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In situ inactivation of infiltrating T cells in the central nervous system with autoimmune encephalomyelitis. The role of astrocytes

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SUMMARY

Our previous study using bromodeoxyuridine (BrdU) has shown that T cells in lesions of experimental autoimmune encephalomyelitis (EAE) in the rat central nervous system (CNS) lose their proliferating capability immediately after infiltration into the CNS. To characterize the nature of this phenomenon in more detail, we have isolated T cells from EAE lesions and examined their surface phenotype and response to encephalitogenic antigen, myelin basic protein (MBP). By flow cytometry (FCM) analysis, it was revealed that compared with peripheral blood lymphocytes, up-regulation of interleukin-2 (IL-2) receptors (0·06% → 3·73%) and the lymphocyte function-associated antigen-1 (LFA-1) molecules (0·76% → 17·6%) on spinal cord T cells (SCT) was observed. In spite of the latter finding suggesting that SCT are activated, SCT recovered from rats with full-blown EAE responded very poorly to MBP. The addition of thymocytes or thymocytes plus astrocytes did not alter the low responsiveness of SCT. More importantly, astrocytes strongly suppressed the response of lymph node T cells to MBP. Using MBP-specific T-line cells, it was revealed that T-cell suppression might be induced by incomplete presentation of MBP and release of suppressive humoral factors by astrocytes. Since the response of SCT was still poor when assayed after three and 12 rounds of stimulation with the antigen and propagation with IL-2, this phenomenon is long lasting. These findings are consistent with the findings obtained by the BrdU study that infiltrating T cells into the CNS do not proliferate vigorously. Taken together, the poor response of infiltrating T cells to MBP would be induced by co-existing cells such as astrocytes although the T cells are in an active form as judged by their surface phenotype. The present study suggests that activation of non-haematopoietic parenchymal cells in each organ by infiltrating T cells and subsequent inactivation of the T cells are important healing processes for organ-specific autoimmune diseases.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated autoimmune disease inducible in several species either by immunization with brain-specific antigens such as myelin basic protein (MBP) or by adoptive transfer of lymphoid cells sensitized with the antigen. After immunization, the frequency of MBP-reactive T cells increases in both the regional lymph nodes and spleen 2 or 3 days before onset of the disease.[1] Radiolabelling of MBP-reactive T cells and their subsequent transfer experiments have revealed that labelled T cells accumulate in the central nervous system (CNS) 4 days after the transfer,[2] and this accumulation occurs in an antigen-restricted manner.[3] It was previously believed that infiltrating T cells would proliferate further in the CNS for a considerable period because cultured brain cells such as astrocytes[4,5] and microglia[6] were reported to have an ability to present encephalitogenic antigens and to stimulate T-cell proliferation. However, our recent studies using bromodeoxyuridine (BrdU) have demonstrated that T cells *in situ* in the CNS lose their proliferating ability immediately after the infiltration into the CNS.[7,8] Furthermore, cultured microglia up-regulate, whereas astrocytes down-regulate, T-cell proliferation under *in vivo*-mimicking conditions. More importantly, mixed glial cells comprising microglia and astrocytes were shown to have weak antigen-presenting ability.[9] These findings suggest that brain cells, probably astrocytes, suppress T-cell proliferation or induce low responsiveness of infiltrating T cells.

In the present study, we isolated infiltrating T cells from the spinal cord with EAE and characterized their functions. It has been revealed: (1) that spinal cord T cells (SCT) are activated in terms of the expression of interleukin-2 (IL-2) receptors and adhesion molecules; (2) that SCT react poorly with MBP; and

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; IFN-γ, interferon-γ; GPBP, guinea-pig MBP; LNC, lymph node cells; MBP, myelin basic protein; SCT, spinal cord T cells.

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(3) that astrocytes suppress T-cell proliferation by the induction of antigen-driven T-cell unresponsiveness and secretion of suppressive humoral factors such as prostaglandin. These observations indicate that the proliferation of SCT are suppressed by brain cells and further suggest that parenchymal cells such as brain cells may play a significant role in the healing processes of organ-specific autoimmune diseases.

MATERIALS AND METHODS

Rats

Lewis rats were obtained from Charles River Japan (Kanagawa, Japan). All rats except for brain cell cultures were used at the age of 8–12 weeks.

Monoclonal antibodies (mAb) and reagents

The following mAb were used in the present study: W3/25 (anti-CD4);[10] OX8 (anti-CD8);[11] R73 (anti-T-cell receptor $\alpha\beta$);[12] OX39 (anti-IL-2 receptor);[13] OX6 (anti-RT1.B);[14] and OX42 (anti-complement receptor type 3)[15] which labels macrophages and microglia. These mAb were purchased from Serotec (Blackthorn, Bicester, U.K.). Monoclonal antibodies against lymphocyte function-associated antigen-1α (LFA-1α), LFA-1β and ICAM-1 (WT.1, WT.3 and 1A29)[16,17] were obtained from Seikagaku Corp. (Tokyo, Japan). Neutralizing antibodies against transforming growth factor β1 (TGF-β1) and -β2 were obtained from R & D System Inc. (Minneapolis, MN) and that against TGF-β (1+2) was purchased from King Brewing Co. (Kakogawa, Japan). Purified rat natural IL-2 and indomethacin were obtained from Collaborative Research Inc. (Bedford, MA) and Sigma (St Louis, MO), respectively. Guinea-pig MBP (GPBP) and a synthetic peptide corresponding to the 68–88 sequence of GPBP were prepared as described previously.[18]

Immunoperoxidase staining

Immunoperoxidase staining was performed as described previously.[19] Briefly, frozen sections were air dried and fixed in ether. After incubation with normal sheep serum, sections were allowed to react with mAb, biotinylated sheep anti-mouse Ig (Amersham International, Amersham, U.K.) and Horseradish peroxidase (HRP)-labelled streptavidin (Serotec). HRP-binding sites were detected in 0·05% diaminobenzidine and 0·01% hydrogen peroxide.

FCM analysis

Leucocytes in the spleen, popliteal lymph node, blood [peripheral blood leucocytes (PBL)] and spinal cord (see below) were collected and stained with mAb, followed by fluorescein isothiocyanate (FITC)-labelled anti-mouse Ig (Amersham). For two-colour analysis, cells were incubated with the first unlabelled mAb followed by phycoerythrin (PE)-conjugated anti-mouse IgG (Biomeda, Foster City, CA). To saturate free binding-sites of the secondary antibody, cells were incubated with normal mouse serum. Then, FITC-conjugated mAb in the second step was applied. Ten thousand cells were analysed in each sample by FACScan flow cytometry.

