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SPONTANEOUS REMISSION AND ACQUIRED RESISTANCE TO AUTOIMMUNE ENCEPHALOMYELITIS (EAE) ARE ASSOCIATED WITH SUPPRESSION OF T CELL REACTIVITY: SUPPRESSED EAE EFFECTOR T CELLS RECOVERED AS T CELL LINES[1]

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Experimental autoimmune encephalomyelitis (EAE) is a disease that can be induced in Lewis rats by inoculating them with myelin basic protein (BP) emulsified in complete Freund's adjuvant. Rats spontaneously recover from EAE and subsequently become refractory to attempts to induce a second bout of EAE. Mechanisms involved in spontaneous recovery and acquired refractoriness to EAE were elucidated by investigating a number of variables as a function of the clinical stage of disease. We studied the differential proliferative responses of T lymphocytes to specific antigenic determinants of BP, detected the presence of suppressor cells by their influence on antigen-specific T lymphocyte lines *in vitro*, and isolated suppressed effector T lymphocytes by growing them as cell lines. The following results were obtained: 1) Onset of EAE and spontaneous recovery correlated with the rise and fall of T lymphocyte responses in the draining lymph nodes to specific encephalitogenic determinants of BP. The responses to other determinants of BP were not related to the clinical stage of EAE. 2) Depression of this T lymphocyte response to critical antigenic determinants was associated with the appearance of immunospecific suppressor cells, most prominent in the spleen and thymus. 3) Thymic suppressor cells appeared to recognize specifically T cells with anti-BP receptors. 4) Suppressed EAE effector T lymphocytes were rescued from the thymus, spleen, and lymph nodes of rats recovered from and refractory to EAE by selecting and propagating these cells *in vitro* as lines. Upon i.v. inoculation, cells of these rescued T lymphocyte lines produced EAE in naive recipients. 5) Suppressor cells and suppressed EAE effector T lymphocytes were also detected in rats that had been injected with nonencephalitogenic BP in incomplete Freund's adjuvant. Thus, spontaneous restoration of self-tolerance to BP and acquired resistance to EAE were regulated by reversible suppression of specific effector T lymphocytes reactive against critical self-antigenic determinants.

Experimental autoimmune encephalomyelitis (EAE)[2] is an autoimmune disease that can be induced in genetically susceptible animals by inoculating them with myelin basic protein (BP) in a suitable adjuvant such as complete Freund's adjuvant (CFA) (1). Recently we demonstrated that EAE can be mediated by isolated T lymphocytes that are reactive only against BP and that were propagated for over a year as a T cell line (2). This confirms previous indications that EAE is caused by T lymphocytes rather than by antibodies (3). EAE in rats is characterized by acute onset of paralysis, chiefly of the hind limbs, and perivascular infiltration by mononuclear cells in the central nervous system. Spontaneous recovery from clinical paralysis usually occurs after 4 to 6 days, particularly if the rats are given easy access to food and water during disease. Rats that have recovered from an episode of EAE acquire resistance to further attempts to induce EAE by inoculation with BP/CFA (4, 5). In view of the fact that BP, the self-antigen, is continuously present in the animal (6), it is unlikely that termination of EAE results from the disappearance of potentially stimulating antigen. Rather, it is conceivable that spontaneous recovery and acquired resistance to EAE might involve regulation of the autoimmune response that reestablishes self-tolerance to BP. In support of this notion is our recent observation that full recovery from EAE did not occur in rats that had undergone splenectomy or thymectomy before inoculation with BP/CFA (7). Thus, spontaneous recovery would appear to require regulatory processes related to functions of the spleen or thymus. Reestablishment of self-tolerance to BP could occur through permanent deletion (8) of self-reactive anti-BP clones of lymphocytes or, alternatively, by some form of long-term suppression of such clones (9). Active suppression of EAE is inducible (10, 11) by multiple injections of BP in incomplete Freund's adjuvant (ICFA); however, active suppression has not yet been demonstrated as the cause of spontaneous remission and refractoriness after clinical EAE. The experiments described in this article were designed to explore cellular mechanisms involved in spontaneous recovery and acquired resistance to EAE, and in resistance to EAE induced by injection of BP/ICFA.

