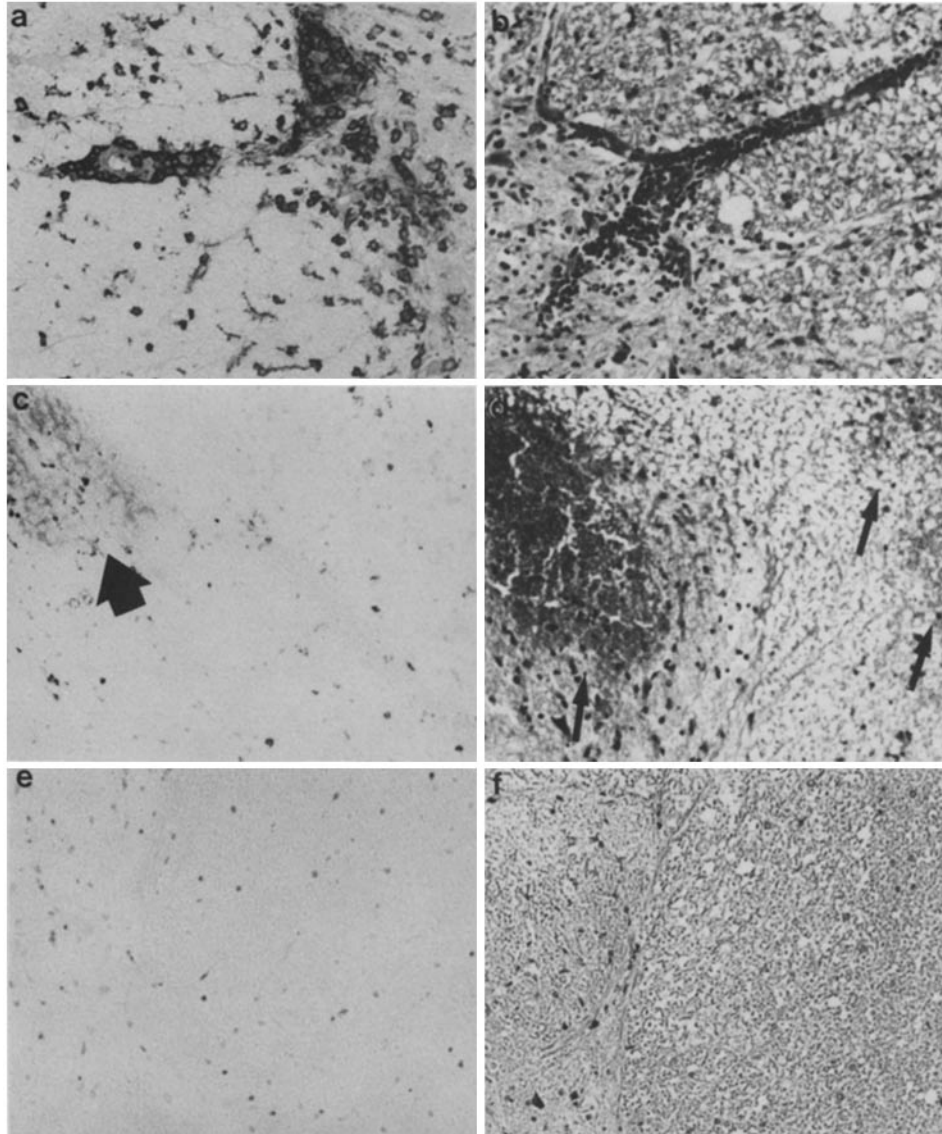
FIGURE 1. Microscopic appearance of spinal cord from irradiated recipient rats. Shown are sections of spinal cord from rats 5–7 d after receiving MBP- or OVA-stimulated splenocytes. (a, c, and e) Cryostat sections stained for leukocytes by using mAbs MRC OX-1 and MRC OX-30 (see Materials and Methods); (b, d and f) paraffin sections stained with H and E. (a and b) Nonirradiated recipients of MBP-reactive cells (× 160); (c and d) irradiated recipients of MBP-reactive cells (× 100 and × 130, respectively); (e and f) irradiated recipients of OVA-reactive cells (× 130). (c and d) Arrows designate areas of hemorrhage. Spinal cord in a–d is from animals with severe clinical signs of EAE (at least unilateral hind limb paralysis), while that in e and f is from animals with no signs of disease.