EAE induction

EAE was induced in Lewis rats as described previously.[8] Each rat was injected in hind footpads with an emulsion containing 100 μg MBP and complete Freund's adjuvant (CFA). Immunized rats were observed daily for clinical signs of EAE which were graded into four categories.[20]

Isolation of infiltrating T cells from the spinal cord

SCT were isolated on either days 12 or 13 post-immunization when rats developed full-blown EAE. A group of rats (usually five rats) was killed under ether anaesthesia by bleeding from the heart. The spinal cords were removed and minced with scissors. After passing through a stainless mesh screen, the cell suspension was mixed with an equal volume of Ficoll–Isopaque gradient medium ($\rho = 1{\cdot}085$) and spun at 1200 g for 10 min. By this procedure, myelin cake was floated on top of the mixture of Ficoll–Isopaque and culture media, and infiltrating inflammatory cells were in the pellet. The pellet was harvested and passed through a nylon wool column to enrich T cells. By this procedure, $1\text{–}2 \times 10^6$ cells/rat were usually obtained. In initial experiments, minced spinal cord tissue was further digested with a cocktail of enzymes (final concentrations: 0·1% trypsin, 100 U/ml DNase and 0·2% collagenase) to obtain a large number of inflammatory cells. However, this treatment of the tissue resulted in damage of T-cell function as evidenced by the finding that enzyme-treated, but not untreated, lymph node cells (LNC) from MBP-immunized rats did not respond *in vitro* to MBP.

Establishment of MBP-reactive T-cell lines from LNC and SCT

MBP-specific T-cell lines were established as described previously.[1] Briefly, SCT or LNC from MBP/CFA-immunized rats were stimulated with GPBP for 3 days, and then propagated in the medium containing concanavalin A (Con A) supernatant. Stimulation with antigen in the presence of irradiated thymocytes as antigen-presenting cells (APC) and propagation of T cells were repeated 10–20 times, after which the cells reacted only with the selected antigen. All the GPBP-specific T-cell lines used in the present study were $CD4^+$. In some experiments, SCT were divided into two parts. One part of T cells was stimulated with GPBP without exogenous IL-2 at the first stimulation, and the other part of T cells were stimulated with the antigen in the presence of a suboptimal dose of IL-2 (2 U/ml).

Brain cell culture

Primary mixed glial cell cultures were prepared from cerebra of newborn rats. Microglia and astrocytes were isolated from these cultures as described previously.[9] In brief, microglia were isolated from primary mixed glial cell cultures by hand tapping of culture flasks. This procedure allowed us to obtain highly purified microglia (more than 95% of cells isolated were positive for OX42 and there was no contamination of GFAP-positive astrocytes). Astrocytes were obtained by passaging primary cultures three to five times after which microglia were no longer present in the cultures. Before an assay for antigen-presenting function, portions of mixed glial cells, microglia and astrocytes were treated with interferon-γ (IFN-γ) to induce Ia antigens.[9]

Proliferation assay

SCT, LNC (2×10^5 cells/well) and T-line cells (3×10^4 cells/well) were cultured in flat-bottom microtitre plates with GPBP (10–100 μg/ml), GPBP plus a suboptimal dose of IL-2 (2 U/ml) or Con A (5 U/ml) in the presence or absence of irradiated thymocytes ($3\text{–}5 \times 10^5$ cells/well) or brain cells ($1\text{–}30 \times 10^3$ cells/well). The microwell cultures were incubated for 3–5 days, with

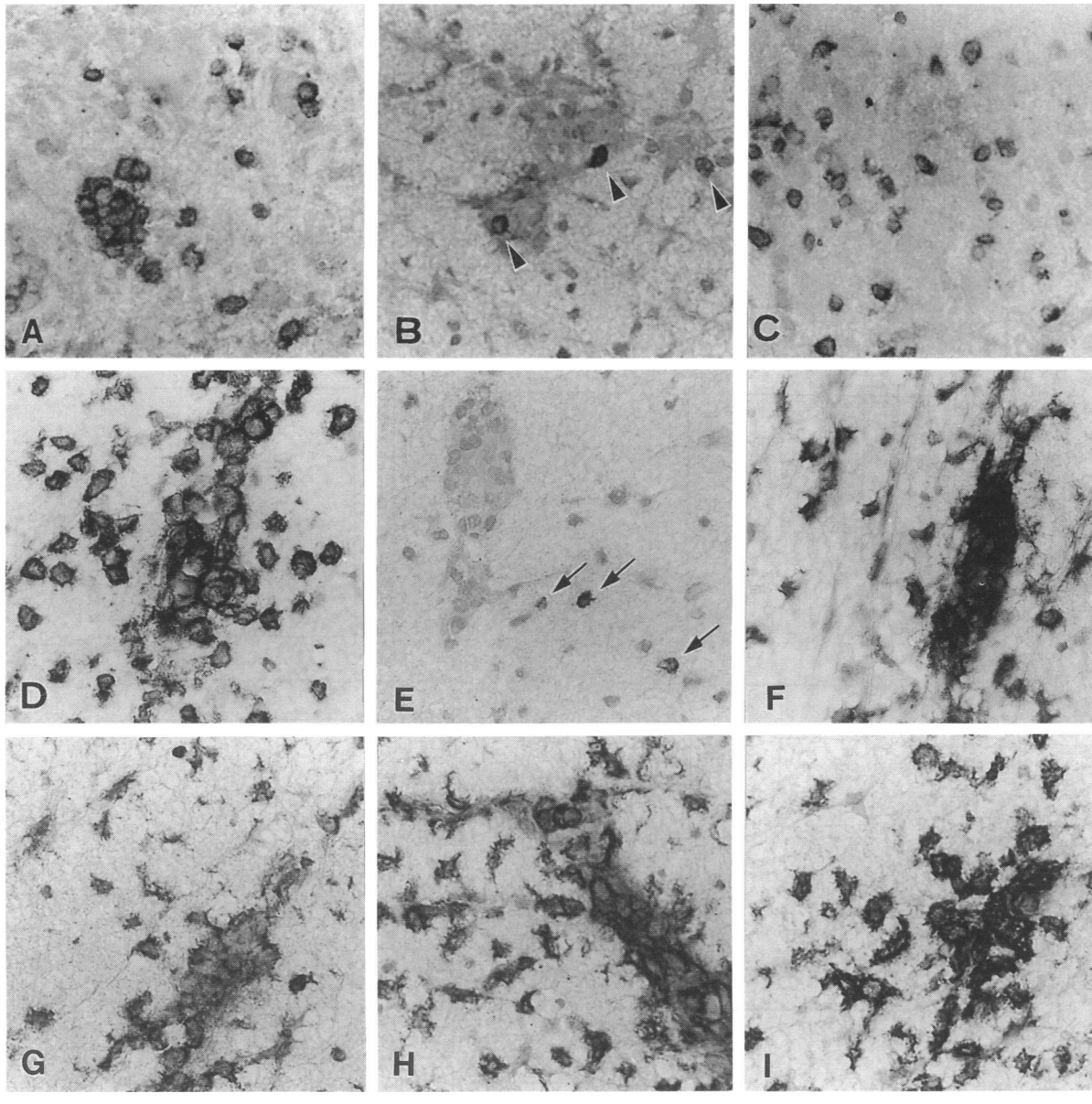

Figure 1. Immunohistochemical staining of infiltrating T cells and other inflammatory cells in the spinal cord with EAE lesions. (A) W3/25 (anti-CD4); (B) OX8 (anti-CD8); (C) R73 (anti-TcR); (D) OX6 (anti-Ia); (E) OX39 (anti-IL-2R); (F) anti-ICAM-1; (G) anti-LFA-1α; (H) OX42 (anti-macrophage and microglia); (I) anti-LFA-1β. Magnification $\times 211{\cdot}5$.

the last 18 hr in the presence of 0·5 μCi [^{3}H]thymidine (Amersham). The cells were harvested on glass fibre filters, and the label uptake was determined using the standard liquid scintillation techniques.

