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REGULATION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

II. Appearance of Suppressor Cells during the Remission Phase of the Disease[1]

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Lewis rats are susceptible to experimental autoimmune encephalomyelitis (EAE). Most rats recover from paralysis and are subsequently resistant to the disease. In an adoptive transfer system, we found that lymph node cells (LNC) from rats that had recovered from EAE protect syngeneic recipients from the disease when the latter are challenged with encephalitogenic myelin basic protein and adjuvant after receiving donor cells. Suppression is antigen-specific and requires viable LNC. In contrast to the suppressor cells we previously studied in tolerized rats, which were nonadherent T lymphocytes, the suppressor cells found in rats that have recovered from EAE adhere to glass wool. However, they are not retained on Sephadex G-10 columns to which macrophages adhere. Suppressor activity is enriched in the nylon wool-adherent LNC population (which consists of approximately 80% Ig^+ cells). Our findings suggest that activation of adherent suppressor cells may be implicated in recovery from EAE. These may be adherent T cells, or B cells that produce anti-BP blocking antibodies.

Inbred Lewis rats are very susceptible to experimental allergic encephalomyelitis (EAE),[2] a T cell-mediated (1–4) autoimmune disease of the central nervous system (5). Approximately 10 to 12 days after challenge with guinea pig myelin basic protein (BP) emulsified in complete Freund's adjuvant (CFA), virtually every Lewis rat develops severe hind limb paralysis, often accompanied by urinary incontinence. The disease can be transferred to syngeneic recipients with lymph node cells (LNC) from challenged donors (6, 7). The optimal time of transfer is 9 days after donor challenge, and recipients exhibit neurologic signs 4 to 6 days post-transfer. Unlike other species that succumb to this disease, most Lewis rats recover from EAE by day 18 post-challenge or day 9 post-transfer and are resistant to further attempts to reinduce EAE (8, 9, and present report). Willenborg (9) recently postulated that suppressor cells might regulate EAE in these resistant rats, although no supporting evidence was presented.

Previously, we reported that antigen-specific suppressor T cells regulate EAE in Lewis rats rendered specifically unresponsive to this disease by pretreatment with BP in nonencephalitogenic form (10–13). Since suppressor cells seem to play a role in this tolerance state, we were also interested in the possibility that the activation of suppressor cells might also account for the recovery of Lewis rats from EAE. Accordingly, the present study was initiated to elucidate the mechanism of recovery from this neurologic autoimmune disease.

MATERIALS AND METHODS

Animals. Female 8- to 10-week-old Lewis rats (Microbiological Associates, Walkersville, Md.) were used in all experiments.

Antigens. BP was prepared from frozen guinea pig or rat spinal cords (Pel Freez, Rogers, Ark.) and purified by Sephadex gel chromatography (Pharmacia, Uppsala, Sweden) as previously described (14). Equine heart cytochrome *c* (Calbiochem, San Diego, Calif.) served as the control antigen.

Induction of EAE. Active EAE: Rats were challenged in one hind foot with 0.05 ml of an emulsion containing 25 μg guinea pig BP and 100 μg *Mycobacterium butyricum* in the adjuvant.

Adoptive EAE: Draining lymph nodes were removed from donors 9 days post-challenge (6, 7, 10). Single cell suspensions were prepared, washed twice in Hanks' balanced salt solution, and viability was determined by exclusion of trypan blue. LNC (5×10^8 viable cells) were injected i.v. into the lateral tail vein of syngeneic recipients, as previously described (10, 12). Thymocyte (Thy) suspensions were similarly prepared after removal of the parathymic lymph nodes.

Evaluation of EAE. Rats were observed daily for signs of EAE, and were scored as follows: 0, no EAE; 1, loss of tail tonicity; 2, paresis; 3, hind limb paralysis often accompanied by incontinence. Clinical signs usually appeared 10 to 12 days after challenge with BP + CFA, or 4 to 6 days after adoptive transfer of donor LNC. Parasagittal sections of spinal cord were stained with hematoxylin-eosin and evaluated for mononuclear infiltration without knowledge of origin. Histologic EAE was graded as follows: 0, no lesions; 1, approximately 5 lesions on an entire section; 2, a few lesions in each of a few fields (low power magnification); 3, lesions in many fields; 4, many lesions in almost all fields (15). Basically, scores of 1 to 2 denote mild pathologic changes, whereas scores of 3 and 4 indicate severe inflammation.