MATERIALS AND METHODS

Rats. Pure-bred Lewis rats were supplied by the Animal Breeding Center of this Institute and were used at 2 to 4 mo of age. Rats were matched for age and sex in each experiment.

Antigens. BP was prepared as described (12) from the spinal cords of guinea pigs (G-BP) or rats (R-BP), without the step of purification by column chromatography. The encephalitogenic peptide (EP) composed of residues 68-88 of G-BP was prepared, purified, and kindly donated by Dr. F. C.-H. Chou of Emory University, Atlanta, GA (13). Chicken egg ovalbumin (OA) was purchased from Sigma Chemicals (St. Louis, MO), and purified protein derivative of tuberculin (PPD) from Statens Serum Institut (Copenhagen, Denmark).

Immunizations. Rats were inoculated in each of 4 footpads with 0.05 ml containing 25 μg antigen in phosphate-buffered saline (PBS), or PBS alone, emulsified in an equal vol of CFA containing 200 μg *Mycobacterium tuberculosis* H37Ra (CFA; Difco, Detroit, MI) or in ICFA (Difco). Inoculated rats

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[1] This work was supported by a grant from the Stiftung Volkswagenwerk, Hannover.
[2] Abbreviations used in this paper: BP, basic protein of myelin; CFA, complete Freund's adjuvant; EAE, experimental autoimmune encephalomyelitis; EM, Dulbecco's modification of Eagle's medium; EP, encephalitogenic peptide; G-BP, guinea pig basic protein of myelin; ICFA, incomplete Freund's adjuvant; LN, lymph nodes; OA, chicken egg albumin; PPD, purified protein derivative of tuberculin; R-BP, rat basic protein of myelin; SI, stimulation index.

were observed for development of EAE or were used on day 9 after immunization as donors of lymphoid organs from which cells were grown as lines or were tested for proliferative responses *in vitro*.

Diagnosis of EAE. Overt paralysis of the tail and hind legs was considered as the minimal sign of clinical EAE. Pathologic evidence of EAE, consisting of perivascular infiltration of mononuclear cells, was determined in 5- to 8-μ microscopic sections of brains and spinal cords that had been fixed with 10% formalin and stained with hematoxylin and eosine (14).

Proliferative response of lymphocytes from primed rats. Suspensions of lymphocytes were prepared by pressing lymph nodes, spleens, or thymuses through a fine wire mesh; the washed cells were seeded in flat-bottom microtiter plates (Costar, Cambridge, MA) in quadruplicate wells. Each well contained 10^6 lymph node or spleen cells or 2×10^6 thymus cells in 0.2 ml of culture medium, with antigens in the optimal concentrations determined by dose-response experiments: R-BP, 100 μg/ml; G-BP, 50 μg/ml; EP, 5 μg/ml; OA, 125 μg/ml; and PPD, 25 μg/ml. Concanavalin A (Con A; Miles-Yeda, Rehovot, Israel), 2.5 μg/ml, was used in some cultures. Proliferation culture medium was composed of Dulbecco's modification of Eagle's medium (EM) supplemented with 2-mercaptoethanol (5×10^{-5} M), sodium bicarbonate (0.024 M), L-glutamine (2 mM), antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml; and garamycin, 40 μg/ml), and 1% fresh autologous rat serum. The cultures were incubated for 72 hr at 37°C in humidified air plus 7.5% CO_2, and each well was pulsed with 1 μCi of ^{3}H-thymidine (specific activity 10 Ci/mmol; Nuclear Research, Negev, Israel) for the final 18 hr. The cultures were then harvested on fiberglass filters, and the proliferative response was expressed as the mean cpm or delta cpm (mean cpm of test cultures minus mean cpm of control cultures without antigens), or as a stimulation index (SI; mean cpm of test cultures divided by mean cpm of control cultures). The standard errors were always less than 10% of the mean.