distributed throughout the white and, to a lesser extent, the grey matter of the spinal cord. The association of these cells with structural elements in the CNS, in particular the vasculature, was difficult to establish with the single staining
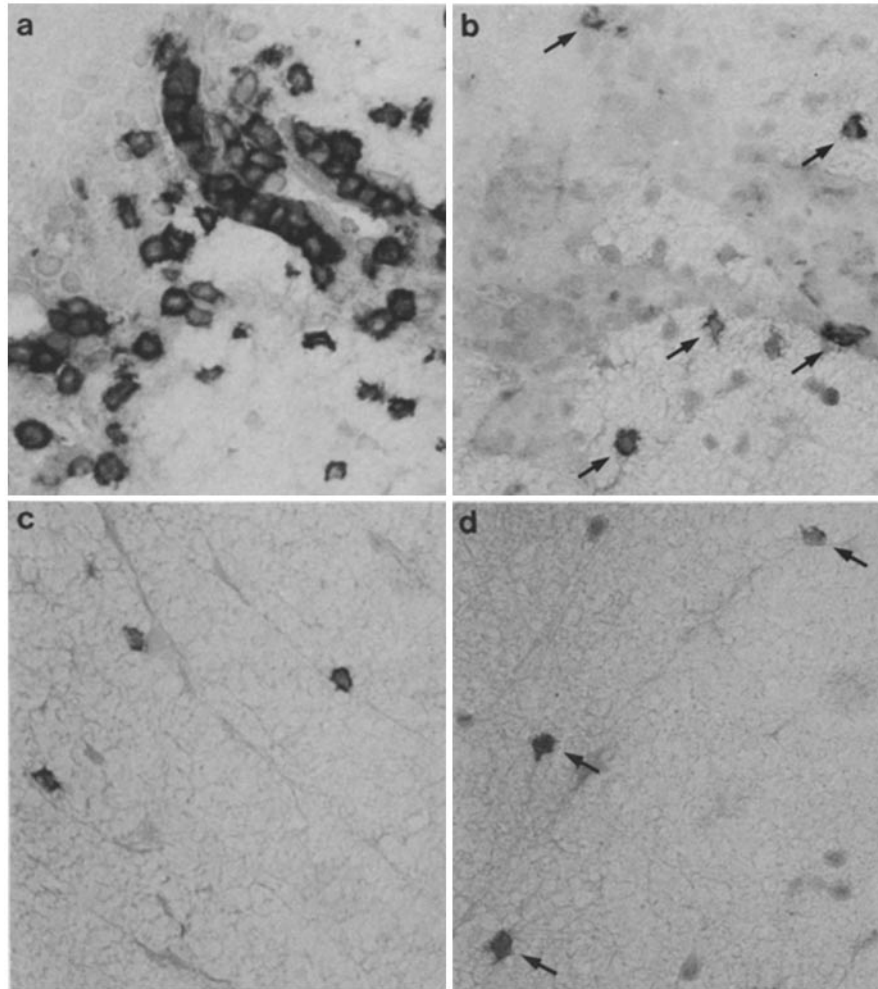
FIGURE 2. IL-2-R⁺ cells infiltrating the spinal cord of irradiated and nonirradiated recipient animals with EAE. Shown are cryostat sections of thoracic spinal cord from nonirradiated (a and b) and irradiated (c and d) rats 5–7 d after receiving MBP-stimulated splenocytes. The sections are stained for T cells (a and c) and for the IL-2-R (b and d). Arrows indicate IL-2-R⁺ cells. × 410.

procedure used here. Dual staining of infiltrating lymphocytes and the vasculature, the latter with an mAb that exclusively labels the vascular endothelium in the rat brain (26), should help resolve this uncertainty.

This reduction in leukocyte infiltration in irradiated recipients was reflected in the numbers of cells reactive with all but one of the mAbs used to define the phenotypes of the cells present in the spinal cords of these animals. The notable exception was that the numbers of IL-2-R⁺ cells were very similar in irradiated and nonirradiated recipients of MBP-reactive cells. Thus, while there was a considerable T cell infiltrate in nonirradiated recipient animals, relatively few of these expressed the IL-2-R as illustrated in the serial sections of Fig. 2, a and b. In contrast, the spinal cord of irradiated recipients contained only low numbers

of infiltrating T lymphocytes (Fig. 2 c) and cells expressing the IL-2-R were present at about the same frequency (Fig. 2 d and Table I, group A).

It should be noted that the features summarized in Table I are not characteristic solely of the spinal cord of these animals. Comparable investigations of the brain stem and cerebellum of both irradiated and nonirradiated recipient animals revealed similar patterns of infiltrating leukocytes to those seen in the spinal cord, although in general, cerebellar leukocyte infiltrate tended to be less than that seen in the spinal cord and brain stem; cerebral leukocyte infiltrate was virtually absent (data not shown).

