Immunology 1994 **81** 420–427

Identification of CD4$^-$ CD8$^-$ $\alpha\beta$ T cells in the subarachnoid space of rats with experimental autoimmune encephalomyelitis. A possible route by which effector cells invade the lesions

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SUMMARY

Experimental autoimmune encephalomyelitis (EAE) was induced in Lewis rats to elucidate the origin of effector T cells and the route by which they invade lesions. Since mouse studies have suggested that some autoimmune diseases are induced by extrathymic T cells in the liver, we focused our attention on the properties of mononuclear cells (MNC) isolated from the liver and other organs in rats with EAE. A small but significant proportion of LFA-1$^+$ $\alpha\beta$ T cells was identified in the liver as early as day 7 after immunization with myelin basic protein (MBP). Such LFA-1$^+$ $\alpha\beta$ T cells were also abundant among MNC attached to the spinal cord (i.e. subarachnoid space), and MNC infiltrated the spinal cord in rats with EAE (day 12). In electron microscopy, MNC attached to the spinal cord were found to be quite unique in terms of their large cell size with well-developed microvilli. More importantly, they were comprised of a considerably large proportion of double-negative CD4$^-$ CD8$^-$ T cells as well as single-positive CD4$^+$ T cells. However, the cells which infiltrated the spinal cord were mainly CD4$^+$. The present results raise the possibility that the subarachnoid space might be a major site for the expansion of extrathymic T cells in rats with EAE, and that only a limited population of CD4$^+$ T cells invade the spinal cord directly through the outer layer and elicit EAE.

INTRODUCTION

In addition to a regular pathway of T-cell differentiation in the thymus, extrathymic pathways of T-cell differentiation were recently demonstrated to exist at several sites in humans and mice. Such extrathymic T cells include intermediate T-cell receptor (TcR) in the liver[1-6] and intraepithelial lymphocytes (IEL) in the intestine.[7-9] Although extrathymic T cells contain a considerably large proportion of $\gamma\delta$ T cells, extrathymic $\alpha\beta$ T cells with unique properties also reside in the corresponding organs. In particular, extrathymic T cells in the liver have been found to have TcR of intermediate intensity (i.e. intermediate TcR cells), to contain double-negative (DN) CD4$^-$ CD8$^-$ cells, and to constitutively express high levels of interleukin-2 receptor β-chain (IL-2Rβ) and lymphocyte function-associated antigen-1 (LFA-1) similar to the case with natural killer (NK) cells.[3-6] Moreover, extrathymic T cells have been found to comprise a significant proportion of self-reactive forbidden clones as revealed by a system using minor lymphocyte stimulatory (Mls) and various anti-Vβ monoclonal antibodies (mAb).[4,10] Congenitally athymic nude mice and neonatally thymectomized mice were found to have TcR of relatively dull intensity and to contain self-reactive forbidden clones.[11,12] These T cells correspond to the intermediate TcR cells in our studies. In mice, the conditions of ageing,[13,14] bacterial infection,[4,15] malignancies[2,16,17] and autoimmune diseases[1,5,18] have been revealed to activate the extrathymic pathways in the liver and other organs. Severe thymic atrophy is always observed in mice under these conditions. Interestingly, we observed that similar thymic atrophy was induced in rats with experimental autoimmune encephalomyelitis (EAE) at the peak of their diseased state.

In the light of these findings, we investigated whether extrathymic T cells with similar properties existed in the liver or other organs of rats with or without EAE. Although intermediate TcR cells could not be detected in any organs tested in rats, unique T cells with properties similar to those of extrathymic T cells seen in autoimmune mice were demonstrated in the livers of aged rats without EAE and of rats with EAE. In particular, T cells attached to the spinal cord (i.e. subarachnoid space) displayed unique properties, including LFA-1$^+$ and DN CD4$^-$ CD8$^-$ phenotype, in rats with EAE. Such DN T cells were rarely seen in the other organs in normal and diseased rats. On the other hand, only CD4$^+$ T cells were found to infiltrate the spinal tissue. Until the present study, many investigators in

Received 7 June 1993; revised 4 August 1993; accepted 25 October 1993.

Abbreviations: DN, double-negative; EAE, experimental autoimmune encephalomyelitis; IEL, intraepithelial lymphocytes; IL-2Rβ, interleukin-2 receptor β-chain; LFA-1, lymphocyte function-associated antigen-1; MBP, myelin basic protein; Mls, minor lymphocyte stimulatory; MNC, mononuclear cells.

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this and other fields seem to have believed that effector T cells are possibly regular T cells of thymic origin which invaded the lesions by way of the blood vessels. We propose the possibility that the spinal cavity might be a major site for the expansion of extrathymic T cells in the case of these organ-specific autoimmune diseases.

MATERIALS AND METHODS

Rats

Lewis rats were obtained from Charles River Japan Inc., Atsugi, Kanagawa, Japan. All animals were fed under conventional conditions and used at the age of 3–63 weeks.