Lines of T lymphocytes. The Z1a anti-BP line and the C1a anti-OA line were derived and maintained as described (2). Additional lines of lymphocytes reactive against BP were developed from suspensions of lymphoid cells in the absence of density separation (2) by culturing the cells in supplemented EM for 72 hr at a concentration of 5×10^6/ml in 60-mm petri dishes (Falcon, Oxnard, CA; 6 ml/dish) with G-BP (60 μg/ml). The lymphoblasts were then seeded at a concentration of 2×10^5/ml in propagation medium. The propagation medium was the proliferation medium described above, minus autologous serum and supplemented with 15% (v/v) of supernatant of Con A-stimulated lymphocytes, prepared as described (15), 10% horse serum (Gibco, Grand Island, NY), sodium pyruvate (1 mM), and nonessential amino acids (1% of X100; Bio-Lab, Jerusalem, Israel). The cultures were transferred every 3 to 4 days for 8 wk before study of their proliferative response to antigens or their ability to mediate EAE.

Proliferative responses of T cell lines. The method to determine proliferative responses of T cell lines to various antigens was like that described for cells from primed rats except that each culture well contained 2.5×10^4 line cells plus 10^6 accessory cells in the form of irradiated (1500 R) syngeneic lymph node or spleen cells or 2×10^6 thumus cells. After 24 hr of incubation, each well was pulsed with ^{3}H-thymidine for an additional 18 hr, and the wells were harvested and counted.

Assay of suppressor cells. Suppressor cells in lymph nodes, spleens, or thymuses were assayed by their effect on the proliferative responses of the Z1a or C1a line cells. We added 10^6 or 2×10^6 test cells (irradiated by 1500 R) to the proliferative response cultures of 2.5×10^4 cells of the Z1a or C1a lines. The degree of suppression was measured by comparing the proliferative responses to those obtained using these lines in the presence of accessory cells from normal rats, or from rats that had been inoculated with CFA alone.

EAE mediated by cell lines. Cells (2×10^5/ml) of lines were restimulated by incubation for 3 days with G-BP (10 μg/ml) in the presence of syngeneic irradiated (1500 R) thymus cells (15×10^6/ml) as accessory cells. The lymphoblasts were collected and washed 2 times in PBS, and 5×10^6, 10×10^6, or 20×10^6 in 1 ml PBS were inoculated i.v. into Lewis rats. At the time of inoculation about 85 to 95% of the cells were seen to be lymphoblasts, the accessory cells having largely disintegrated. The recipient rats were observed daily for development of EAE.

RESULTS

Induction of EAE. Rats were injected with G-BP, R-BP, or EP emulsified in CFA or in ICFA to investigate the encephalitogenicity of these inocula. Table I shows the results. None of the rats inoculated with the antigens in ICFA developed clinical EAE. In contrast, rats inoculated with G-BP/CFA or EP/CFA developed paralysis about 12 or 13 days after inoculation, and all rats spontaneously recovered 5 to 6 days later. Injection of R-BP/CFA also led to development of clinical EAE, but, as has been previously noted (16), the disease tended to be milder in that the latent period was longer and paralysis was judged to be less severe than that induced by injections of G-BP/CFA.