Hemorrhage in the CNS of Irradiated Rats with Passive EAE.   Both the macroscopic and microscopic appearance of the spinal cord in irradiated recipient animals of MBP-reactive cells differed markedly from that seen in nonirradiated rats. In the latter group, the macroscopic appearance of the spinal cord was unremarkable while at the microscopic level the only visible pathology was the considerable, predominantly perivascular leukocyte infiltrate described in the previous section. In irradiated recipients of MBP-reactive cells, however, at the macroscopic level the surface of the spinal cord was considerably altered from normal, with multiple areas of hemorrhage. At the microscopic level these areas were also observed in cryostat sections extending inward from the surface of the cord (Fig. 1 c, arrow). As expected from the presence of hemoglobin they were weakly stained nonspecifically by the immunoperoxidase process. Subsequent examination of spinal cord stained with H and E confirmed the presence of erythrocytes in the white and grey matter of spinal cord of only those irradiated animals that received MBP-reactive cells (Fig. 1 d). Spinal cord from irradiated recipients of OVA-reactive cells (Fig. 1, e and f), like nonirradiated recipients of MBP-reactive cells (the latter with both clinical signs and considerable cellular infiltrate, Fig. 1 b), did not show any hemorrhagic lesions at either the macroscopic or microscopic level. Spinal cord from nonirradiated recipients of OVA-reactive cells (not shown) was identical in appearance to that of their irradiated counterparts (Fig. 1, e and f).

Other CNS tissues in irradiated recipients of MBP-reactive cells were also examined (data not shown). Areas of white matter hemorrhage were present in the brain stem, although to a lesser extent than that seen in the spinal cord. Scattered erythrocytes were occasionally observed in the parenchyma of the cerebellar white matter of these animals while hemorrhage was absent in the cerebrum.

Effect of Injected Anti-CD4 mAb on Clinical and Histological Signs of EAE in Irradiated Recipients.   Recent studies in this laboratory have established that both actively (5) and passively (11) induced EAE in the rat can be prevented by the in vivo administration of the mouse W3/25 mAb against the rat CD4 antigen. To assess whether passively transferred EAE in irradiated animals was similarly susceptible to this form of treatment, irradiated or nonirradiated recipient rats were injected intravenously with either anti-CD4 (W3/25) IgG or the control mAb MRC OX-21 for seven consecutive d after transfer of MBP-stimulated splenocytes (for details see legend to Table II and reference 11). The animals were assessed for clinical signs of EAE during and after mAb treatment and

TABLE II

Anti–Rat CD4 mAb Prevents Clinical EAE and Hemorrhage in Irradiated Recipient Animals

Group	Treatment of recipients	Animal	Clinical score (days after cell transfer)											Leukocyte infiltrate*	Hemorrhage‡
			0	1	2	3	4	5	6	7	8	9	10		
A	Irradiated plus MBP-activated	1	—	—	—	—	—	—	—	—	—	—	Dead	ND	—
	cells day 0 plus anti-CD4 IgG	2	—	—	—	—	—	—	—	—	—	—	Dead	ND	—
	day 0–6 inclusive	3	—	—	—	—	—	—	—	—§				—	—
		4	—	—	—	—	—	—	—	—§				—	—
B	Irradiated plus MBP activated	1	—	—	—	—	—	—	—	1	2	Dead		ND	ND
	cells day 0 plus MRC OX-21	2	—	—	—	—	—	—	—	1	1	3§		ND	+
	mAb day 0–6 inclusive	3	—	—	—	—	—	—	—	1	4§			+/−	+
		4	—	—	—	—	—	—	1	1	5§			+/−	+
C	Not irradiated plus MBP acti-	1	—	—	—	—	—	—	—	—	—	—	—	ND	ND
	vated cells day 0 plus anti-	2	—	—	—	—	—	—	—	—	—	—	—§	ND	—
	CD4 IgG day 0–6 inclusive	3	—	—	—	—	—	—	—	—§				+/−	—
		4	—	—	—	—	—	—	—	—§				+/−	—
D	Not irradiated plus MBP acti-	1	—	—	—	—	—	1	3	5	1	1	—	ND	ND
	vated cells day 0 plus MRC	2	—	—	—	—	—	—	2	5	2	1	—§	ND	—
	OX-21 mAb day 0–6 inclu-	3	—	—	—	—	—	1	4§					+++	—
	sive	4	—	—	—	—	—	1	4§					+++	—

Irradiated or nonirradiated rats received MBP-reactive cells on day 0 and either 0.3 mg sterile W3/25 (anti-CD4) IgG or 0.5 ml sterile MRC OX-21 ascites (ascites diluted to ~0.6 mg specific antibody per milliliter) intravenously on days 0–6 (inclusive). Animals were assessed for clinical signs of EAE and some from each group subsequently culled for histological examination of the CNS.

* Leukocyte infiltrate is that observed in cryostat sections of the spinal cord, brain stem, and cerebellum stained for the L-CA. (−) None observed; (+/−) minimal infiltrate, no perivascular cuffing (see Table I, group A); +++, extensive perivascular and parenchymal infiltrate.