Induction of EAE

The procedure has been described in detail previously.[19,20] In brief, active EAE was induced in Lewis rats by immunization with guinea-pig myelin basic protein (MBP) in conjunction with *Mycobacterium tuberculosis* H37Ra-enriched complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). Rats were observed daily for clinical signs of EAE. Severity of clinical signs was graded into the following categories: flaccid tail, grade 1; mild paresis, grade 2; severe paresis, grade 3; and tetraparesis or moribund condition, grade 4.

Cell preparation

Mononuclear cells (MNC) in the thymus, spleen, liver and popliteal lymph nodes were collected by forcing corresponding organs through a 200-gauge stainless steel mesh. Spleen cells were haemolysed in 0·17 M Tris buffer supplemented with 0·83% NH_4Cl. Liver MNC were isolated from parenchymal hepatocytes by Ficoll–Isopaque density (1·121) gradient centrifugation at 2500 r.p.m. for 30 min.[21] Cells attached to the surface of the spinal cord were obtained by shaking the removed spinal cord in physiological saline (0·8% NaCl, 0·04% KCl, 0·1% glucose, 0·035% $NaHCO_3$, pH 7·4) supplemented with 0·015% trypsin and 0·015% collagenase. Cells that had infiltrated the spinal cord were obtained by forcing it through a 200-gauge mesh. Such cells were overlaid on the same volume of Ficoll–Isopaque and centrifuged for removal from the myeline tissue debris. Then, cells were isolated by passage through a nylon wool column. MNC in the peripheral blood were isolated by Ficoll–Isopaque (1·094) gradient centrifugation at 2000 r.p.m. for 20 min.

Immunofluorescence test

The surface phenotype of cells was identified by using mAb in conjunction with the two-colour immunofluorescence test.[21,22] Anti-CD11a mAb against LFA-1α was obtained from Seikagaku Kogyo Inc., Tokyo, Japan. FITC-conjugated anti-CD4 (W3/25), anti-CD8 (OX8), FITC- and phycoerythrin (PE)-conjugated anti-TcRαβ (R73) were obtained from Serotec Co., Blackthon, Bicester, U.K. Monoclonal antibody against rat CD3 (1F4) was kindly provided by Dr T. Masuko (Department of Hygienic Chemistry, Pharmaceutical Institute of Tohoku University, Japan). Monoclonal antibody against TcR Vβ8.2, 8.5 and 10 were kindly provided by Dr Hünig, Institute for Virology and Immunobiology, University of Wurzburg, Germany. Monoclonal antibody against Vβ16 was purchased from Serotec Co. For two-colour analysis, cells were first incubated with unlabelled mAb, followed by incubation with PE-conjugated anti-mouse IgG antibody (Biomedical Co., Forster City, CA). To ensure free binding sites of the secondary antibody, cells were incubated with normal mouse serum. FITC-conjugated mAb was then applied as the second step. Polyclonal rabbit anti-asialo-GM_1 antibody was obtained from Wako Co. (Tokyo, Japan), and was stained with FITC-conjugated donkey anti-rabbit IgG (H+L) antibody (Jackson Immunoresearch Lab. Inc., West Grove, PA). Ten thousand cells were analysed in each sample by FACScan flow cytometry (Becton Dickinson & Co., Mountain View, CA).

Preparation of tissue specimens for scanning electron microscopy

Tissue specimens were washed three times with saline, fixed with 2·5% glutaraldehyde, and dehydrated in graded ethanol. The specimens were critical-point dried, mounted and coated with gold. They were examined by using an Hitachi S-450 scanning electron microscope (Hitachi Seisakusho, Hitachi, Japan).

Adoptive transfer of cells in the subarachnoid space

MNC were isolated from rats on day 10 post-immunization, as described above, and 10^7 cells in 0·1 ml saline was injected into the subarachnoid space of the lumber spinal cord of syngeneic naive rats. To do this, a midline skin incision was made and a 27-gauge needle inserted into the intervertebral regions. For control experiments, MNC taken from the spleen of normal rats were used.

RESULTS

Appearance of LFA-1⁺ T cells in the liver of rats with EAE

In the case of mice, a small but significant proportion of T cells expressing TcR (and CD3) of intermediate intensity, and LFA-1 of high intensity were identified as extrathymic T cells in the liver.[23,24] A further examination therefore carried out to determine whether a similar population was present in the liver of rats. In this experiment, single- and two-colour stainings of liver MNC were performed in rats at various ages. Liver MNC from young rats (3 and 15 weeks old) and old rats (62 weeks old) were examined (Fig. 1). Two-colour staining for CD3 and LFA-1 and single-colour staining for LFA-1 demonstrated that LFA-1⁺ T cells appeared in the liver of aged rats. A small population with the same phenotype was already detected in rats at the age of 15 weeks. In the case of mice, almost all LFA-1⁺ T cells seen in the liver at resting conditions had CD3 of intermediate intensity (i.e. intermediate TcR cells). However, the intensity of CD3 on LFA-1⁺ T cells in the liver of rats was comparable to that on LFA-1⁻ T cells.