RESULTS

In situ phenotyping of infiltrating inflammatory cells in the spinal cord

Using a panel of mAb, the surface phenotype of infiltrating T cells and other inflammatory cells was determined by immunohistochemistry. As shown in Fig. 1, many CD4$^+$ (W3/25$^+$) cells were detected in EAE lesions (Fig. 1A), whereas CD8$^+$ (OX8$^+$) cells were very few in number (Fig. 1B). Although CD4$^+$ cells formed clusters around blood vessels in most cases, T-cell receptor (TcR) $\alpha\beta^+$ (R73$^+$) cells were evenly distributed in the parenchyma (Fig. 1C). This difference is probably attributable to the fact that in contrast to R73, W3/25 recognizes not only T cells, but also CD4$^+$ macrophage and microglia.[21] Most inflammatory cells including T cells expressed Ia antigens (Fig. 1D), whereas IL-2 receptor-expressing cells were few in number compared with cells bearing other T-cell markers (Fig. 1E). With regard to adhesion molecules, LFA-1α (CD11a), OX42 (CD11b), LFA-1β (CD18) and intracellular adhesion molecule-1 (ICAM-1) were examined immunohistochemically. Most inflammatory cells as well as reacting microglia expressed LFA-

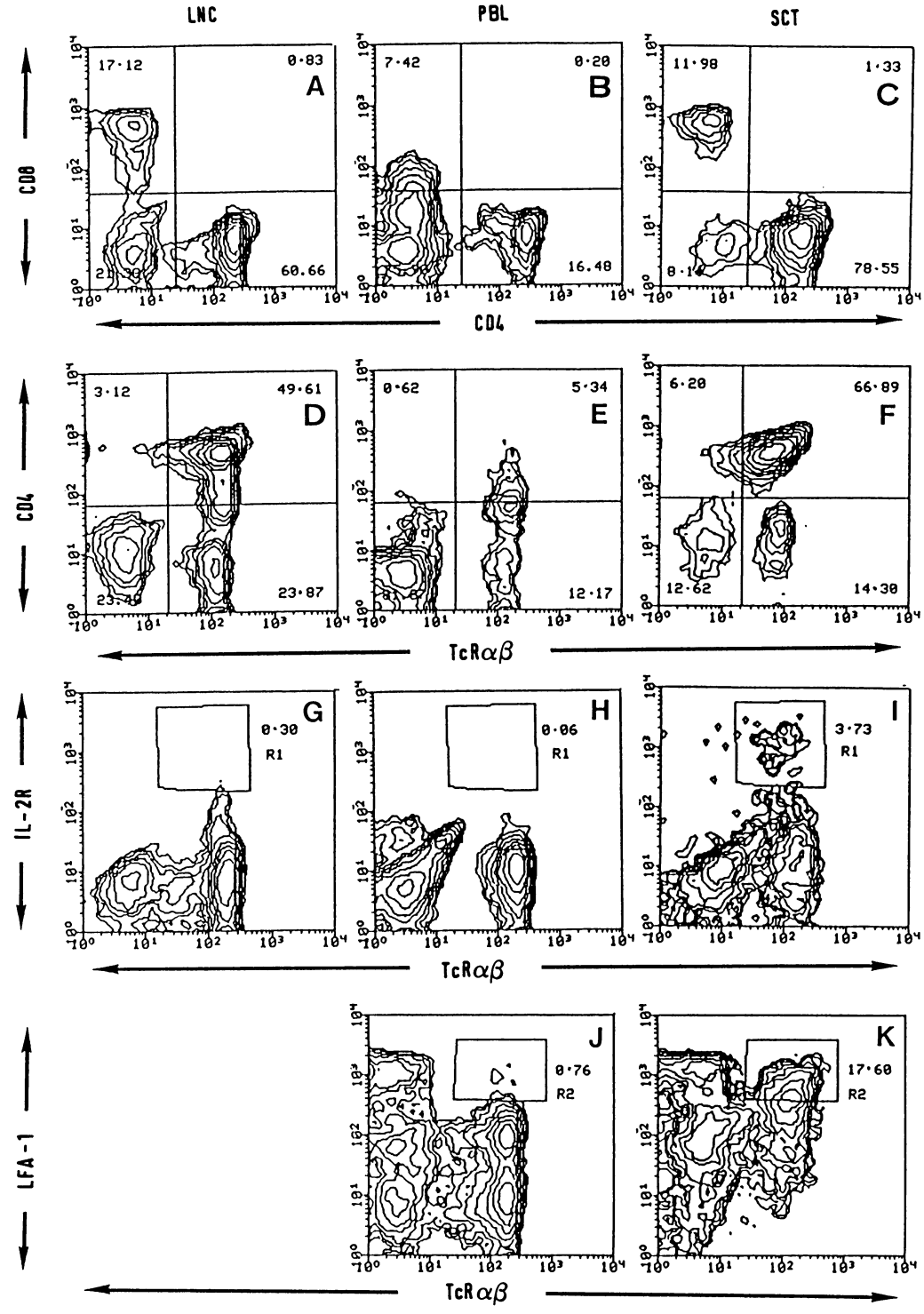

Figure 2. FCM analysis of LNC (A, D and G), PBL (B, E, H and J) and SCT (C, F, I and K) taken from rats with full-blown EAE. Samples (LNC, PBL and SCT) for each combination of staining were taken from the same rats. Green fluorescence is shown on the *x*-axes and red fluorescence is on the *y*-axes.

1α, OX42 and LFA-1β (Fig. 1G, H and I). Astrocytes, on the other hand, did not express detectable levels of these adhesion molecules (data not shown). Although ICAM-1 was localized on vascular endothelial cells under normal conditions, inflammatory cells and reactive microglia also expressed ICAM-1 in the CNS with EAE (Fig. 1F). These findings indicate that infiltrating T cells in EAE lesions, most of which are positive for CD4 and bear αβ chain of TcR, express Ia antigens and various types of adhesion molecules, suggesting that they may be activated.