‡ Refers to the presence (+) or absence (−) of hemorrhage in the spinal cord and brain stem of these animals. Rats 1 and 2 in group A and rat 2 in groups B, C, and D were assessed for hemorrhage only at the macroscopic level. Rats 3 and 4 in each group were examined for hemorrhage at both the macroscopic and microscopic level, the latter by staining of paraffin sections with H and E.

§ Sampled for histological examination.

subsequently culled and examined for the presence of infiltrating leukocytes and hemorrhage in the CNS (Table II).

Animals treated with anti-CD4 mAb, whether irradiated (group A) or not (group C), did not show any clinical signs of EAE, in contrast to those receiving the control mAb, MRC OX-21 (groups B and D). Of those irradiated animals treated with anti-CD4 mAb that were not culled for histology in this (group A, rats 1 and 2) and other comparable experiments, no disease was observed up to 10–12 d after cell transfer, at which time the rats died, presumably as a direct result of the lethal dose of irradiation they had received. Furthermore, of particular interest was the complete absence of hemorrhagic lesions in irradiated recipients receiving anti-CD4 mAb (group A).

Those irradiated animals receiving control mAb (group B) typically showed signs of EAE 5–7 d after receiving cultured cells, and they showed hemorrhage in both the spinal cord and brain stem. There was no indication of hemorrhage at either the macroscopic or microscopic level in nonirradiated recipient rats (groups C and D).

The extent of leukocyte infiltrate was variable. In keeping with previous observations, irradiated animals receiving control mAbs (group B) had only a minimal infiltration of leukocytes into the spinal cord, brain stem, and cerebellum, while there was extensive perivascular cuffing in similarly treated nonirradiated recipient rats (group D). As has been observed in earlier studies (11), anti-CD4 mAb treatment of nonirradiated recipient rats (group C) resulted in a
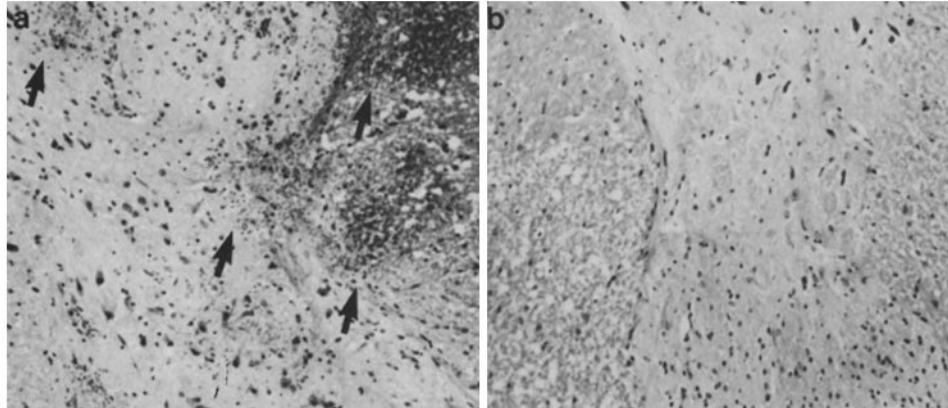
FIGURE 3.   Hemorrhage in the spinal cord of leukopenic recipient rats after treatment with cytotoxic drugs. Recipient rats were treated with a combination of two cytotoxic drugs (see Materials and Methods), and some were then injected with MBP-reactive splenocytes. 5–7 d later, at a time when the cell recipients showed signs of EAE (at least limp tail), all animals were culled for histological examination. Shown are paraffin sections of spinal cord stained with H and E from animals that received MBP-stimulated cells (a, × 100) or did not (b, × 100). Areas of hemorrhage are indicated by arrows.

substantial reduction in the numbers of cells infiltrating the CNS, and hence it was not surprising that the spinal cord, brain stem, and cerebellum in irradiated recipient animals receiving anti-CD4 mAbs, were completely devoid of infiltrating leukocytes (group A).

Hemorrhage Is Not an Irradiation Artefact.   Given the appearance of hemorrhage only in those rats that received MBP-reactive cells (Fig. 1) and its prevention by anti-CD4 mAb (Table II, group A), it was considered highly unlikely that the observed hemorrhage was artefactual, but rather, the visible signs of specific damage to the vasculature by the injected MBP-reactive, CD4$^+$ effector cells.

The levels of radiation used herein may affect both the integrity of the microvasculature (27) and severely deplete the numbers of circulating platelets (28). It was therefore possible that the specific vascular damage caused by the MBP-reactive cells was accentuated by the absence of effective clotting mechanisms (no platelets), and by the presence of already irradiation-damaged microvasculature, the net result being the extravasation of erythrocytes into the parenchyma.