Two-colour staining of liver MNC for TcRαβ and LFA-1 was then performed in rats injected with CFA alone and with MBP/CFA (Fig. 2). A unique population of LFA-1 bright positive αβ T cells appeared in rats with EAE 7 days after MBP/CFA injection. This population gradually became small in the course of the disease. On days 13 and 20 post-immunization, there was only a LFA-1 dull positive population. In contrast, the population was not apparently seen in rats injected with CFA alone, although the staining pattern was slightly changed.

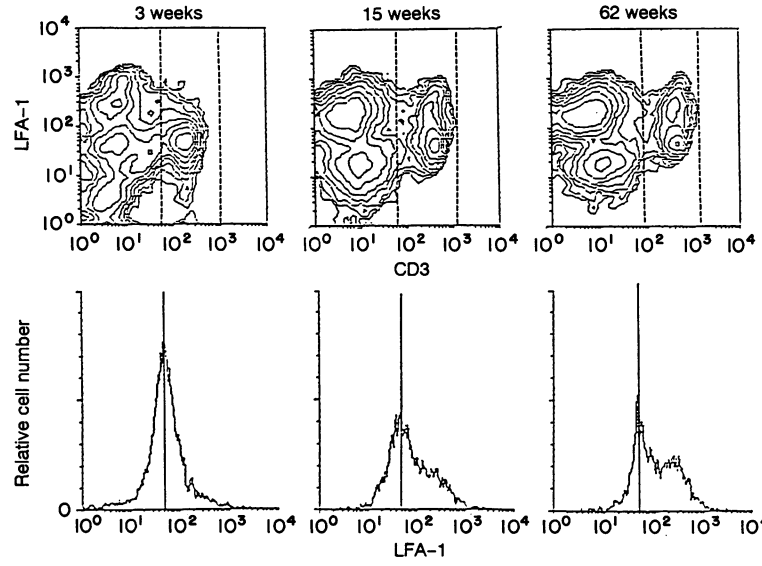

Figure 1. Identification of LFA-1$^+$ T cells in the liver of rats. Hepatic MNC were isolated from rats aged 3, 15 and 62 weeks. Two-colour staining for CD3 and LFA-1, and single-colour staining for LFA-1, were performed. Representative data from three experiments are depicted.

Time kinetics of the number of MNC obtained from the thymus and liver in rats with EAE

The clinical course of EAE induced by our procedure, which was essentially the same throughout all the experiments, is represented in Fig. 3. In rats immunized with MBP/CFA, encephalomyelitis was elicited 10 days after immunization and reached its peak on day 12 (see the clinical score). The diseased state continued to day 15 and then decreased gradually. The number of MNC obtained from the thymus and liver in these rats was examined in parallel, since the thymus and liver are known to be

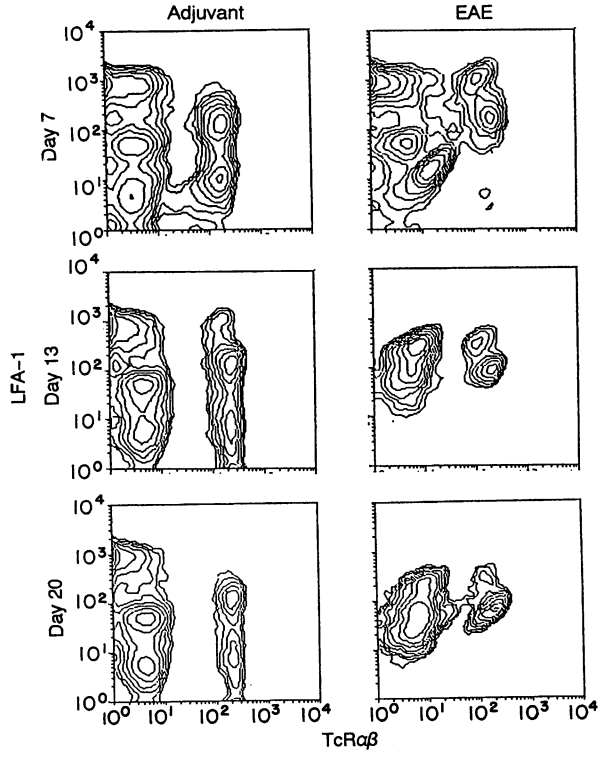

Figure 2. Appearance of LFA-1$^+$ T cells in the liver of rats with EAE. Rats aged 8 weeks were injected with CFA alone or with MBP/CFA, and hepatic MNC were isolated at the indicated days after injection. Representative data from three experiments are depicted.