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[2] Abbreviations: EAE, experimental allergic encephalomyelitis; BP, myelin basic protein; LNC, lymph node cells; Thy, thymocytes; CMI, cell-mediated immunity.

Cell separation methods. Adherent LNC (primarily B cells and macrophages) were removed by filtration through glass wool columns, according to the method of Folch *et al.* (16) as previously reported (11). Macrophages were removed by passage through Sephadex G-10 columns, as reported by Ly and Mishell (17). Briefly, 2×10^8 LNC were loaded into columns consisting of 10 ml Sephadex G-10 in MEM (with 5% fetal bovine serum (FBS)) and incubated for 1 hr at 37°C. Nonadherent cells were eluted with 37°C MEM + FBS. T and B lymphocytes were separated on nylon wool columns (Fenwal Leukopack) according to the method of Julius *et al.* (18). The columns were equilibrated with MEM + FBS at 37°C, and approximately 4×10^8 LNC were added and incubated for 1 hr at 37°C. Nonadherent cells were collected by elution with MEM + FBS, and adherent cells were obtained after manipulation of the nylon wool.

The surface immunoglobulin-bearing (Ig^+) LNC were determined by direct immunofluorescence with fluorescein-conjugated rabbit anti-rat IgG, as previously described (19). The percentage of macrophages was determined by uptake of neutral red (20).

RESULTS

Maximal disease developed in almost all rats challenged with BP + CFA. When provided ready access to food and water, approximately 90% of these rats recovered. Only rarely did we observe relapses after recovery from EAE. In order to confirm whether rats remained susceptible to EAE after recovery, we rechallenged 16 animals at various times after primary challenge. As shown in Table I, only one rat exhibited a second episode of EAE (severity = 1). The histologic severity was comparable to that of rats challenged only once and sacrificed at a similar time. Since it has been reported that CFA pretreatment inhibits subsequent development of EAE in guinea pigs (21), we included a group of rats that had received CFA alone. These animals also failed to develop EAE when challenged, whereas all untreated rats developed severe disease when given the same emulsions (Table I).

Since the administration of CFA alone interfered with the induction of EAE, we designed the following experiment in an effort to overcome this adjuvant effect. Donor rats were given BP + CFA, and 5×10^8 LNC were transferred to syngeneic recipients 9 days later. Thus, the recipients were not directly exposed to CFA. To test the argument that small amounts of CFA could be carried over with the washed LNC and still interfere with EAE, controls included recipients of LNC from donors given CFA alone. Of 40 recipients given LNC from BP + CFA-challenged donors, 39 exhibited clinical EAE. Severity varied, and all recovered by day 8. When these recipients were subsequently challenged with BP + CFA, 23 rats failed to develop EAE, and 17 exhibited only mild clinical signs (maximum severity = 1) (Table II). In contrast, recipients of LNC from CFA-treated donors or from untreated donors developed severe EAE (Table II), thus revealing an immunologically specific component of the post-recovery suppression phenomenon.

To directly test the postulate that suppressor cell activation may be involved in recovery from EAE and subsequent resistance to challenge, we challenged donor rats with BP + CFA and transferred LNC to normal recipients at various times. Recipients of LNC obtained 9 or 12 days after donor challenge developed EAE when they were challenged 24 hr after receiving the cells (Fig. 1). At this time, effector cells are present in donor

TABLE I

Resistance after recovery from active EAE

Primary EAE	Rechallenge	Secondary EAE	
		Incidence[a]	Histologic[b]
no.	*day*		*mean*
6/6	18	0/6	2.8
2/2	19	1/2	n.d.[c]
2/2	24	0/2	1.3
2/2	37	0/2	1.0
4/4	58	0/4	1.1
Controls:			
CFA alone	18	0/9	1.0
Not treated		14/14	1.2–3.8

[a] No. of rats with clinical signs of EAE/total.