To test this hypothesis, rats were depleted of circulating leukocytes with two cytotoxic drugs and then injected with MBP-stimulated splenocytes (for details see Materials and Methods). Drug-treated animals receiving cells developed both clinical signs of EAE 5–6 d after cell transfer and macroscopic and microscopic hemorrhage in the spinal cord (Fig. 3a). Drug treatment alone resulted in neither clinical signs nor hemorrhage (Fig. 3b). The severity of the clinical disease was not as marked as that seen in either irradiated or nonirradiated recipient animals receiving equivalent numbers of MBP-stimulated splenocytes, nor was the hemorrhage as extensive as that occurring in irradiated animals. This may possibly reflect killing of some of the injected cells by low levels of cytotoxic drugs still present in the recipient animal at the time of cell transfer. Nevertheless, clinical

TABLE III

Absence of a Classic DTH Reaction in Irradiated Recipient Animals

Group	Treatment of recipient	Number of animals	Ear injected with:		Change in ear thickness*	Histology
			MBP	OVA		
			mm	*mm*	*mm*	
A	Irradiated plus MBP activated cells	10	0.65 ± 0.02[‡]	0.56 ± 0.06	0.09	Fig. 4 *a*
B	No irradiation plus MBP activated cells	9	0.99 ± 0.09	0.59 ± 0.06	0.40	Fig. 4, *c* and *e*
C	Irradiated plus OVA activated cells	5	0.59 ± 0.01	0.63 ± 0.07	0.04	Fig. 4 *b*
D	No irradiation plus OVA activated cells	5	0.61 ± 0.01	1.0 ± 0.1	0.39	Fig. 4, *d* and *f*

Irradiated or nonirradiated animals received MBP- or OVA-reactive cells then were ear tested for DTH responsiveness as described in Materials and Methods. Some animals from each group were subsequently sampled for histological examination (Fig. 4) while the remainder were observed for the development of clinical signs of EAE.

* Mean ear thickness (test antigen) − mean ear thickness (control antigen).

[‡] Mean ear thickness ± 1 SD.

EAE (at least a limp tail and usually also, hind limb weakness) and hemorrhage in the spinal cord were seen in all recipients of MBP-reactive cells, but never in those animals treated with drugs alone. Analysis of infiltrating cells in the spinal cord of drug-treated, recipient rats revealed a similar picture to that seen in irradiated animals (Table I), but with even fewer leukocytes detected. Numbers of cells counted in a cross-sectional area of 0.13 mm² (as per Table I) in two rats receiving drugs and MBP-reactive cells were: class II–positive, three and four; L-CA⁺, six and nine; pan T–positive, two and one; CD4⁺, four and five; CD8⁺, one and two; IL-2-R⁺, one and two; OX-42⁺, seven and seven.

Transfer of DTH Responsiveness with MBP- and OVA-Reactive Cells. EAE is generally considered to be the clinical manifestation of a DTH reaction in the CNS (see Introduction); however, it is also recognized that it is not possible to passively transfer DTH reactions into irradiated recipient animals (29). DTH responsiveness in such animals is dependent on hematopoietic stem cell replacement, indicating a requirement for host leukocytes in this reaction (29).

To assess whether the induction of DTH in recipient animals of OVA- and MBP-reactive cells was dependent on the presence of host leukocytes (and thus different from passively transferred EAE), in vitro–stimulated cells were transferred to irradiated or nonirradiated animals and DTH responsiveness in the ears of recipient rats was tested. Ear swellings were then measured (Table III) and ears were assessed histologically (Fig. 4) for evidence of a classic DTH reaction.