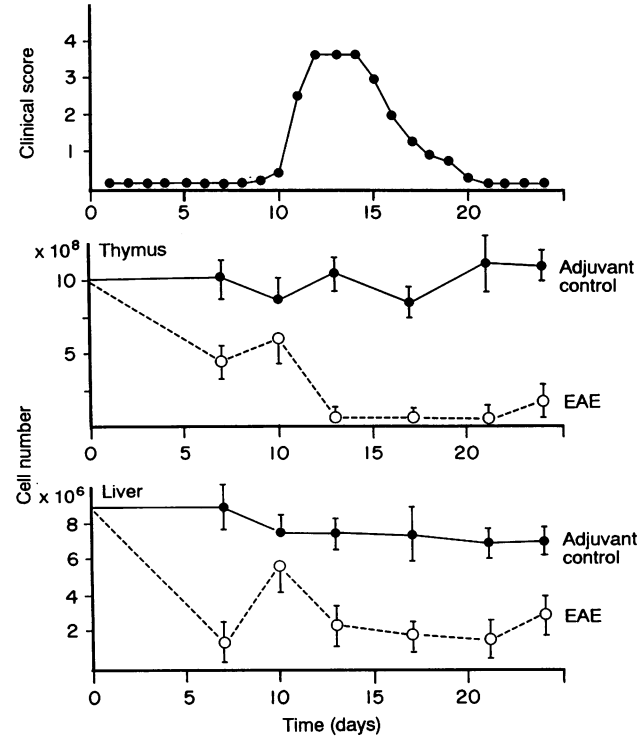

Figure 3. Time kinetics of the cell numbers yielded from the thymus and liver in rats with EAE. The cell numbers are enumerated for the indicated days after MBP/CFA injection. The mean ± SD from four rats are depicted. The clinical score is represented in parallel.

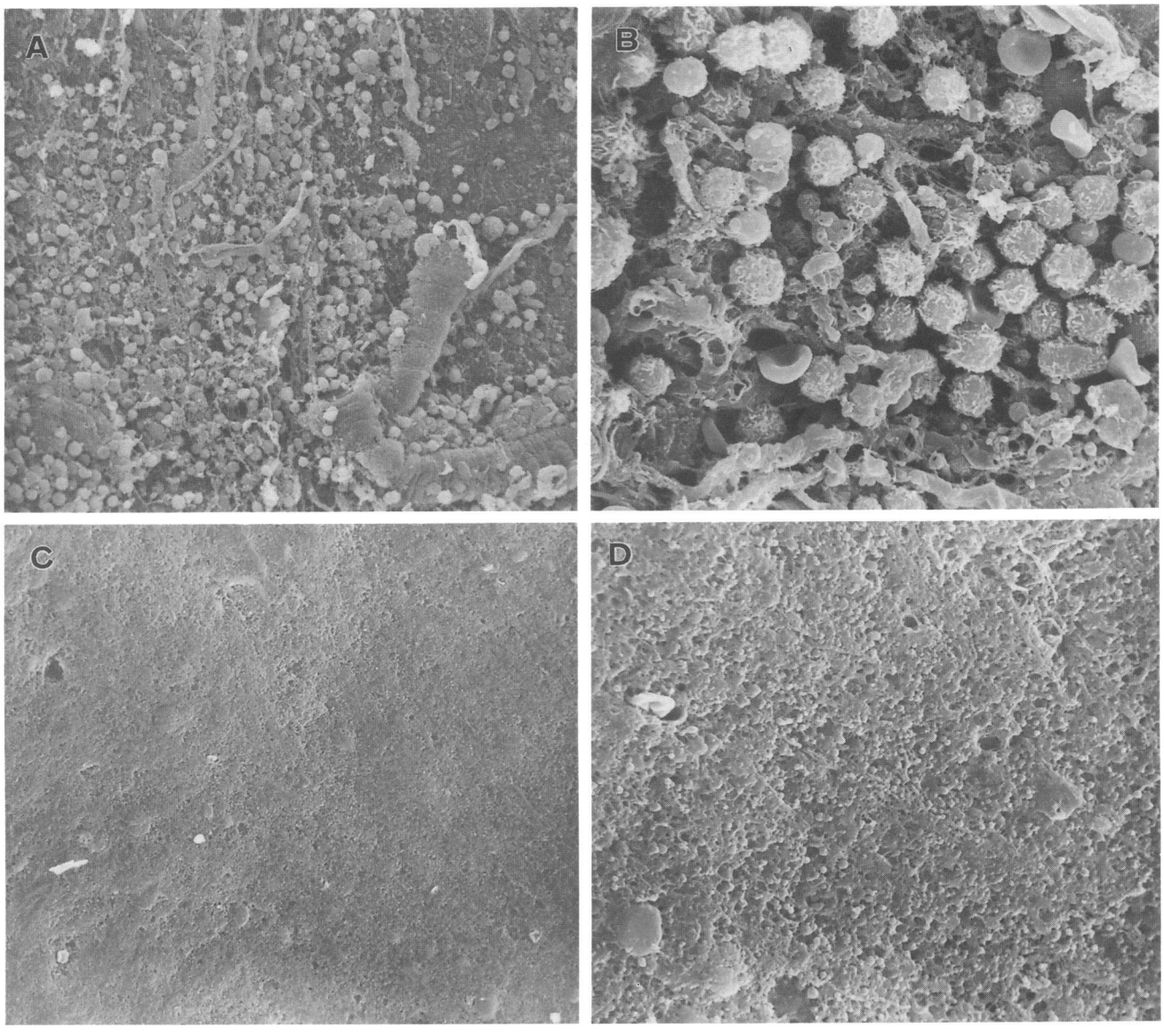

Figure 4. Accumulation of activated lymphocytes in the outer surface of the spinal cord. (A) (× 300) and (B) (× 1200), the spinal cord of EAE rats (day 9); (C) (× 300) and (D) (× 1200), the spinal cord of control rats.