[b] Almost all rats exhibited mild histologic EAE when sacrificed after rechallenge. This probably represents lesions incurred during primary episode of EAE that had not completely resolved. Similar lesions were seen in untreated control rats, in which severity decreased with increasing time between challenge and sacrifice (e.g., controls sacrificed 53 days postchallenge exhibited mild lesions; average = 1.2).

[c] Not determined.

TABLE II

Resistance after recovery from passive EAE

Primary EAE	Challenge	Secondary Clinical EAE		
		Incidence	Severity[a]	Histologic EAE
no.	*day*			*mean*
7/7	10	2/7	0.2	1.9
8/8	15	4/8	0.3	1.9
7/7	25	2/7	0.2	0.9
7/8	28	4/8	0.5	3.1
10/10	49–50	5/10	0.6	1.8
LNC from CFA-treated rats	16	7/7	3.0	2.1
LNC from untreated rats	29	2/2	3.0	3.5

[a] Mean clinical severity of the group.

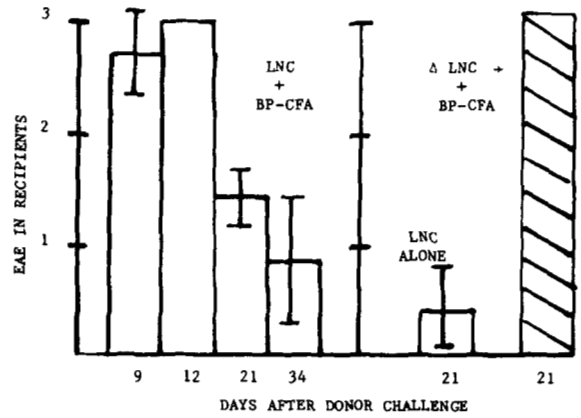

Figure 1. Correlation of suppressor activity with recovery from EAE. *Left panel*: mean clinical severity ± S.D. of EAE in groups of four to seven recipients given LNC at different times after donor challenge, as indicated on horizontal axis. Recipients were challenged 24 hr post-transfer. *Right panel*: deficiency of LNC transferred 21 days postchallenge with respect to ability to transfer EAE to naive recipients (*open bar*); inability of heat-killed LNC to suppress EAE in recipients challenged 24 hr later (*hatched bar*).

lymph nodes (Table II). By day 21, donor LNC were deficient in effector cells, since only mild EAE could be transferred at that time. In contrast, viable LNC transferred after recovery had suppressor activity, since recipients were protected when challenged 24 hr post-transfer, whereas LNC transferred before or at time of onset did not exhibit suppressor activity. Nor were heat-killed LNC transferred on day 21 effective (Fig. 1).

Several additional experiments were carried out in order to ascertain the optimal conditions for demonstrating suppressor cell activity after recovery from EAE. The most consistent results were obtained with cells transferred 30 days after donor challenge, and representative findings are presented in Table III. Recipients of 5×10^7 or 5×10^8 LNC were protected against EAE, whereas 5×10^6 LNC or thymocytes were ineffective. Suppression appeared to be specific, since five of six recipients of LNC from donors given cytochrome *c* +ᵏCFA developed severe EAE when challenged; the sixth recipient did not exhibit clear-cut signs of EAE.

In an effort to identify the suppressor cell, LNC from rats that had recovered from EAE were passed through glass wool columns before transfer. These findings are also presented in Table III. Glass wool nonadherent LNC, which were depleted of Ig^+ cells, were essentially devoid of suppressor activity. Since B cells and macrophages adhere to glass wool, we ascertained the role of macrophages in suppression of EAE by passing the LNC from recovered rats through Sephadex G-10 columns. The nonadherent cells were depleted of macrophages, as determined by neutral red uptake, although Ig^+ cells were not removed. As also shown in Table III, these macrophage-depleted nonadherent cells still exhibited significant suppressor activity when transferred to syngeneic recipients, suggesting that the suppressor cells are not macrophages.