In nonirradiated recipient animals (Table III, groups B and D), DTH was transferred by both MBP- and OVA-reactive splenocytes and the reaction was antigen specific. The degree of specific swelling with the two antigens was virtually identical. Histological examination revealed the presence of a considerable leukocyte infiltrate in the ear that received the antigen to which the
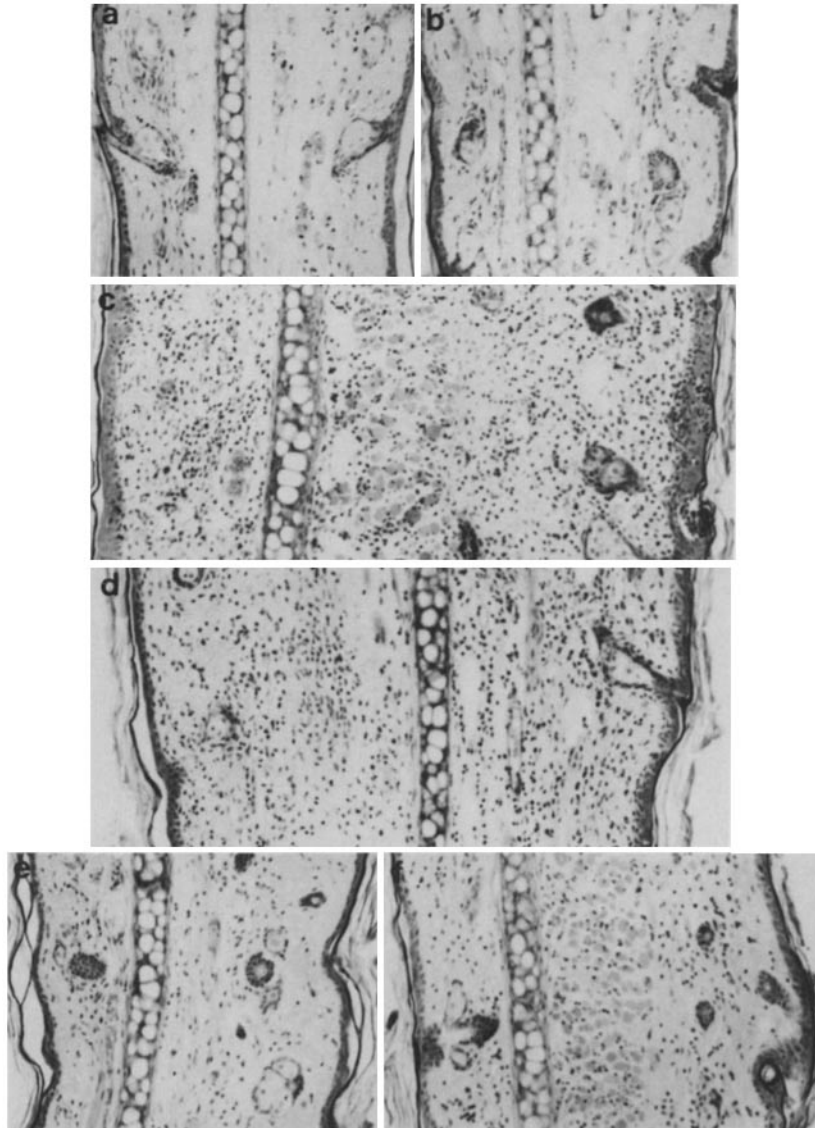
FIGURE 4.   Histological evidence of absence of a classic DTH reaction in irradiated recipient animals. Ear swelling in animals treated as per Table III was determined 24 h after injection of either MBP or OVA into the ears. Representative animals from each group were then sampled for histological examination of the ears. Shown are paraffin sections stained with H and E (× 100) of: (a) MBP-injected ear from irradiated recipient of MBP reactive cells; (b) OVA-injected ear from irradiated recipient of OVA-reactive cells; (c) MBP-. and (e) OVA-injected ear from nonirradiated recipients of MBP-reactive cells; (d) OVA- and (f) MBP-injected ear from nonirradiated recipients of OVA-reactive cells.

transferred cells were preactivated (Fig. 4, c and d), but not in the contralateral ear which was injected with control antigen (Fig. 4, e and f). Infiltrating cells were by no means completely absent in the control ears, however (Fig. 4, e and f), suggesting some level of nonspecificity. MBP in particular appeared to cause

a mild inflammatory reaction even in normal (nonirradiated, non–cell recipient) animals, which may indicate the presence of some form of irritant in our MBP preparation. The extent of this nonspecific inflammation was variable and never greater than that in Fig. 4*f*. However, the degree of ear swelling in irradiated animals was markedly reduced in comparison, and in recipients of either MBP- or OVA-reactive cells, was not significantly above that seen in the contralateral ear receiving control antigen (Table III, groups A and C). Leukocyte infiltrate in these animals was considerably less than that seen in non-irradiated rats (compare Fig. 4, *a* and *c* with *b* and *d*).

Particularly interesting was the observation that all recipients of MBP-reactive cells developed some (albeit mild) clinical signs of EAE 3–4 d after ear testing, irrespective of the development of DTH in the antigen-challenged ears. Furthermore, hemorrhage was seen in the spinal cord of irradiated recipient rats but not in the MBP-injected ears of these same animals.

Discussion

The demonstration of the essential role that CD4$^+$ T cells play in the induction of EAE in rats and mice (see also references 5–8) has not shown whether these cells act alone or in collaboration with other infiltrating cells to cause the nerve conduction defects characteristic of this disease.