the major organs for intrathymic and extrathymic pathways of T-cell differentiation, respectively. The number of MNC obtained from the thymus and liver was counted at the indicated points of time. The number of thymocytes began to decrease on day 7 after the immunization with MBP/CFA and reached a base line on day 13. At this stage, only $3–5 \times 10^6$ thymocytes remained in the thymus, accompanied with profound thymic atrophy (> 95%). A pattern of variation similar to that seen in the thymus was also observed in the liver, although the variation was not so drastic (~80%) as that in the thymus.

The identification of DN CD4⁻ CD8⁻ TcRαβ cells on the surface of the spinal cord

In rats with experimental autoimmune myocarditis, we have previously reported that T cells which expressed high levels of LFA-1 and IL-2R were found in the pericardial effusion and heart tissue. These cells contained a large proportion of DN CD4⁻ CD8⁻ TcRαβ cells and were located in the outer layer of the heart (i.e. the pericardial cavity). In light of these findings, we then examined, by electron microscopy, whether similar lymphocytes existed on the surface of the spinal cord (Fig. 4). In control rats, no attached lymphocytes were present on the surface of the spinal cord (Fig. 4C and D). However, in the diseased rats, numerous lymphocytes were found on the surface of the spinal cord (Fig. 4A and B).

We analysed the phenotypes of these cells by two-colour staining for CD4 plus CD8 and TcRαβ (Fig. 5). These lymphocytes were obtained from the surface of the spinal cord of rats with EAE at the early phase of the disease (on day 9 post-immunization). MNC infiltrating the spinal cord and lymph node cells were examined in parallel. A large proportion of TcRαβ⁺ cells in the subarachnoid space were DN CD4⁻ CD8⁻. In sharp contrast, MNC which infiltrated the spinal tissue as

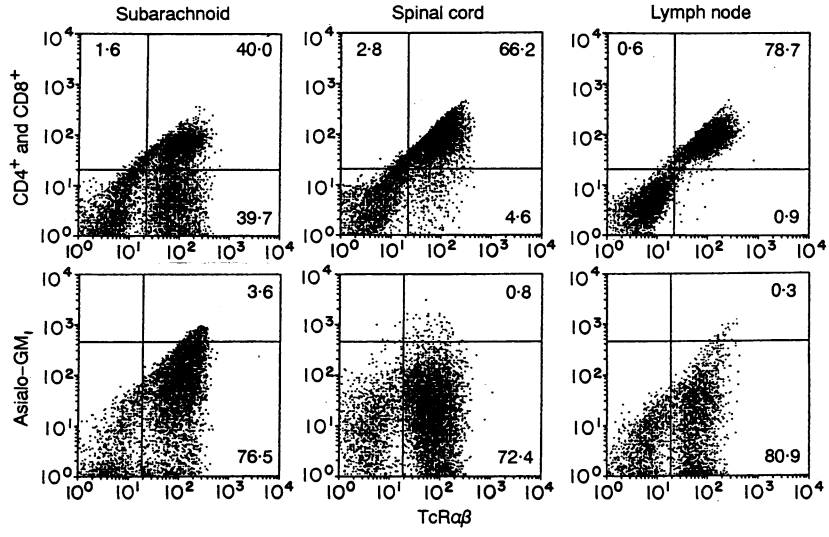

Figure 5. Identification of DN CD4$^-$CD8$^-$ TcR$\alpha\beta$ cells in MNC attached to the spinal cord. MNC were isolated from the subarachnoid, spinal cord and lymph nodes in rats with EAE (day 9). Two-colour staining with anti-TcR$\alpha\beta$ mAb (green) and a mixture of anti-CD4 and -CD8 mAb was performed (top of the figure). To show that our cell preparations did not contain any asialo-GM$_1^+$ NK cells (in the TcR$\alpha\beta^-$ cell fraction), two-colour staining for TcR$\alpha\beta$ and asialo-GM$_1$ antigen was performed.

well as MNC in the lymph nodes contained hardly any such DN TcR$\alpha\beta^+$ cells. These experimental results were confirmed by using five rats with EAE (Table 1). Interestingly, more than 50% of TcR$\alpha\beta^+$ cells in the subarachnoidal space were of the DN CD4$^-$ CD8$^-$ phenotype.

In the present experiments, we applied the nylon-wool column method to isolated lymphocytes from the tissue debris of the subarachnoid space and spinal cord. In this regard, T cells were enriched and NK cells were inversely depleted in the cell preparation. In this situation, when MNC were stained for TcR$\alpha\beta$ and asialo -GM$_1$ antigen, asialo-GM$_1^+$ NK cells were very few in each preparation (see the bottom of Fig. 5). Activated rat T cells had a tendency to express a considerable level of asialo-GM$_1$ antigen (our unpublished observation). Lymph node cells are well known to lack a NK cell population.