To further characterize the suppressor cells, LNC from recovered donors were separated into nonadherent and adherent fractions on nylon wool columns. Yields of adherent LNC were low, and in each experiment the adherent and nonadherent cells were compared at similar concentrations. The Ig^+-enriched adherent LNC exhibited significant suppressive activity relative to the nonadherent cells, although the latter were not entirely devoid of activity (Table III).

TABLE III

Summary of postrecovery suppressor cell experiments. Cells were transferred 28 to 32 days postchallenge, and recipients were challenged with BP + CFA 8 days post-transfer

Cells Transferred	Clinical EAE Severity					Histologic
	0	1	2	3	Mean	
						mean
5×10^8 thymocytes				6	3.0	3.6
5×10^8 LNC	6				0.0[a]	1.3
5×10^7 LNC	1	3	1	1	1.3[a]	1.8
5×10^6 LNC				4	3.0	2.8
1.5–2 $\times 10^8$ Sephadex G-10 nonadherent LNC (25% Ig^+; <2% stained with neutral red)	3	3	2	1	1.1[a]	2.6
5×10^7 glass wool nonadherent LNC (7% Ig^+)				6	3.0	1.8
6–10 $\times 10^7$ nylon nonadherent LNC (<6% Ig^+)	3	5	1	8	1.8[a]	3.3
6–10 $\times 10^7$ nylon adherent LNC (>73% Ig^+)	6	3		3	1.0[a]	2.6
Controls:						
LNC from donors given cytochrome-*c* + CFA	1		1	4	2.3	2.6
No cells transferred			3	35	2.9	N.D.[b]

[a] Significantly different from controls that received no cells ($p < 0.001$).

[b] N.D., not determined.

DISCUSSION

After recovery, Lewis rats were resistant to attempts to reinduce EAE for at least 8 weeks, the longest interval studied. However, rats given CFA alone before challenge with BP + CFA were also resistant to EAE. This is in agreement with a similar observation by Lisak and Zweiman (21) in guinea pigs, although Willenborg (9) recently reported that adjuvant-induced suppression may be transient.

Since we were not able to evaluate the relative contributions of specific *vs* nonspecific factors in resistance to EAE after recovery from actively induced disease, we employed an adoptive transfer system to demonstrate immunologically specific resistance to EAE. After recovery from adoptively transferred EAE, 58% of the recipients were completely resistant when challenged, even when the challenge was delayed for 7 weeks. The remaining rats exhibited only mild clinical signs. Resistance was specific, since recipients of LNC from donors given CFA alone were not protected.

Evidence in support of the role of suppressor cells in recovery from EAE derives from transfer experiments in which viable lymph node cells from recovered donors inhibited active induction of EAE in syngeneic recipients. In contrast, LNC transferred before or at onset of EAE did not exhibit suppressor activity. Recipients of these cells developed EAE, suggesting the sequential activation of effector cells, followed by suppressive cells, in the response to BP + CFA. The appearance of suppressor activity coincided with recovery from EAE and persisted until day 34 post-challenge, the longest interval studied. This contrasts markedly with the transient activity reported by Adda *et al.* (22), who observed that LNC from rats challenged with BP + CFA displayed suppressor activity 17 days later, but not on day 25.

One interpretation of our findings is that immunization of Lewis rats with BP + CFA stimulates the production of effector T lymphocytes, which elicit EAE and CMI to the antigen. These are probably short-lived cells, since adoptive transfer of EAE can only be achieved for a limited time (Fig. 1, Reference 5). Antigen-specific suppressor cells are also generated after challenge but appear at a slower rate than the effector cells. These suppressor cells, which are relatively long lived, can prevent the continued production of effector cells but are unable to inhibit previously generated effector cells. This would account for the transient nature of EAE in the rat and rapid recovery from the disease.

Cell separation studies (Table III) indicate that significant suppressor activity is associated with adherent cells, although presumably not with macrophages, since they do not adhere to Sephadex G-10 (17, 23). The adherent cells with suppressive activity could be isolated in low yield from nylon wool columns. This population was enriched in Ig^+ cells, but still contained approximately 20% of cells lacking detectable surface Ig.