Table 1. Proliferative responses of inflammatory cells and T cells freshly isolated from the spinal cord with EAE lesions*

Group (cell no.)†	Proliferation (c.p.m. $\times 10^{-3}$)‡	
	Medium	GPBP (stimulation index)
(A) CNS inflammatory cells§ (2×10^5)	0·2	0·2 (1·0)
CNS inflammatory cells (2×10^6)	0·2	0·4 (2·0)
(B) Nylon wool-passed T cells (SCT)¶ (2×10^5)	1·7	3·7 (2·1)
(C) Nylon wool-passed T cells + thymocytes (3×10^5)	1·5	4·8 (6·3)
(D) Nylon wool-passed T cells + astrocytes** (3×10^4)	0·7	7·6 (1·5)
Nylon-wool passed T cells + IFN-γ-treated†† astrocytes (3×10^4)	1·4	7·7 (2·0)

* This experiment was repeated five times and essentially the same results were obtained.

† The cell number in each well in the proliferation assay.

‡ Each value indicates mean c.p.m. $\times 10^{-3}$ of triplicate wells. The SD were within 10% of the means.

§ CNS inflammatory cells were isolated from EAE lesions by spinning a mixture of the spinal cord tissue suspension and Ficoll–Hypaque solution at a volume ratio of 1:1.

¶ SCT were obtained by passing CNS inflammatory cells through a nylon wool column.

** Astrocytes were obtained by passaging the primary mixed glial cell culture three to five times. Cultures contained more than 95% of astrocytes as determined by staining for glial fibrillary acidic protein and were free from microglia.

†† Astrocytes were treated with 100 U/ml IFN-γ for 48 hr before use to induce Ia antigens.

Table 2. Proliferative responses of T cells freshly isolated from the lymph nodes*

Group (cell no.)†	Proliferation (c.p.m. $\times 10^{-3}$)‡		
	Medium	GPBP	PPD
(A) Nylon wool-passed T cells (2×10^5)§	3·8	24·2 (100%)	47·8 (100%)
(B) Nylon wool-passed T cells + thymocytes (3×10^5)	2·2	57·5 (238%)	106·2 (222%)
(C) Nylon wool-passed T cells + astrocytes¶ (3×10^4)	3·5	3·1 (12%)	34·7 (72·6%)
Nylon wool-passed T cells + IFN-γ-treated** astrocytes (3×10^4)	4·7	4·4 (18%)	49·2 (103%)

* † ‡ ¶ ** See footnotes to Table 1.

§ LNT were obtained by passing LNC through a nylon wool column.

Flow cytometry (FCM) analysis of spinal cord T cells

In order to characterize further and quantify infiltrating T cells, two-colour FCM analysis was performed and the profile of SCT was compared with that of LNC and PBL (Fig. 2). $CD4^+$ cells accounted for approximately 80%, whereas $CD8^+$ cells accounted for 12% (Fig. 2C). By two-colour analysis with mAb against CD4 and TcR$\alpha\beta$, it was revealed that 70% of $CD4^+$ cells bore $\alpha\beta$ TcR (Fig. 2F). $CD4^-$ TcR$\alpha\beta^+$ cells (15%) would be $CD8^+$ T cells. These findings indicate that $CD4^+$ T cells are the major component of SCT. PBL, on the other hand, contained much fewer $CD4^+$ $CD8^-$ cells (17%; Fig. 2B) and $CD4^+$ TcR$\alpha\beta^+$ cells (5%; Fig. 2E). A decrease in $CD8^+$ cells was frequently observed in PBL taken from rats with full-blown EAE (Fig. 2B). We further examined the phenotype of SCT using mAb against IL-2 receptors and LFA-1 molecules (Fig. 2G–K). It was clearly demonstrated that the expression of IL-2 receptors was up-regulated on SCT compared with that of LNC and PBL. There were virtually no $IL\text{-}2R^+$ cells in the latter. Similarly, LFA-1 expression on SCT was augmented (Fig. 2K). In summary, SCT were mainly composed of $CD4^+$ TcR$\alpha\beta^+$ T cells, the IL-2R and LFA-1 expression of which was augmented.

SCT responded poorly to MBP

We then isolated SCT from rats on days 12 and 13 post-immunization and measured their proliferative responses to MBP under various conditions. As shown in Table 1, inflammatory cells (approximately 30% T cells and 70% non-T cells including brain cells) isolated from EAE lesions did not respond to GPBP at all (Table 1, group A). Brain cells and some macrophages were then depleted by passing inflammatory cells through a nylon wool column. At this stage, T cells were enriched to more than 80% of the total cells (Fig. 2F). However, proliferative responses of SCT to GPBP were still weak (Table 1, group B). The addition of thymocytes augmented SCT proliferation (Table 1, group C), but the response was low compared with that of lymph node T cells (LNT) (see below). Similarly, the addition of astrocytes with or without IFN-γ treatment did not augment T-cell proliferation significantly (Table 1, group D). All groups of T cells except group A responded well to Con A (data not shown).

It is of interest to note the effect of astrocytes on the reactivity of LNT to the antigens. Compared with SCT, LNT responded well to both GPBP and PPD without accessory cells (Table 2, Group A) presumably because of the presence of a

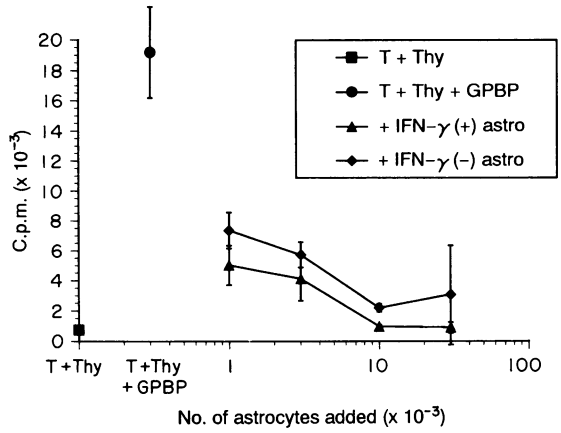

Figure 3. Suppression of GPBP-driven T-cell proliferation by astrocytes which had been treated with or without IFN-γ. Astrocytic cultures were treated with 100 U/ml IFN-γ or left untreated for 48 hr. They were then tripsinized, washed, irradiated and added at the indicated numbers to GPBP-specific T-line cells (3×10^4 cells/well) in the presence of GPBP (25 μg/ml) and irradiated thymocytes (5×10^5 cells/well). Per cent suppression for IFN-γ-treated astrocytes ranged from 75% to 95% and that for untreated astrocytes ranged from 60% to 85%. The representative results of three experiments are shown.