Further phenotypic characterization of MNC in various organs of rats with EAE

Detailed phenotypic characterization of MNC was extended to various organs in rats with EAE (day 9), including the blood, spleen, lymph nodes and spinal cord (Fig. 6). MNC attached to the spinal cord were also examined. When two-colour staining for TcR$\alpha\beta$ and LFA-1 was performed, a unique population of LFA-1$^+$ $\alpha\beta$ T cells was identified in the MNC attached to the spinal cord and in those which infiltrated the spinal cord. The LFA-1$^+$ $\alpha\beta$ T cells were also detected in MNC in the lymph node cells obtained from the inguinal lymph nodes at the side of antigenic injection. The MNC obtained from the spinal cord, but not from other organs, contained IL-2R$^+$ cells, as revealed by two-colour staining for TcR$\alpha\beta$ and IL-2R. Two-colour staining for CD4 and CD8 showed that the MNC which infiltrated the spinal cord, as well as those attached to the spinal cord, mainly comprised CD4$^+$ cells.

Oligoclonality of $\alpha\beta$ T cells attached to the spinal cord as well as those infiltrating the spinal cord

It is known that extrathymic T cells constitute the predominance of Vβ8$^+$ cells in mice. In this regard, it was examined whether $\alpha\beta$ T cells among MNC in the spinal cord of rats with EAE had some degree of oligoclonality (Table 2). The mAb used here only became available recently and were used to characterize MNC in rats with EAE. Interestingly, both MNC which infiltrated the spinal cord and those which were attached to the spinal cord showed a relatively higher level of Vβ8.2$^+$ cells. Neither MNC

Table 1. Abundance of DN CD4$^-$ CD8$^-$ $\alpha\beta$ T cells in MNC obtained from the subarachnoid space of EAE rats

	Subarachnoid	Spinal cord	Lymph node
Total T cells	81·9 ± 5·6	76·2 ± 10·2	72·8 ± 7·6
DN CD4$^-$ CD8$^-$ T cells	48·2 ± 5·4	4·7 ± 0·6	0·3 ± 0·1
Ratio of DN T cells/total T cells	58·1 ± 2·4	6·5 ± 1·2	0·5 ± 0·1

MNC were isolated from the subarachnoid, spinal cord and lymph nodes in five rats with EAE (day 9). Total T cells (TcR$\alpha\beta^+$ cells) and DN CD4$^-$ CD8$^-$ cells (TcR$\alpha\beta^+$ CD4$^-$ CD8$^-$ cells) were identified as described in Fig. 5. The mean ± SD are represented.