The post-recovery suppressor cell is clearly different from the suppressor cell found in the lymph nodes of tolerant rats, which is a nonadherent T lymphocyte (11). Perhaps the post-recovery suppressive cell is an adherent T lymphocyte of the type described by Waksman and his colleagues (16, 24). Support for this possibility derives from the finding that thoracic duct lymphocytes from challenged rats also suppress EAE (25).

Moreover, Cooke *et al.* (26) recently observed long-lived suppressor T lymphocytes that control autoantibody production in mice with hemolytic anemia induced by cross-reacting rat erythrocytes. The authors suggested that these suppressor cells are responsible for the self-limiting nature of experimental autoimmune hemolytic anemia in normal mouse strains. It is also conceivable that adherent and nonadherent cells are both required for most effective suppression, as suggested by Doyle *et al.* (27).

Alternatively, the possibility must also be considered that post-recovery suppression is mediated by antibodies produced by B cells transferred to the recipient rats. Since it is known that Lewis rats have relatively high titers of anti-BP antibodies after recovery from EAE (28, 29), it is conceivable that we transferred B cells that synthesized anti-BP blocking antibodies in the recipients. This would be consistent with the early report of Paterson and Harwin (30), who found that pooled serum from rats that had been previously challenged with guinea pig spinal cord homogenate and adjuvant protected recipients against EAE. Moreover, Sy *et al.* (31) recently reported that antibodies regulate the duration of contact sensitivity in mice. Those antibodies appeared to possess anti-idiotypic activity.

The present findings strongly indicate that suppression of EAE is mediated by an adherent cell present in the lymph nodes of rats that have recovered from the disease. Further work is necessary before it can be ascertained whether these cells mediate suppression directly, or function indirectly via synthesis of antibody. These studies are in progress.