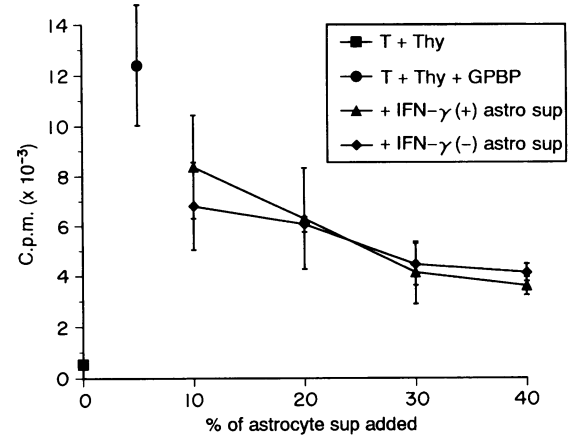

Figure 4. Suppression of GPBP-driven T-cell proliferation by supernatant from astrocytic cultures treated with or without IFN-γ. Supernatant from astrocytic cultures was filtered though a 0·22-μm filter and added to the culture in which T-line cells were stimulated with GPBP in the presence of irradiated thymocytes. Per cent inhibition ranged from approximately 40% to 60%. The representative results of three experiments are shown.

small amount of APC (non-T cells in the LNT population accounted for less than 5% by FCM analysis). Addition of thymocytes, however, resulted in a twofold increase in responses to both antigens (Table 2, group B). On the other hand, astrocytes either treated or not treated with IFN-γ suppressed the responses of LNT to GPBP, whereas the response to PPD was relatively preserved (72·6% and 103% of the response of LNT alone).

Characterization of immunosuppressive effects of astrocytes

To characterize the functions of astrocytes in more detail, IFN-γ-treated or untreated astrocytes were added to the cultures in which MBP-specific T cells established from LNC of GPBP-immunized rats were stimulated in the presence of GPBP and irradiated thymocytes. As shown in Fig. 3, addition of astrocytes (▲, ◆) resulted in a marked reduction in the proliferative response of T cells. Ia-expressing astrocytes after IFN-γ treatment suppressed the response more strongly than untreated and Ia-negative astrocytes. Furthermore, supernatant harvested from the astrocytic cultures suppressed T-cell proliferation in a dose-dependent manner (Fig. 4). In this case, supernatant from both IFN-γ-treated and untreated astrocytic cultures showed the same suppressive activity. Since it was reported that astrocytes release several suppressive cytokines,[22-24] we next examined if these cytokines were involved in this immunosuppression. To do this, indomethacin and anti-TGF-β (1+2) antibody were added to the culture (Fig. 5). By addition of indomethacin (1 μg/ml), responses of T cells partially recovered, whereas anti-TGF-β (1+2) antibody did not alter the astrocytic suppression under the conditions tested [Fig. 5 (⊠)]. The finding that anti-TGF-β antibody did not eliminate the immunosuppression was quite unexpected because TGF-β is thought to be a final inhibitor of immune responses in the CNS.[25,26] We therefore repeated the same experiment using different antibodies against TGF-β1 and -β2. As shown in Fig. 6, saturating

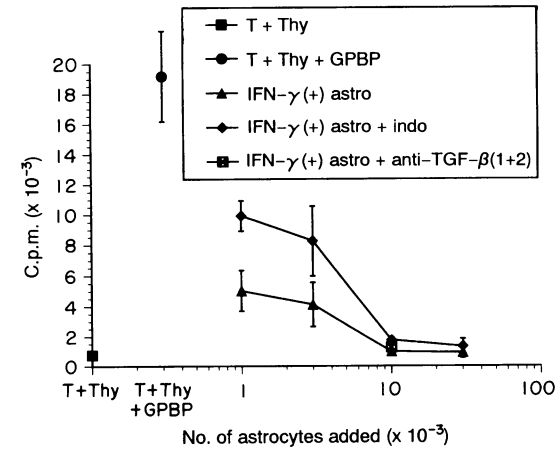

Figure 5. Effect of indomethacin and anti-TGF-β (1+2) antibody on astrocyte-driven immunosuppression of T-cell proliferation to GPBP. Addition of indomethacin [1 μg/ml (◆)] to the culture containing GPBP-reactive T-line cells, GPBP, thymocytes and astrocytes resulted in partial recovery of T-cell proliferation. Per cent inhibition shifted from 75% to 50% in the presence of 10^3 astrocytes and from 80% to 55% in the presence of 10^4 astrocytes. On the other hand, anti-TGF-β (1+2) antibody [10 μl/well (⊠)] did not eliminate the suppressive activity of astrocytes under the conditions tested.

doses of the neutralizing antibodies were added to the assay. Again, addition of both the antibodies did not augment the T-cell responses which were suppressed by IFN-γ-treated (Fig. 6) and untreated (data not shown) astrocytes.

Reactivity of T-cell lines derived from spinal cord T cells to MBP

Finally, we examined whether the low responsiveness of SCT to GPBP is a short-term or long-lasting phenomenon. For this purpose, SCT were divided into two parts. One part of SCT was

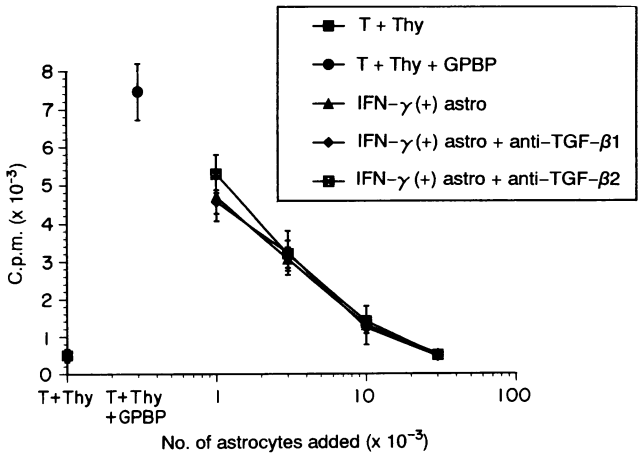

Figure 6. Effect of anti-TGF-β1 and anti-TGF-β2 antibodies on astrocyte-driven immunosuppression of T-cell proliferation. Neutralizing antibodies against TGF-β1 (◆) and TGF-β2 (⊠) at a final concentration of 5 μg/ml (saturating dose) were added to the culture. As well as anti-TGF-β (1+2) antibody, these antibodies did not inhibit astrocytic immunosuppression.

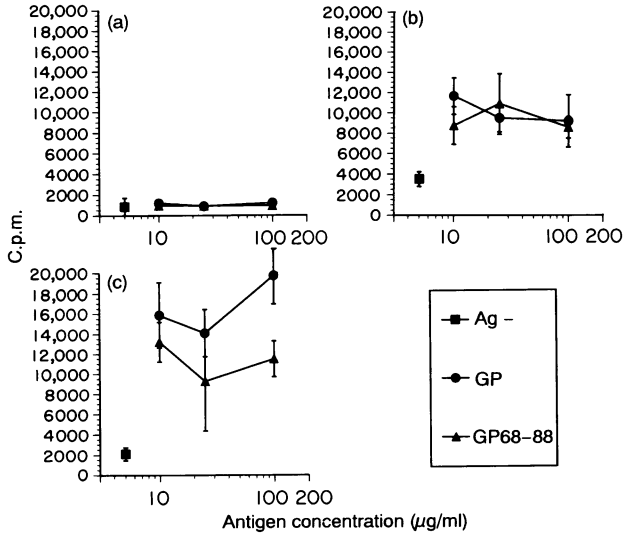

Figure 7. Proliferative responses of SCT (a and b) and LNC (c) at the third stimulation with antigen. SCT were isolated from the spinal cord with EAE lesions and divided into two parts. One part of SCT (a) and LNC (c) was first stimulated with GPBP without exogenous IL-2 in the presence of thymocytes as APC and the other part of SCT (b) was stimulated with GPBP and IL-2 plus APC. After two more stimulations with the antigen and APC followed by propagation with Con A supernatant, the proliferative responses of three types of T-line cells to GPBP and a synthetic peptide corresponding to the 68–88 sequence of GPBP (GP68–88) were measured. The representative results of three experiments are shown.

