Autoimmune Effector Cells

VII. Cells Isolated From Thymus and Spinal Cord of Rats With Experimental Allergic Encephalomyelitis Transfer Disease

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Effector cells mediating experimental allergic encephalomyelitis (EAE) were recovered from the thymus glands and spinal cords of Lewis rats immunized with myelin basic protein (BP) and complete Freund's adjuvant (CFA), as demonstrated by adoptive transfer to syngeneic recipients following *in vitro* activation with BP. The thymic effector cells (TCs) were lymphoblasts. Sequential transfer studies suggested that the effector TCs probably recirculated back to the thymus from the periphery. The transfer of EAE with cells derived from the spinal cords of paralyzed donors indicates that disease-inducing effector cells are present in the target organ. (Am J Pathol 1986, 122:218–222)

THE FINDING that the lymphocyte-mediated adoptive transfer of experimental allergic encephalomyelitis (EAE) is markedly enhanced by activating effector cells from immunized donor animals *in vitro* in the presence of concanavalin A (Con A),[1] myelin basic protein (BP),[2–5] or allogeneic spleen cells (SpC)[6] has provided a unique opportunity to study the mechanism of T-cell-mediated autoimmune diseases. Effector cells which mediate EAE have been found in the spleens and lymph nodes of Lewis rats immunized previously with BP in complete Freund's adjuvant (CFA)[1–6] and in peritoneal exudates from Strain 13 guinea pigs.[7]

The cells activated in culture are T cells which presumably matured in the thymus and migrated to the peripheral lymphoid tissues.[8] Although it is generally believed that the emigration of T cells from thymus to periphery is unidirectional,[8] Naparstek et al[9] reported that rat EAE effector line cells, when transferred to syngeneic recipients, can migrate back to the thymus. We have conducted studies to follow up this unique finding. Specifically, we were interested in ascertaining whether EAE effector cells were present in the thymus glands of BP-CFA-immunized rats.

Because mononuclear cell infiltration of the central nervous system is a characteristic feature of EAE, we also sought to determine whether the inflammatory cells present in the spinal cords of rats with EAE were recruited bystander cells or EAE effector cells by virtue of their ability to be activated to adoptively transfer the disease. Burns et al[10] recently established mononuclear cell lines from rats with EAE. After culture for 10–14 days with BP followed by interleukin-2, these cells transferred EAE to syngeneic recipients. We have now confirmed and extended their finding in a larger group of animals. The present report describes our investigation of effector cell activity in the thymus glands and spinal cords of rats with EAE.

Materials and Methods

Animals

Female Lewis rats were purchased from Harlan Sprague-Dawley (Walkersville, Md) and were used at 8 weeks of age.

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Immunization

Donor rats were immunized with guinea pig BP in CFA in the footpad of one hind foot. Each rat received 25 μg BP and 100 μg *Mycobacterium butyricum* in a total volume of 0.05 ml.

Cell Suspensions

Spleen cells (SpCs) and thymus cells (TCs) were obtained from immunized donors, and cell suspensions were prepared as described previously.[3] In some experiments donor rats were given intraperitoneal injections of India ink (Pelikan) 1–2 hours before sacrifice for facilitation of identification and dissection of parathymic lymph nodes.

Cell suspensions were prepared from spinal cords of rats with clinical EAE. Three experiments were performed; in each experiment, inflammatory cells were obtained from the spinal cords of 30 rats immunized 13 days earlier with BP-CFA. The spinal cords were obtained aseptically, and the tissue was minced with scissors and forceps for preparation of a cell suspension. Mononuclear cells were separated by centrifugation on 60% Percoll (density, 1.077 g/ml) for 45 minutes at 14,500*g* in a Sorvall RC2-B refrigerated centrifuge, according to the method of Burns et al.[10] As a control, spinal cords were taken from naive rats, and cells were obtained in a similar fashion.

Cell Culture

SpCs or TCs were cultured for 72 hours at 37 C in 5% CO_2 in RPMI 1640 in the presence of 2 μg/ml BP as previously described.[3] Spinal cord-derived cells were similarly cultured except that irradiated SpCs from naive rats (2000 rads, Gammacell 40 ^{137}Cs irradiator, Atomic Energy of Canada, Ottawa) were added as a source of accessory cells (1:1 ratio). The final cell density in all cultures was 2×10^6/ml.