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REFERENCES

1. Gonatas, N., and J. C. Howard. 1974. Inhibition of experimental allergic encephalomyelitis in rats severely depleted of T cells. Science 186:839.
2. Lennon, V. A., and W. J. Byrd. 1973. Role of T lymphocytes in the pathogenesis of experimental allergic encephalomyelitis. Eur. J. Immunol. 3:243.
3. Shaw, C. M., E. C. Alvord, Jr., J. Kaku, and M. W. Kies. 1965. Correlation of experimental allergic encephalomyelitis with delayed-type skin sensitivity to specific homologous encephalitogen. Ann. N.Y. Acad. Sci. 122:318.
4. Ortiz-Ortiz, L., R. M. Nakamura, and W. O. Weigle. 1976. T cell requirement for experimental allergic encephalomyelitis induction in the rat. J. Immunol. 117:576.
5. Paterson, P. Y. 1966. Experimental allergic encephalomyelitis and autoimmune disease. Adv. Immunol. 5:131.
6. Paterson, P. Y. 1960. Transfer of experimental allergic encephalomyelitis in rats by means of lymph node cells. J. Exp. Med. 111:119.
7. Levine, S., E. M. Hoenig, and M. W. Kies. 1970. Allergic encephalomyelitis: immunologically specific inhibition of cellular passive transfer by encephalitogenic basic protein. Clin. Exp. Immunol. 6: 503.
8. McFarlin, D. E., S. E. Blank, and R. F. Kibler. 1974. Recurrent experimental allergic encephalomyelitis in the Lewis rat. J. Immunol. 113:712.
9. Willenborg, D. O. 1979. Experimental allergic encephalomyelitis in the Lewis rat: studies on the mechanism of recovery from disease and acquired resistance to reinduction. J. Immunol. 123:1145.
10. Swierkosz, J. E., and R. H. Swanborg. 1975. Suppressor cell control of unresponsiveness to experimental allergic encephalomyelitis. J. Immunol. 115:631.
11. Welch, A. M., and R. H. Swanborg. 1976. Characterization of suppressor cells involved in regulation of experimental allergic encephalomyelitis. Eur. J. Immunol. 6:910.
12. Swierkosz, J. E., and R. H. Swanborg. 1977. Immunoregulation of experimental allergic encephalomyelitis: conditions for induction of suppressor cells and analysis of mechanism. J. Immunol. 119:1501.
13. Welch, A. M., J. E. Swierkosz, and R. H. Swanborg. 1978. Regulation of self tolerance in experimental allergic encephalomyelitis. I. Differences between lymph node and spleen suppressor cells. J. Immunol. 121:1701.
14. Swanborg, R. H., J. E. Swierkosz, and R. G. Saieg. 1974. Studies on the species-variability of experimental allergic encephalomyelitis in guinea pigs and rats. J. Immunol. 112:594.
15. Levine, S., and E. J. Wenk. 1963. Encephalitogenic potencies of nervous system tissues. Proc. Soc. Exp. Biol. Med. 114:220.
16. Folch, H., M. Yoshinaga, and B. H. Waksman. 1973. Regulation of lymphocyte response *in vitro*. III. Inhibition by adherent cells of the T lymphocyte response to phytohemagglutinin. J. Immunol. 110:835.
17. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. J. Immunol. Methods 5:239.
18. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645.
19. Swanborg, R. H., U. Hellström, H. Perlmann, S. Hammerström, and P. Perlmann. 1977. A new rat lymphocyte surface marker: characterization and separation of cells with receptors for *Helix pomatia* hemagglutinin. Scand. J. Immunol. 6:235.
20. Cohen, Z. A., and E. Wiener. 1963. The particulate hydrolases of macrophages. I. Comparative enzymology, isolation, and properties. J. Exp. Med. 118:991.
21. Lisak, R. P., and B. Zweiman. 1974. Immune responses to myelin basic protein in mycobacterial-induced suppression of experimental allergic encephalomyelitis. Cell. Immunol. 14:242.
22. Adda, D. H., E. Beraud, and R. Depieds. 1977. Evidence for suppressor cells in Lewis rats' experimental allergic encephalomyelitis. Eur. J. Immunol. 7:620.
23. Koren, H. S., and R. J. Hodes. 1977. Effect of tumor cells on the generation of cytotoxic T lymphocytes *in vitro*. I. Accessory cell functions of mouse tumor cells in the generation of cytotoxic T lymphocytes *in vitro*: replacement of adherent phagocytic cells by tumor cells or 2-mercaptoethanol. Eur. J. Immunol. 7:394.
24. Bash, J. A., A. M. Singer, and B. H. Waksman. 1976. The suppressive effect of immunization on the proliferative responses of rat T cells *in vitro*. II. Abrogation of antigen-induced suppression by selective cytotoxic agents. J. Immunol. 116:1350.
25. Frost, H. 1979. Suppression of experimental allergic encephalomyelitis with thoracic duct lymphocytes. Fed. Proc. 38:4531. (Abstr.)
26. Cook, A., P. R. Hutchings, and J. H. L. Playfair. 1978. Suppressor T cells in experimental autoimmune haemolytic anemia. Nature 273:154.
27. Doyle, M. V., D. E. Parks, and W. O. Weigle. 1976. Specific, transient suppression of the immune response by HGG tolerant spleen cells. II. Effector cells and target cells. J. Immunol. 117:1152.
28. Day, E. D., and O. M. Pitts. 1974. The antibody response to myelin basic protein in Lewis rats: the effect of time dosage of BP, and dosage of *Mycobacterium butyricium*. J. Immunol. 113:1958.
29. Swierkosz, J. E., and R. H. Swanborg. 1977. Antibody response to myelin basic protein: comparisons between Lewis rats with experimental allergic encephalomyelitis, and tolerant rats. Immunol. Commun. 6:499.
30. Paterson, P. Y., and S. M. Harwin. 1973. Suppression of allergic encephalomyelitis in rats by means of antibrain serum. J. Exp. Med. 117:755.
31. Sy, M. S., J. W. Moorhead, and H. N. Claman. 1979. Regulation of cell-mediated immunity by antibodies: possible role of antireceptor antibodies in the regulation of contrast sensitivity to DNFB in mice. J. Immunol. 123:2593.