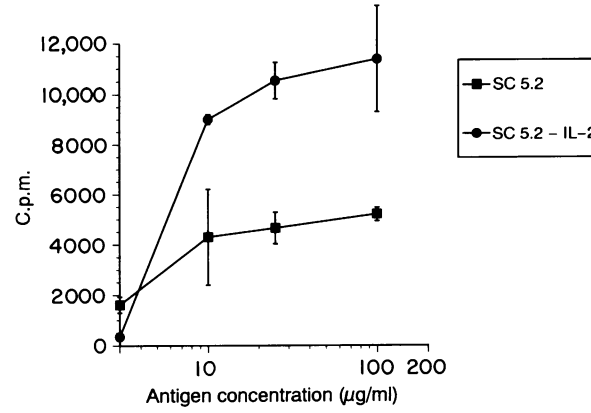

Figure 8. Proliferative responses of SCT-derived T-line cells to GPBP after twelfth stimulation with GPBP. T-line cells first stimulated with GPBP without IL-2 (a cell line shown in Fig. 7a) or with GPBP plus IL-2 (a cell line shown in Fig. 7b) were maintained *in vitro* by repeated antigen stimulation (SC 5.2 and SC 5.2–IL-2, respectively). After the twelfth stimulation, proliferative responses to indicated concentrations of GPBP were measured. The representative results of three experiments are shown.

cultured with GPBP and irradiated thymocytes as APC in the presence of a suboptimal dose of IL-2 at the beginning of the culture. The other part of SCT was stimulated with GPBP and APC without exogenous IL-2. After the first antigen stimulation, they were propagated in the medium containing Con A supernatant. The subsequent stimulation and propagation procedures for the above-mentioned groups were exactly the same. Then, the proliferating responses of these two types of SCT to GPBP and the encephalitogenic synthetic peptide corresponding to the 68–88 sequence of GPBP (GP68–88) were measured at the third antigen stimulation and compared with those of LNC-derived T-line cells (Fig. 7). Even after three rounds of MBP stimulation, SCT-derived T-line cells stimulated with GPBP without IL-2 (Fig. 7A) did not respond to GPBP and GP68–88 at all. In contrast, T-line cells stimulated first with GPBP and IL-2 (Fig. 7B) responded to the antigens although the magnitude of the response was half as strong as that of LNC-derived T-line cells (Fig. 7C). We maintained these T-cell lines by stimulating them with the antigen followed by propagation with Con A supernatant, and measured antigen reactivity at the twelfth antigen stimulation (Fig. 8). A T-cell line stimulated with GPBP without IL-2 at the beginning (SC 5.2) recovered the proliferating ability slightly. However, its response was still lower than that of a line stimulated with the antigen plus IL-2 (SC 5.2–IL-2). These findings indicate that low responsiveness of SCT is a long-lasting phenomenon, but is partially overcome by the addition of exogenous IL-2 in the cultures.

DISCUSSION

EAE is a T cell-mediated autoimmune disease characterized by the presence of inflammatory cells mainly comprising T cells and macrophages in the CNS. In active EAE, infiltration of inflammatory cells into the CNS begins around day 10 post-immunization and reaches a maximum on day 12. Between days 12 and 15, inflammation is at a plateau state and then most lesions subside by day 21.[8,21] Although these processes are well documented, the kinetics of infiltrating T cells in terms of

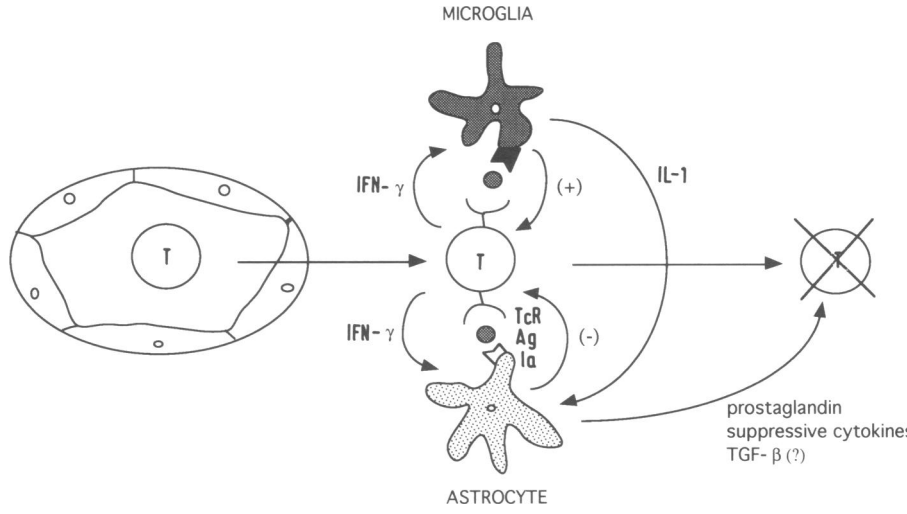

Figure 9. Proposed mechanism of local immune regulation in the CNS with EAE lesions.

antigen reactivity and proliferating ability is poorly understood. Since it was previously reported that Ia$^+$ brain cells such as astrocytes[4,5] and microglia[6] have an ability to present MBP to encephalitogenic T cells, it has been presumed that brain cells *in situ* present brain-specific antigens to infiltrating T cells to induce further T-cell proliferation. However, our previous studies using immunohistochemistry[7,8] have shown that infiltrating T cells do not proliferate vigorously in the CNS. Furthermore, cultured mixed glial cells (comprising microglia and astrocytes) have a weak antigen-presenting ability even after treatment with IFN-γ to induce Ia antigen expression.[9] These findings prompted us to investigate the nature of infiltrating T cells in the spinal cord with EAE lesions.