Lethal irradiation of recipient animals of MBP-reactive cells (Table I and Fig. 2) has provided the most direct evidence that the great majority of leukocytes found in the CNS of normal (nonirradiated) rats with EAE play no essential role in the pathogenesis of this disease. Few infiltrating cells of any type are found in irradiated recipient rats even though clinical signs of EAE are present. Macrophages, which are thought to play some role in demyelinating forms of the disease induced with whole brain homogenate (9, 30), were present at extremely low levels in irradiated recipient animals, suggesting that the development of clinical signs of EAE in the current model is not dependent on their presence, at least at the levels normally seen in nonirradiated animals. Similarly, the frequency of CD8$^+$ cells was significantly reduced in irradiated rats, suggesting that the bulk of these cells in nonirradiated animals are not important in pathogenesis. Furthermore, we have recently shown (Sedgwick, J. D., and D. W. Mason, manuscript in preparation) that the CD8$^+$ population can be depleted in vivo by administration of MRC OX-8 (anti-CD8) mAb and that such animals acquire and recover from both actively and passively transferred EAE with the same tempo as rats with a normal compliment of these cells. Given, also, that the only cell types not reduced in irradiated animals were the infrequent ones expressing the IL-2-R (Table I and Fig. 2), it is tempting to speculate that it is these cells that are directly responsible for the transfer of EAE in both irradiated and nonirradiated rats, and that the bulk of leukocytes observed in the CNS of nonirradiated passive EAE recipients are host leukocytes that migrate secondarily to the tissue damage caused by these IL-2-R$^+$ effector cells. The phenotype of the IL-2-R$^+$ cells is currently being examined by double-staining techniques; however, given the demonstration that only anti-CD4 mAbs can prevent EAE in actively (5, 11) and passively induced EAE in both nonirradiated (5) and irradiated (Table II) recipient rats, it is highly likely that these cells prove to be CD4$^+$.

These data provide the first in vivo evidence that (unless cells normally resident in the CNS play a collaborative role in pathogenesis) it is the CD4$^+$ IL-2-R$^+$ cells themselves that are the actual mediators of the disease process. What the data clearly demonstrate is that the CD4$^+$ cells are not simply acting in the capacity of DTH lymphocytes that attract other leukocytes (e.g., macrophages), which ultimately are responsible for tissue damage (31).

How may these CD4$^+$, IL-2-R$^+$ T cells alone produce the clinical signs of EAE? There is considerable indirect evidence that acute EAE (that form of disease where clinical signs are relatively short-lived and recovery is spontaneous) is associated with an increase in vascular permeability in the CNS and that the transient paralysis observed in this disease is a direct sequel of tissue edema resulting from this permeability change (32, 33). Acute relapses in MS may be associated with a similar phenomenon (34). The grossly visible hemorrhage seen in irradiated recipients receiving MBP-reactive cells (Fig. 1) and in those recipients made leukopenic with cytotoxic drugs (Fig. 3) provides direct evidence that vascular damage does occur under these circumstances, although to observe such damage in the form of hemorrhage it is clearly necessary to compromise the animal's ability to repair the vasculature. In the present case, this was achieved by reducing platelet numbers with gamma radiation or cytotoxic drugs. The doses of drugs and irradiation used here have both been shown to reduce platelet numbers to <20% of normal in the rat (12, 13, 28). Importantly, the induction of hemorrhage in these rats was highly specific as only MBP- (but not OVA-) reactive cells were capable of inducing these changes (Fig. 1). Moreover, the ability of injected anti-CD4 mAb to not only inhibit EAE in irradiated rats, but also to prevent hemorrhage (Table II), confirms that the effector mechanisms that induced hemorrhage were identical to and representative of active EAE and passive EAE in nonirradiated animals. Thus, it appears that CD4$^+$ cells, possibly acting in a cytotoxic capacity, may cause damage to the blood vessels within the CNS, which in turn results in local edema and temporary nerve conduction defects. Consistent with this hypothesis is the recent demonstration in vitro that MBP-specific, CD4$^+$ rat T cell lines are cytotoxic and can lyse MBP-presenting astrocytes (35) and that it is only those lines that induce EAE in vivo that are also cytotoxic in vitro.

A number of gaps still remain in our understanding of the pathogenesis of this disease. First, which class II–positive cell population in the CNS is responsible for stimulating the CD4$^+$ cells in vivo given the absence of MHC class II antigens in the CNS of rats before EAE induction (36)? Second, if indeed the CD4$^+$ effector cell is cytotoxic for class II–positive targets, what is the target cell(s)? It is clear, at least in vitro, that CD4$^+$ T cells involved in the induction of EAE respond only when the antigen is presented by an appropriate class II MHC antigen–positive accessory cell (7, 8, 37), and there is evidence both in the rat (8) and the mouse (7) that the actual effector cell responsible for the clinical signs of disease displays class II MHC antigen restriction. What is evident from the studies herein, however, is that the majority of the MHC class II–positive cells seen in the CNS of nonirradiated rats with EAE are superfluous to disease induction and that if any such cells are required, then the few remaining in irradiated rats (Table I) are obviously sufficient.