Induction of EAE is associated with a response to specific antigenic determinants of BP. To investigate factors influencing the response to specific antigenic determinants of BP, we inoculated rats with G-BP, R-BP, or EP in CFA or in ICFA and assayed the T cell proliferative responses of cells obtained from the draining lymph nodes 9 days later (Table II). Lymph node cells from rats inoculated with nonencephalitogenic G-BP/ICFA responded to G-BP *in vitro* almost as well as did lymph node cells obtained from rats inoculated with encephalitogenic G-BP/CFA. This suggests that CFA and ICFA both functioned grossly as adjuvants supporting a response to BP. However, the nature of the adjuvant used appeared to influence selection of the specific antigenic determinants against which the immune response was directed. Inoculation of encephalitogenic G-BP/CFA led to high *in vitro* responses to R-BP and to EP, compared to the responses to these antigens obtained after inoculation of nonencephalitogenic G-BP/ICFA. Thus, specific determinants presented on R-BP and EP were more effectively antigenic after inoculation of G-BP/CFA than after inoculation of G-BP/ICFA.

It can also be seen in Table II that G-BP was more immunogenic than R-BP. Injection of G-BP/CFA led to greater *in vitro* proliferative responses to R-BP or G-BP than did injection of R-BP/CFA. This finding is compatible with the relatively greater encephalitogenicity of G-BP/CFA noted above (Table I).

A consistent finding was a moderate response against PPD of cells obtained from rats that had been injected with BP or EP in ICFA that contained no Mycobacteria and, hence, no PPD. However, this can be explained by the observation that BP and EP share cross-reactivity with some determinants of PPD (17, 18).

TABLE I

Induction of EAE and spontaneous recovery[a]

Immunization		Clinical EAE		
Antigen	Adjuvant	Incidence of overt paralysis % (No. of rats)	Mean day of onset	Mean day of recovery
G-BP	CFA	100 (50)	12	17
	ICFA	0 (10)	—	—
R-BP	CFA	90 (20)	16	18
	ICFA	0 (10)	—	—
EP	CFA	100 (10)	13	18
	ICFA	0 (10)	—	—

[a] Lewis rats were inoculated with G-BP, R-BP, or EP, emulsified in CFA or ICFA and observed for the appearance of paralysis as a sign of EAE. All rats that came down with EAE recovered spontaneously.

TABLE II

Proliferative responses of rats inoculated 9 days earlier with G-BP, R-BP or EP[a]

Antigen	Adjuvant	Proliferative Responses (cpm $\times 10^{-3}$)				
		No antigen	G-BP	R-BP	EP	PPD
G-BP	CFA	15.7	78.1	55.8	72.3	89.0
	ICFA	11.3	69.8	17.7	28.1	44.5
R-BP	CFA	12.7	37.3	29.8	35.2	90.4
	ICFA	16.4	24.9	21.6	30.3	48.7
EP	CFA	21.9	95.3	83.7	92.1	102.4
	ICFA	27.2	64.1	52.6	59.2	71.5
None	CFA	16.2	27.3	17.1	18.9	86.6

[a] Female Lewis rats were immunized in the hind foot pads and 9 days later the draining lymph node cells were assayed for their proliferative responses to the antigens by measuring their ^{3}H-thymidine incorporation.

Spontaneous recovery from EAE is associated with a depression of the response against specific antigenic determinants of R-BP. As shown above (Table I), rats usually recover from acute EAE induced by injection of BP/CFA or EP/CFA. To gain some insight into the mechanisms responsible for spontaneous recovery from EAE, we studied the proliferative responses of T lymphocytes from various lymphoid organs to specific antigenic determinants as a function of the clinical state of EAE. Figure 1 shows the results of an experiment comparing the degree of responsiveness to R-BP, G-BP, and PPD after an encephalitogenic injection of G-BP/CFA (Fig. 1*A*, *B*, *C*) or a nonencephalitogenic injection of G-BP/ICFA (Fig. 1*D*, *E*, *F*). These responses were measured on days 9 (onset of EAE), 18 (recovery), and 26, 33, 40, and 50 (after recovery from EAE). A number of conclusions can be made upon inspection of Figure 1: a) The onset of EAE at day 9 was associated with a response to R-BP in the draining lymph node (Fig. 1*C*) but not in the spleen or thymus. A second peak of lymph node reactivity to R-BP was found to occur on day 26. No response to R-BP was detected after the nonencephalitogenic injection of BP/ICFA (Fig. 1*F*). b) The period of recovery from EAE, day 18, was marked by a decrease in the response to R-BP of cells from the draining lymph node (Fig. 1*C*). At this time, no decrease was noted in the response to G-BP (Fig. 1*B*) or in the response to the nonrelevant antigen, PPD (Fig. 1*A*). Therefore, the response in the draining lymph nodes to R-BP-specific determinants was correlated with the course of disease; it rose with the onset of EAE and fell with spontaneous recovery. c) The response of lymphocytes to G-BP-specific determinants was not associated with disease. It occurred after a nonencephalitogenic injection of BP/ICFA (Fig. 1*E*), and it persisted in cells obtained from the lymph node after recovery from EAE (Fig. 1*B*). d) Mild responses to BP were detected in cells of the spleen and thymus only after recovery from EAE.