Adoptive Transfer

After culture the cells were washed three times with Hanks' balanced salt solution (HBSS), and injected intraperitoneally into syngeneic recipients. The recipients were evaluated daily for clinical EAE as follows: 0, no EAE; 1, loss of tail tonicity; 2, paresis; 3, hind limb paralysis often accompanied by incontinence. Histologic EAE was evaluated in hematoxylin and eosin (H&E)-stained longitudinal sections of spinal cord, by a "blinded" observer. Mononuclear cell infiltration was scored as follows: O, no lesions; 1, 5 lesions/section; 2, focal lesions in a few low power fields; 3, lesions in many fields; 4, many lesions in almost all fields.

Lymphoblast Separation

BP-cultured TCs were fractionated on discontinuous Percoll gradients as previously described.[3] Briefly, the cultured TCs were suspended in 3 ml 70% Percoll (density, 1.082 g/ml). This was overlayered with 3 ml 50% Percoll (density, 1.062 g/ml) and topped with 3 ml HBSS. The gradients were centrifuged for 15 minutes at 470*g* at room temperature.

Immunofluorescence

B cells were enumerated by direct immunofluorescence with fluorescein-conjugated Fab'_2 fragment of rabbit anti-rat IgG with heavy and light chain specificity (Cappel). The percentage of helper T cells and suppressor/cytotoxic T cells was ascertained with monoclonal antibody W3/25 and OX-8, respectively, and Ia^+ cells were enumerated with monoclonal antibody OX-3, with the use of indirect immunofluorescence as previously described.[11]

Results

Effector Cells in Thymus

In preliminary experiments, we observed that TCs obtained 12 days after immunization transferred EAE to recipient rats after *in vitro* culture with BP (results not shown). To confirm this surprising finding, we conducted a series of experiments in which the effector activity of SpCs and TCs was compared. A special effort was made to ensure that parathymic lymph nodes did not contaminate the thymus preparations, including India ink treatment of the donor rats to better visualize these lymph nodes, careful removal of questionable portions of tissue, and immunofluorescence analysis of the thymus cell suspensions with fluorescein-conjugated anti-rat Ig Fab'_2 to identify B cells derived from lymph nodes which might contaminate the preparations (<0.5% Ig^+ cells were found). Thus, we consider it highly unlikely that our TC suspensions were contaminated with lymph node cells.

As shown in Table 1, TCs obtained 12 days after immunization and cultured with BP transferred mild clinical EAE. Histologic lesions were observed in the spinal cords of rats given TCs obtained 5 days after donor immunization. In contrast, effector cell activity occurred earlier in SpCs; cells obtained 5 days after immunization transferred severe clinical disease. Effector activ-

Table 1—Effector Cell Activity in Thymus and Spleen of BP-CFA-Immunized Lewis Rats

Day* after immunization	Cells transferred (5×10^7)	EAE in recipients			
		Incidence†	Onset (day)	Severity‡	Histology†
2	TC	0/8	–	–	0/2
	SpC	0/9	–	–	0/2
5	TC	0/10	–	–	4/4
	SpC	7/7	6.6	2.1	nd§
9	TC	0/12	–	–	5/6
	SpC	6/6	6.0	2.8	nd
12	TC	10/10	5.5	1.3	nd
	SpC	6/6	5.0	2.8	nd
19	TC	5/5	5.2	1.8	nd
	SpC	3/3	5.0	3.0	nd
27	TC	10/10	5.0	1.5	nd
	SpC	6/6	5.0	3.0	nd
naive	TC	0/4	–	–	0/4

* Day of donor sacrifice and cell culture.
† Number of positive/total examined.
‡ Average clinical severity (graded 0–3).
§ Not done; histologic EAE is found in rats with clinical signs.

ity persisted in TCs and SpCs for at least 27 days, and SpCs always caused more severe disease. Neither cultured TCs from naive donors nor cultured TCs or SpCs from donors immunized 2 days earlier exhibited effector activity, which excludes the possibility that BP carryover accounted for EAE (Table 1).