In the present study, it was clearly shown that T cells isolated from the spinal cord of rats with full-blown EAE responded poorly to MBP. More importantly, this low responsiveness might be induced by astrocytes for the following reasons. Inflammatory cells isolated from EAE lesions which contained brain cells did not respond to GPBP unless brain cells were removed by passing them through a nylon wool column. Conversely, the addition of astrocytes resulted in suppression of the proliferation of GPBP-specific T cells. *In vitro* study using cultured astrocytes with or without IFN-γ treatment and GPBP-specific T-line cells has suggested that several mechanisms may operate in this immunosuppression. First is the antigen-driven T-cell unresponsiveness which occurs after TcR occupancy and lack of co-stimulatory signals.[27-29] This possibility was supported by the finding that Ia$^+$ astrocytes suppressed T-cell proliferation more strongly than Ia$^-$ astrocytes (Fig. 3). In addition, astrocytes suppressed T-cell response to MBP more markedly than that to purified protein derivative (PPD) (Table 2). These findings suggest that this suppression may take place in the process of antigen presentation. Furthermore, low responsiveness of SCT was partially overcome by adding a low dose of IL-2. All the findings fit this model well. Second, it is possible that activated astrocytes in response to inflammatory lesions[30] release immunosuppressive factors such as TGF-β[31] and prostaglandin.[22] In fact, supernatant from the cultures of both IFN-γ-treated and untreated astrocytes suppressed T-cell proliferation in a dose-dependent manner (Fig. 4). Furthermore, addition of indomethacin to the cultures resulted in partial recovery of suppression of T-cell proliferation by astrocytes (Fig. 5), suggesting that prostaglandin released by astrocytes may be involved in this suppression. On the other hand, anti-TGF-β antibodies did not eliminate T-cell suppression (Figs 5 and 6). This was quite an unexpected result because TGF-β is reported to be a leading candidate for the end-organ inhibitor.[25,26] We therefore repeated the same experiment using different neutralizing antibodies against TGF-β1, -β2 and -β(1+2). Even when the antibodies were used at saturating doses (according to the manufacturer's information), the suppressive effect by astrocytes was not eliminated. There are, at least, two possibilities. One is that TGF-β may not be involved in this immunosuppression. The other is that TGF-β released by astrocytes would be effective only when astrocytes attach to T cells. In the latter case, neutralization of TGF-β released into the culture medium by the antibodies may not interfere with the actual function of this cytokine. To examine the mechanism of astrocyte-driven immunosuppression in more detail, we are currently investigating *in situ* expression of TGF-β in the CNS with EAE at the protein and message level. In the present study, it was revealed that more than one mechanism, i.e. antigen-driven T-cell unresponsiveness and secretion of suppressive humoral factor(s) might operate in inactivation of infiltrating T cells in the CNS. Taken together, the immune regulation in the CNS may take place as follows (Fig. 9). Infiltrating T cells release IFN-γ, which induce Ia antigens on microglia and to a lesser degree on astrocytes.[9] Ia$^+$ microglia, in turn, present antigen to infiltrating T cells to induce T-cell proliferation (indicated by + in Fig. 9), whereas Ia$^+$ astrocytes give negative signals in the same manner (indicated by −) as shown in the present study. Astrocytes also release prostaglandin or suppressive cytokines to inactivate infiltrating T cells after cognate interaction with T cells and probably after stimulation with cytokines such as IL-1 which is released by microglia. All these negative signals mainly provided by astrocytes might cease T-cell proliferation in the CNS.

The finding that T cells isolated from the CNS with EAE lesions responded poorly to MBP was observed by several investigators.[32-35] In general, it was presumed that this was due to the low frequency of MBP-reactive T cells in the SCT population[34,35] compared with that in the lymph node and spleen.[1,34,36] However, recent studies have shown that although

there are many T cells which are potentially reactive with MBP, their reactivity is suppressed, which results in the seemingly low frequency of MBP-reactive T cells. Hershkoviz *et al.* applied the improved assay system in which IL-2 was added at the onset of the cell culture and found that the actual frequency of MBP-reactive T cells in the CNS with EAE lesions was higher than that in the spleen and lymph node.[37] In addition, we have recently observed that encephalitogenic T cells bearing Vβ8.2 of TcR preferentially infiltrate the CNS and comprise approximately 20% of the total infiltrating T cells (M. Tsuchida, H. Hanawa, Y. Matsumoto and T. Abo, manuscript submitted for publication). Taken together, it can be concluded that low responsiveness of infiltrating T cells in the CNS to brain-specific antigens results from *in situ* inactivation by brain cells, and not from paucity of the antigen-reactive T cells.

What then is the implication of this phenomenon? It has recently been found that non-haematopoietic resident cells in most organs are able to express Ia antigens both *in vivo* and *in vitro*. Initially, it was presumed that in organ-specific autoimmune diseases, these Ia$^+$ parenchymal cells might stimulate the proliferation of infiltrating T cells in target organs by presenting tissue-specific antigens.[21] However, it is currently believed that resident parenchymal cells on which Ia antigens are induced *in situ* in inflammatory lesions or *in vitro* IFN-γ treatment rather suppress T-cell proliferation and other immune responses. Such parenchymal cells of non-haematopoietic origin reported so far are Müller cells in the retina,[38] keratinocytes,[39] thyrocytes in the thyroid gland[40] and astrocytes.[9] Therefore, it is reasonable to speculate that in organ-specific autoimmune diseases, T cells responsible for the development of the diseases mainly proliferate in the lymphoid organs, and that in the target organs, T-cell activity is down-regulated by parenchymal cells. Parenchymal cells in each organ may play a pivotal role in local immune regulation and healing processes of organ-specific autoimmune diseases.