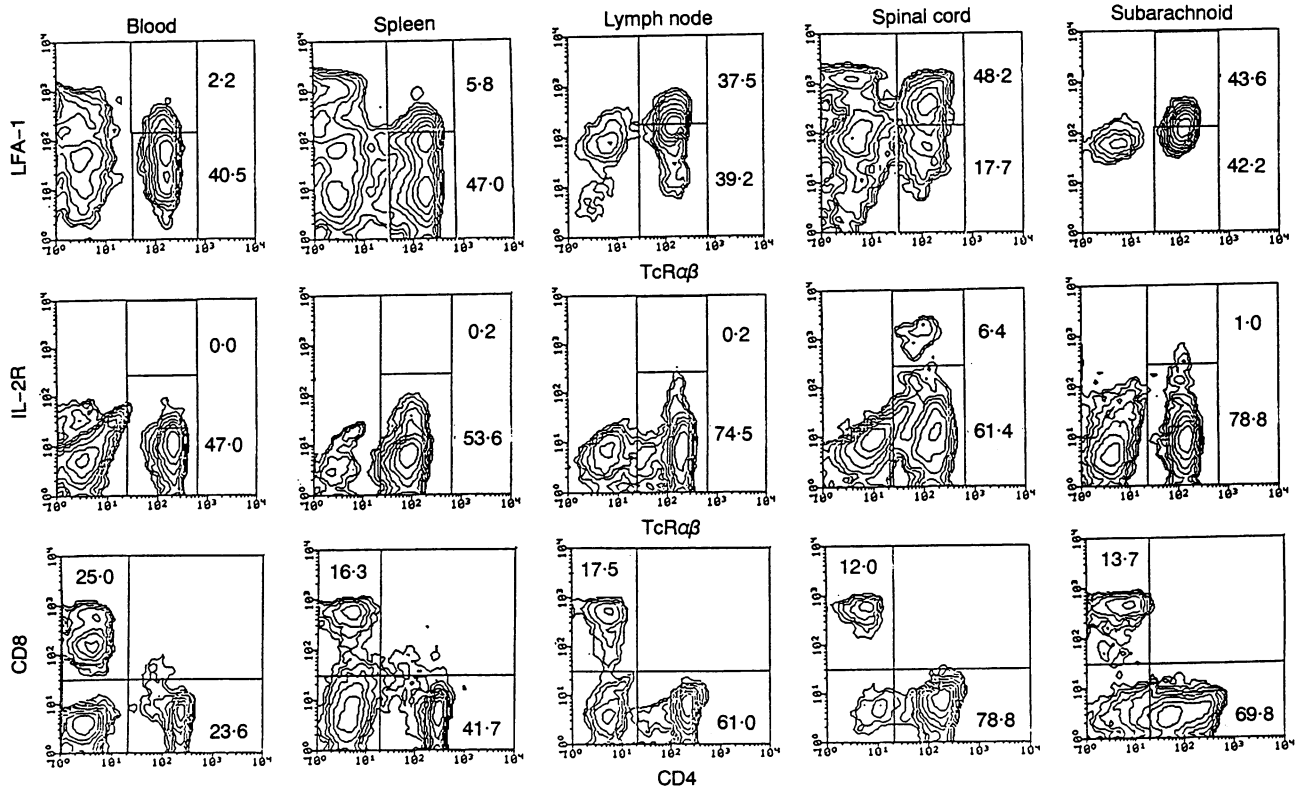

Figure 6. Phenotypic characterization of MNC in various organs of EAE rats. MNC were isolated from various organs of EAE rats (day 9). Two-colour stainings for TcR$\alpha\beta$ and LFA-1, TcR$\alpha\beta$ and IL-2R, and CD4 and CD8 were performed. LFA-1$^+$ T cells were identified in the lymph nodes, spinal cord and subarachnoid space. Representative data from three experiments are depicted.

obtained from the lymph nodes in control rats nor those from rats with EAE showed such a high incidence of Vβ8.2$^+$ cells.

Adoptive transfer of EAE by using MNC attached to the spinal cord

Thus far, experiments have revealed that MNC attached to the spinal cord have many properties similar to those of extrathymic T cells seen in mice and appear to contain actual effector cells which evoke EAE. It was therefore examined whether the injection of such MNC into rats would elicit EAE (Fig. 7). Although apparent signs of EAE were not observed in these rats, some activated lymphocytes were demonstrated to adhere to the surface of the spinal cord on day 3 after injection. Such a feature was not produced by using MNC obtained from the spleen in rats with or without EAE (data not shown).

Table 2. A comparison of the ratio of Vβ^+ T cells/total T cells between control and EAE rats

	Control	EAE		
Vβ	Lymph node	Lymph node	Spinal cord	Subarachoid
Vβ8.2	5·1±0·5	6·8±0·8	20·6±6·8	18·3±5·3
Vβ8.5	6·1±0·6	6·4±1·0	4·6±1·0	6·0±1·0
Vβ10	7·8±1·0	7·6±1·1	5·9±1·1	4·2±1·1
Vβ16	10·1±1·3	10·1±0·4	10·4±2·9	9·7±1·0

MNC were obtained from rats treated with CFA alone (control rats) and with MBP/CFA on day 9 after injection. Vβ^+ T cells were identified by an immunofluorescence test using corresponding mAb. The mean±SD of the experiments using three or four rats are represented.

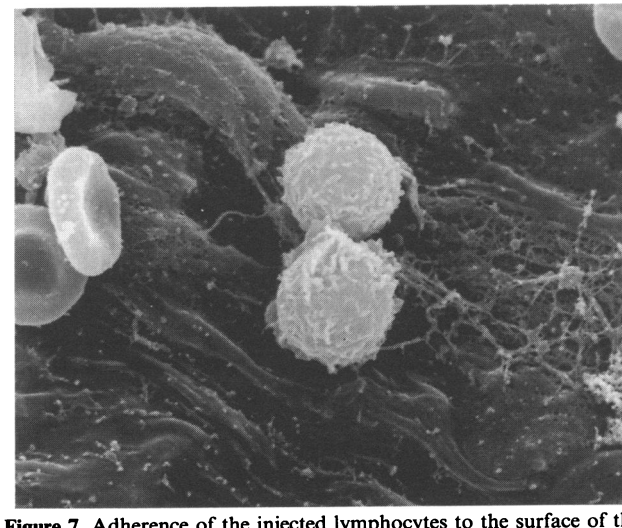

Figure 7. Adherence of the injected lymphocytes to the surface of the spinal cord. The lymphocytes isolated from the subarachnoid space of EAE rats (day 9) were directly injected into the spinal cavity. Activated lymphoblasts with well-developed microvilli were seen on the surface of the spinal cord (×2625).