Characterization of Effector TCs

We previously reported that EAE effector SpCs are T lymphoblasts. Accordingly, we separated BP-cultured TC from immunized donors into blast-enriched and small lymphocyte fractions on Percoll gradients. Only 1–2% of the cells applied to the gradients sedimented as lymphoblasts, but these transferred EAE at low dosages (3×10^6). In contrast, 5×10^7 cells from the small lymphocyte fraction were inactive (Table 2). Thus, it appears that SpC and TC effector cell populations are both lymphoblasts, although many fewer blasts are present in cultured TCs (1–2% versus 30% for SpCs[3]), which may account for the difference in severity of disease mediated by the two cell preparations (Table 1).

Origin of Effector TCs

The presence of effector cells in the thymus could reflect either *in situ* activation of immunocompetent resident thymocytes or recirculation to the thymus of T cells which had encountered antigen in the periphery. To test the latter hypothesis, the following experiment was conducted. Donor rats were immunized with BP-CFA, and 13 days later their SpCs and TCs were cultured separately with BP. These cultured cells were transferred to two groups of recipients (designated 1′ recipients, Table 3). The 1′ recipients of TCs and SpCs developed EAE, as expected, and recovered. On Day 10, we obtained TCs from the 1′ TC recipients and SpCs from the 1′ SpC recipients, cultured them with BP, and transferred them to 2′ recipients. The 2′ recipients of TCs from 1′ TC recipients developed EAE (Table 3), which supports the hypothesis that thymic effector cell activity reflects recirculation back to the thymus, because the 1′ recipients had not been immunized with BP-CFA. Sequential transfer of EAE with SpCs has previously been reported[12] and serves as a positive control for this experiment (Table 3). In other studies, we found that BP-cultured SpCs from 1′ recipients of TCs from sensitized donors also transferred EAE to 2′ recipients, which indicates that effector TCs do not home exclusively to thymus (results not shown).

Effector Cells in Spinal Cords

We prepared cell suspensions from spinal cords of rats paralyzed with EAE and isolated inflammatory cells by centrifugation on Percoll. Three experiments were performed; in each, inflammatory cells were obtained from the spinal cords of 30 donors immunized 13 days earlier with BP-CFA. Recipients of 5×10^7 BP-cultured spinal cord mononuclear cells developed severe EAE in 5–6 days. This time period is consistent with adoptively transferred EAE in the rat,[1–6] and too short to be attributed to active sensitization with BP transferred to the recipients (10–12 days). The results show that clinically there were 15/17 positive clinically, with a severity of 2.6 on a scale of 0–3, and 16/17 positive histologically, with a severity of 2.5 on a scale of 0–4.

In an effort to identify these cells and determine

Table 2—Enrichment of TC Effector Cells on Percoll Gradients

	Percoll density (g/ml)	Cells transferred*	EAE in recipients†	
			Clinical	Histologic
Experiment 1	1.062‡	3×10^6	3/3	3/3
	1.082	5×10^7	0/3	1/3
Experiment 2	1.062‡	4×10^6	2/3	2/3
	1.082	4×10^7	0/3	0/3

* TCs from donors immunized 12 days earlier were cultured with BP for 72 hours, then separated on Percoll gradients.
† Number positive/total.
‡ Corresponds to lymphoblast-enriched fraction.

whether their presence correlates with EAE, groups of 2–5 rats were sacrificed at intervals from 8 to 29 days after immunization, and cells were isolated from the pooled spinal cords and evaluated by immunofluorescence. Spinal cords were similarly obtained from groups of nonimmune rats. Virtually no nucleated cells were found in preparations derived from nonimmune rats or rats killed on Day 8 (early preclinical phase) or on Day 29 (after recovery) of immunization. In contrast, Ia^+ cells and T cells were found in Percoll-isolated preparations obtained 12–21 days after immunization (ie, the clinical and early postrecovery phases). In six separate experiments, it was determined that 39–86% of the cells were T lymphocytes (assessed with monoclonal antibodies W3/25 and OX-8) and 20–48% were Ia^+ (stained with monoclonal antibody OX-3). Thus, we demonstrated that the presence of T lymphocytes and Ia^+ cells in the spinal cord coincides with the clinical phase of EAE.