Similarly unresolved is the possible target in vivo of these putative cytotoxic $CD4^+$ T cells. Contrary to observations in the mouse (2) and guinea pig (38), two thorough studies, including one at the electron microscope level (36, 39), have clearly shown that even in rats with EAE, MHC class II antigens are undetectable on the vascular endothelium and astroglial cells. Thus, even though $CD4^+$ encephalitogenic T cell lines may kill MHC class II–positive astrocytes in vitro (35), it is difficult to see how this process operates in vivo. It is worth noting that the demonstration that the effector cell in rodent EAE is class II MHC antigen restricted (7, 8, 35) is somewhat indirect, as no studies have been made with MHC recombinant animals. The vasculature of rodent brain expresses class I MHC antigens constitutively so that no problem with identifying the vascular endothelium as the target would arise if the final effector cells proved to be restricted by class I MHC antigens. Effector cells expressing CD4 antigens and able to recognize and kill MHC class I–positive targets have been described by others (40, 41).

In addition to the necessity for an appropriate MHC antigen on the target cell, antigen (MBP) would also be required for recognition by the MBP-specific $CD4^+$ T cells. Whether this recognition of antigen occurs on the luminal side of the vasculature due to the presence of MBP bound to the vascular endothelium (42) or in the parenchyma after nonspecific traffic of activated $CD4^+$ T cells through small blood vessels (43) is not known. Studies are currently in progress in an attempt to resolve at least some of these issues.

The data presented in Table III and Fig. 4 on the capacity of both OVA- and MBP-reactive cells to transfer classic DTH reactions (i.e., that reaction where there is swelling of the antigen challenge site together with a large leukocyte infiltrate) to normal (but not irradiated) recipient animals underlines the dissimilarity of this reaction and that which is sufficient to produce clinical signs of EAE in irradiated rats receiving the same MBP-reactive cells. We have interpreted these results as implying that $CD4^+$ T cells are the direct mediators of the disease. Some caution must be exercised in this interpretation, however, as it is not yet possible to exclude the role of cells normally resident in the CNS that may collaborate with the MBP-specific $CD4^+$ encephalitogenic T cells to produce tissue damage. Specifically, microglia are thought by some to be the mononuclear phagocytic cell of the brain and may, for example, secrete IL-1 under appropriate conditions of activation (44). Furthermore, while these cells are also MHC class II–negative in normal rats, unlike vascular endothelium and astroglial cells, activated microglia do express Ia antigen in animals with EAE (36, 39). It is possible, however, that the majority of these Ia^+ cells are actually infiltrating macrophages derived from the bone marrow (45) that appear in response to damage by the $CD4^+$ effector T lymphocytes. Indeed, Ia^+ microglia are not detectable until just before the onset of clinical signs of EAE in normal rats and increase in numbers thereafter (39), so their relevance to the developmental stages of EAE is still in doubt. Moreover, very few Ia^+ cells of any sort are seen in irradiated rats with EAE (Table I), which suggests that large numbers of these putative Ia^+-activated microglia are not central to disease induction. As the origins of microglia and even their designated role as brain macrophages are still areas of some controversy (46), further studies are clearly required to

establish the real function of these cells and whether there is any interaction of microglia and CD4$^+$ T cells in the inductive phase of EAE in rats and mice.

The relevance of these studies to our understanding of the pathogenesis of MS is particularly highlighted by recent evidence that suggests that the IL-2-R (47–49) or other markers of T cell activation (50, 51) may be potentially useful as indicators of the presence of presumed pathogenic T cells in the CNS and peripheral blood of MS patients. In humans as well as animals, therefore, it is clearly necessary to direct our attention toward the elucidations of the mechanisms by which these cells may initiate the disease process.

Summary

One characteristic of experimental allergic encephalomyelitis (EAE) in all species is the presence of a considerable leukocyte infiltrate in the central nervous system (CNS). By adoptive transfer of EAE into irradiated or nonirradiated Lewis strain rats we now show that the bulk (>90%) of infiltrating cells in the CNS are superfluous to the induction of disease, as lethally irradiated recipients, despite having very few infiltrating cells in the CNS, acquire severe paralytic EAE. The reduction in the level of infiltration in irradiated recipients is selective, however, as both irradiated and nonirradiated diseased animals have very similar numbers of cells expressing IL-2-R. Disease in irradiated recipient animals is associated with substantial submeningeal hemorrhage in the spinal cord and brain stem and similar hemorrhages are found in recipients rendered leukopenic with cytotoxic drugs. Clinical signs of disease and hemorrhage are preventable, however, by administration to the recipient rats of mAbs specific for the CD4 antigen. Classic delayed-type hypersensitivity (DTH) reactions are transferable with the same cells that produce EAE in both irradiated and nonirradiated recipient rats, but such transfer of DTH is observed only in nonirradiated recipient animals and not in irradiated rats.

Collectively, the findings reported herein support the conclusion that the paralysis characteristic of acute EAE is mediated by the direct action of very small numbers of activated CD4$^+$ lymphocytes that infiltrate the CNS and produce their effects by inducing vascular damage. The findings are not consistent with reports that the lesions in EAE are produced by a classic DTH reaction.

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