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REFERENCES

1. Matsumoto Y., Kawai K., Tomita Y. & Fujiwara M. (1990) Limiting-dilution analysis of the frequency of myelin basic protein-reactive T cells in Lewis, PVG/c and BN rats. Implication for susceptibility to autoimmune encephalomyelitis. *Immunology*, **69,** 215.
2. Naparstek Y., Ben-Nun A., Holoshitz J., Reshef T., Frenkel A., Rosenberg M. & Cohen, I.R. (1983) T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells. *Eur. J. Immunol.* **13,** 418.
3. Holoshitz J., Naparstek Y., Ben-Nun A., Marquardt P. & Cohen I.R. (1984) T lymphocyte lines induce autoimmune encephalomyelitis, delayed hypersensitivity and bystander encephalitis or arthritis. *Eur. J. Immunol.* **14,** 729.
4. Fontana A., Fierz W. & Wekerle H. (1984) Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature*, **307,** 273.
5. Fierz A., Endler B., Reske K., Wekerle H. & Fontana A. (1985) Astrocytes as antigen-presenting cells. I. Induction of Ia antigen expression on astrocytes by T cells via immune interferon and its effect on antigen presentation. *J. Immunol.* **134,** 3785.
6. Frei K., Siepl C., Groscurth P., Bodmer S., Schwerdel C. & Fontana A. (1987) Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells. *Eur. J. Immunol.* **12,** 237.
7. Matsumoto Y., Kawai K. & Fujiwara M. (1988) Hemorrhagic autoimmune encephalomyelitis induced by adoptive transfer of activated semiallogeneic spleen cells into irradiated rats. *Am. J. Pathol.* **133,** 306.
8. Ohmori K., Hong Y., Fujiwara M. & Matsumoto Y. (1992) *In situ* demonstration of proliferating cells in the rat central nervous system during experimental autoimmune encephalomyelitis. Evidence suggesting that most infiltrating T cells do not proliferate in the target organ. *Lab. Invest.* **66,** 54.
9. Matsumoto Y., Ohmori K. & Fujiwara M. (1992) Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under *in vivo*-mimicking conditions. *Immunology*, **76,** 209.
10. White R.A.H., Mason D.W., Williams A.F., Galfre G. & Milstein C. (1978) T-lymphocyte heterogeneity in the rat: separation of functional subpopulations using monoclonal antibody. *J. exp. Med.* **148,** 664.
11. Brideau R.J., Carter P.B., McMaster W.R., Mason D.W. & Williams A.F. (1980) Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur. J. Immunol.* **10,** 609.
12. Hünig T., Wallny H., Hartley J.K., Lawetzky A. & Tiefenthaler G. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. *J. exp. Med.* **169,** 73.
13. Paterson D.J., Jefferies W.A., Green J.R., Brandon M.R., Corthesy P., Puklavec M. & Williams A.F. (1987) Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blast. *Mol. Immunol.* **24,** 1281.
14. Williams A.F., Galfre G. & Milstein C. (1977) Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell*, **12,** 663.
15. Robinson A.P., White T.M. & Mason D.W. (1986) Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC OX-41 and OX-42, the latter recognizing complement receptor type 3. *Immunology*, **57,** 239.
16. Tamatani T. & Miyasaka M. (1990) Identification of monoclonal antibodies reactive with the rat homolog of ICAM-1, and evidence for a differential involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *Int. Immunol.* **2,** 165.
17. Tamatani T., Kotani M. & Miyasaka M. (1991) Characterization of the rat leukocyte integrin CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur. J. Immunol.* **21,** 627.
18. Matsumoto Y., Kawai K. & Fujiwara M. (1990) Analysis of the T cell repertoire for myelin basic protein in thymus-grafted and other types of chimera: evidence that major histocompatibility complex molecules on accessory cells rather than T cell specificity mainly regulate susceptibility to autoimmune encephalomyelitis. *Eur. J. Immunol.* **20,** 2119.
19. Matsumoto Y., Watabe K. & Ikuta F. (1985) Immunohistochemical study on neuroglia identified by the monoclonal antibody against a macrophage differentiation antigen (Mac-1). *J. Neuroimmunol.* **9,** 379.
20. Matsumoto Y. & Fujiwara M. (1988) Adoptively transferred experimental allergic encephalomyelitis in chimeric rats: identification of transferred cells in the lesions of the central nervous system. *Immunology*, **65,** 23.
21. Matsumoto Y., Hara N., Tanaka R. & Fujiwara M. (1986) Immunohistochemical analysis of the rat central nervous system

during experimental allergic encephalomyelitis, with special reference to Ia-positive cells with dendritic morphology. *J. Immunol.* **136,** 3668.

22. Fontana A., Kristensen F., Dubs R., Gemsa D. & Weber E. (1982) Production of prostaglandin E and an interleukin-1 like factor by cultured astrocytes and C6 glioma cells. *J. Immunol.* **129,** 2413.
23. Constam D.B., Philipp J., Malipiero U.V., ten Dijke P., Chachner M. & Fontana A. (1992) Differential expression of transforming growth factor-β1, -β2, and -β3 by glioblastoma cells, astrocytes and microglia. *J. Immunol.* **148,** 1404.
24. da Cunha A. & Vitkovic L. (1992) Transforming growth factor-beta 1 (TGF-β1) expression and regulation in rat cortical astrocytes. *J. Neuroimmunol.* **36,** 157.
25. Racke M.K., Cannella B., Albert P., Sporn M., Raine C.S. & McFarlin D.E. (1992) Evidence of encogenous regulatory function of transforming growth factor-β1 in experimental allergic encephalomyelitis. *Int. Immunol.* **4,** 615.
26. Karpus W.J. & Swanborg R.H. (1991) $CD4^+$ suppressor cells inhibit the function of effector cells of experimental autoimmune encephalomyelitis through a mechanism involving transforming growth factor-β. *J. Immunol.* **146,** 1163.
27. Jenkins M.K., Pardoll D.M., Mizuguchi J., Chused T.M. & Schwartz R.H. (1987) Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. natl. Acad. Sci. U.S.A.* **84,** 5409.
28. Jenkins M.K. & Schwartz R.H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. exp. Med.* **165,** 302.
29. Schwartz R.H. (1990) A cell culture model for T lymphocyte clonal anergy. *Science*, **248,** 1349.
30. Matsumoto Y., Ohmori K. & Fujiwara M. (1992) Microglial and astroglial reactions to inflammatory lesions of experimental autoimmune encephalomyelitis in the rat central nervous system. *J. Neuroimmunol.* **37,** 23.
31. Bodmer S., Strommer K., Frei K., Siepl C., Tribolet N.D., Heid I. & Fontana A. (1989) Immunosuppression and transforming growth factor-β in glioblastoma. Preferential production of transforming growth factor-β2. *J. Immunol.* **143,** 3222.
32. Hayosh N.S. & Swanborg R.H. (1986) Autoimmune effector cells. VII. Cells isolated from thymus and spinal cord of rats with experimental allergic encephalomyelitis transfer disease. *Am. J. Pathol.* **122,** 218.
33. Lyman W.D., Abrams G.A. & Raine C.S. (1989) Experimental autoimmune encephalomyelitis: isolation and characterization of inflammatory cells from the central nervous system. *J. Neuroimmunol.* **25,** 195.
34. Fallis R.J., Powers M.L., Sy M. & Weiner H.L. (1987) Adoptive transfer of murine chronic-relapsing autoimmune encephalomyelitis. Analysis of basic protein-reactive cells in lymphoid organs and nervous system of donor and recipient animals. *J. Neuroimmunol.* **14,** 205.
35. Cohen J.A., Essayan D.M., Zweiman B. & Lisak R.P. (1987) Limiting dilution analysis of the frequency of antigen-reactive lymphocytes isolated from the central nervous system of Lewis rats with experimental allergic encephalomyelitis. *Cell. Immunol.* **108,** 203.
36. Binder T.A., Clark R.B. & Goleschneider I. (1991) Relative susceptibility of SJL/J and B10.S mice to experimental allergic encephalomyelitis is correlated with high and low responsiveness to myelin basic protein. *J. Neuroimmunol.* **35,** 31.
37. Hershkoviz R., Mor F., Gilat D., Cohen I.R. & Lider O. (1992) T cells in the spinal cord in experimental autoimmune encephalomyelitis are matrix adherent and secrete tumor necrosis factor alpha. *J. Neuroimmunol.* **37,** 161.
38. Capsi R.R., Roberge F.G. & Nussenblatt R.B. (1987) Organ-resident, nonlymphoid cells suppress proliferation of autoimmune T-helper lymphocytes. *Science*, **237,** 1029.
39. Gaspari A.A., Jenkins M.K. & Katz S.I. (1988) Class II-bearing keratinocytes induce antigen-specific TH1 clones. *J. Immunol.* **141,** 2216.
40. Iwatani Y., Iitaka M., Row V.V. & Volpé R. (1988) Effect of HLA-DR positive thyrocytes on *in vitro* thyroid autoantibody production. *Clin. Invest. Med.* **11,** 279.