Discussion

The present findings reveal that effector cells of EAE are present in the thymus glands and spinal cords of rats previously immunized with BP-CFA. Thymic effector activity appears later than effector activity in SpC, and BP-cultured TCs transfer less severe disease. That this reflects the presence of fewer effector cells in thymus than in spleen is supported by the observation that only 1–2% of the BP-cultured TCs sediment as blasts in Percoll gradients, compared with 30% of blasts in the spleen cell population.[3] Nevertheless, this small population of thymic blasts accounts for virtually all EAE effector activity (Table 2). Thus, both thymic and splenic effector cells appear to be lymphoblasts. Precautions were taken to dissect away the parathymic lymph nodes, and we consider it highly unlikely that TC effector activity is the result of contamination with lymph node cells.

Effector activity in TCs of BP-CFA-immunized rats could be explained on the basis of *in situ* induction or migration of effector cells from the periphery to the thymus. It has been thought that traffic of T cells from thymus to peripheral lymphoid tissue is unidirectional: ie, mature T cells do not return to the thymus (reviewed by Stutman[8]). However, our sequential transfer experiments support the hypothesis that effector cells migrate back to the thymus (Table 3). Moreover, our findings are consistent with those of Naparstek et al,[9] who demonstrated that cells from long-term cultures of functionally active T lymphocytes reactive to self or foreign antigens migrate to the thymus after transfer to syngeneic recipients. Nevertheless, we cannot entirely exclude the possibility that EAE effector cells are generated within the thymus glands of BP-CFA-sensitized rats, because Fink et al[13] presented evidence that TCs which mediate cytotoxic T cell responses to minor histocompatibility antigens are primed *in situ*. In addition, they reported that mature peripheral T cells specific for minor histocompatibility antigens home to the thymus and contribute to the response.[13] Whether the persistence of these effector cells in the thymus after recovery from EAE (Table 1 and Naparstek[9]) relates to suppressor cell regulation of this autoimmune

Table 3—Sequential Transfer of EAE with Cells From Primary Recipients

Cells transferred to 1′ recipients	EAE in 1′ recipients (clinical*)	Cells transferred to 2′ recipients†	EAE in 2′ recipients	
			Clinical*	Histologic
5×10^7 TC	6/6 (2.7)	5×10^7 TC	4/4 (1.0)	3/4
5×10^7 SpC	3/3 (3.0)	5×10^7 SpC	2/2 (3.0)	2/2

* Number sick/total; severity in parentheses.
† TCs and SpCs obtained 10 days after transfer from recipients of TCs and SpCs, respectively, cultured with BP for 72 hours, and transferred to 2′ recipients.

disease[14] remains to be determined. It has long been recognized that the thymus is a source of suppressor T cells.[15]

To determine whether specific effector cells are present in the inflammatory lesions of rats with EAE, we isolated mononuclear cells from the spinal cords of paralyzed donors. This is an important issue because it has been shown that isotopically labeled lymph node cells from donors with EAE account for less than 5% of the infiltrating cells in the central nervous system.[16] Thus it is conceivable that EAE lesions are composed predominantly of "bystander" cells that damage the myelin in a nonspecific manner.[17] However, we found that effector cells are present in the central nervous system because they could be activated *in vitro* to transfer EAE. The appearance of these cells in the spinal cord coincided with the paralytic phase of disease, and they were not found in nonimmune rats. Immunofluorescence analyses revealed that the cells isolated from the spinal cords were predominantly T lymphocytes and Ia^+ cells. This is consistent with immunohistologic studies which have also shown that the inflammatory lesions of EAE include T cells and Ia-bearing cells.[18-22]

In conclusion, we have found EAE effector cells in the thymus and spinal cord of BP-CFA-immunized Lewis rats. The role of these cells in the regulation and pathogenesis of EAE is under investigation.

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