DISCUSSION

Extrathymic pathways of T-cell differentiation were recently demonstrated in the liver[1-6] and the intestine of mice.[7-9] In a series of recent studies, we showed that T cells seen uniquely in the liver of mice are characterized as T cells with TcR of intermediate intensity (i.e. intermediate TcR cells) on their surface, including both $\alpha\beta$ and $\gamma\delta$ T cells. They contain DN CD4$^-$ CD8$^-$ cells as well as single-positive CD4$^+$ or CD8$^+$ cells and express high levels of LFA-1 and IL-2Rβ.[23,24] In this study, we examined whether such extrathymic T cells with similar properties existed in the liver of rats. When an extrathymic pathway of T-cell differentiation in the liver is activated in mice, an intrathymic pathway of T-cell differentiation is reciprocally inactivated. Such conditions include ageing,[13,14] bacterial infection,[4,15] malignancies[2,16,17] and autoimmune diseases.[1,5,18] In rats with EAE, profound thymic atrophy was clearly induced. However, the TcR$\alpha\beta$ (and CD3) intensity of LFA-1$^+$ cells was almost the same as that of LFA-1$^-$ cells. With regard to this, we cannot definitely say that TcR$\alpha\beta^+$ LFA-1$^+$ cells in rats are the same as extrathymic T cells in mice, although LFA-1$^+$ T cells seem to have a slightly lower intensity of TcR$\alpha\beta$ than bright TcR$\alpha\beta$ cells. There is a possibility that extrathymic T cells in rats acquire a much higher density of TcR on the surface during their phylogenetic development.

In the present study, we also determined directly the number and phenotype of lymphocytes infiltrating the spinal cord, as well as MNC in other immune organs in rats with EAE. It was demonstrated that a considerable proportion of T cells infiltrating the spinal cord expressed high levels of LFA-1 (and IL-2R). In addition to these properties of T cells infiltrating the spinal cord, T cells attached to the surface of the spinal cord contained a significant proportion of DN CD4$^-$ CD8$^-$ T cells. These properties of T cells attached to the spinal cord were found to be quite similar to those of extrathymic T cells demonstrated previously in autoimmune mice. As shown in our previous reports,[23,24] DN CD4$^-$ CD8$^-$ cells in mice were only confined to the cell fraction of intermediate TcR cells, but not to that of bright TcR cells at all. Although intermediate TcR cells were not identified in the case of rats, LFA-1$^+$ T cells with the DN CD4$^-$ CD8$^-$ phenotype in rats might correspond to the intermediate TcR cells in mice.

In a previous study, we demonstrated in rats with experimental autoimmune pericarditis that LFA-1$^+$ T cells which also comprised DN CD4$^-$ CD8$^-$ cells actively proliferated in the outer surface of the heart (i.e. pericardial cavity) and then directly invaded the cardiac tissue through the outer surface.[25] Reflecting on this situation, lymphocytes infiltrating the heart were found to exist only in the outer layer of the heart tissues. Until this time, the invading lymphocytes in organ-specific autoimmune diseases had been considered to come from the circulation, as shown by cell transfer experiments in which they were injected into veins.[26,27] However, we postulated that in the case of experimental autoimmune myocarditis, extrathymic T cells might be activated in the pericardial cavity and that mainly the CD4$^+$ T cells among them would invade the tissue. Similarly, in the case of EAE, the activation site for extrathymic T cells might be the subarachnoid space. Although only CD4$^+$ T cells, but not DN CD4$^-$ CD8$^-$ cells, invaded the spinal tissue, both these CD4$^+$ and DN CD4$^-$ CD8$^-$ cells share some common properties, including the LFA-1 expression and the high incidence of Vβ8.2 usage. They may belong to the same cell lineage.

In our most recent study, we demonstrated that extrathymic T-cell differentiation, particularly for intermediate TcR cells, in mice occurred not only in the hepatic sinusoids but also in splenic red pulp (i.e. sinusoidal architecture).[24] Furthermore, extrathymic generation of $\gamma\delta$ T cells occurred *in vitro* only under serum-rich conditions.[28] Concerning these results, a serum-rich area, such as the sinusoidal lumen and inflammatory cavity, might be a candidate for the site of extrathymic T-cell differentiation. Indeed, the autoimmune lesions are cross-linked with the inflammatory cavities, including the intra-occular space of uveitis (Sjögren syndrome), the joint space of rheumatoid arthritis, and the pericardial cavity of autoimmune myocarditis.

In murine studies, we demonstrated that extrathymic T cells contained a higher proportion of autoreactive Vβ8$^+$ T cells and forbidden T-cell clones than did regular T cells of thymic origin, especially in their activated states.[5,10] The present results, namely the high incidence of Vβ8.2 among both MNC attached to the spinal cord and MNC which have infiltrated the spinal tissue, seem to be compatible with the notion that such T cells might be of extrathymic origin. In conclusion, we propose the possibility that in the case of organ-specific autoimmune diseases, the cavities which are in close contact with the diseased lesions might become activation sites for extrathymic T cells and that only the selected population (e.g. CD4$^+$ T cells) might directly invade the lesion tissues. The actual reason why only CD4$^+$ T cells invade the tissue remains obscure. We prefer to think that perhaps antigenic sites of disease-inducing antigens preferentially bind with class II MHC antigens on activated macrophages or nerve cells and, therefore, only a limited population of T cells with a CD4$^+$ phenotype recognized these molecules and infiltrates the lesion. The function of DN CD4$^-$ CD8$^-$ cells in the cavity remains to be investigated. Cell separation experiments might be required in conjunction with the method of direct cell injection into the spinal cavity, which was applied here.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, Japan. We wish to thank Ms Hiroko Karasawa and Mr Minoru Kenmotsu for preparing the manuscript and Mr Tetsuo Hashimoto for animal maintenance.

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