Essentially similar results were obtained in each of 3 or 4 repeat experiments of each point studied.

Lymphoid cells from EAE-recovered rats suppress the proliferative response to R-BP of a specific T cell line. The specificity of the rise and fall of the response of lymph node cells to R-BP led us to suspect that spontaneous recovery from EAE might be associated with suppression of T lymphocytes specifically reactive against the antigenic determinants displayed on R-BP. T lymphocytes responsive to other determinants displayed on G-BP appeared not to be depressed (Fig. 1).

As an indicator system to detect this proposed suppression, we used the proliferative responses of 2 lines of T lymphocytes: the Z1a line, responsive to G-BP and to R-BP, and the C1a line, responsive to OA. Each of these T cell lines responds to its specific antigen only in the presence of accessory cells, irradiated cells obtained from lymph nodes, spleens, or thymuses of syngeneic rats (2; Table III). To test whether rats that had recovered from FAE had lymphoid cells capable of suppressing a response to BP, we used lymphoid cells from such rats compared to those obtained from normal rats, as accessory cells for the Z1a and C1a lines of T lymphocytes. We found (Table III) that lymph node, spleen, or thymus cells from normal rats served as accessory cells for the responses of Z1a line cells to R-BP or to G-BP. Normal spleen cells also furnished accessory cells for the response of the C1a line cells to OA. However, lymphoid cells obtained from rats that had recovered from EAE induced 35 days earlier suppressed the proliferative response of the Z1a line but not that of the C1a line. Spleen cells from EAE-recovered rats showed no significant inhibition of the response of C1a line cells to OA (4% inhibition) but strongly inhibited the responses of Z1a line cells to R-BP (81% inhibition) and inhibited somewhat less strongly their responses to G-BP (53% inhibition). Thymus cells showed similar inhibitory effects: 75% inhibition of the response to R-BP and 37% inhibition of the response to G-BP. Lymph node cells were

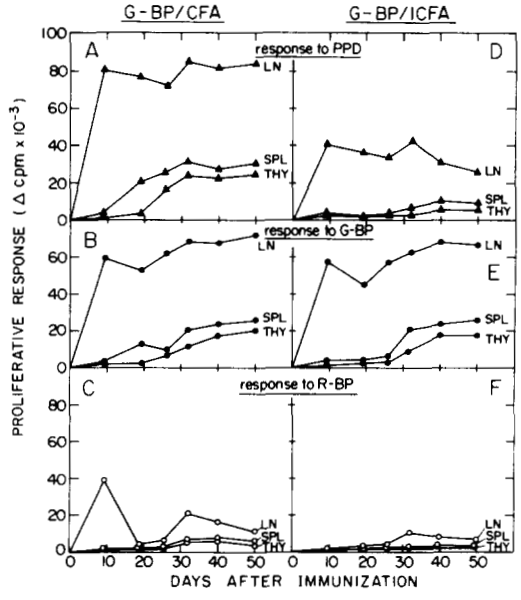

Figure 1. The kinetics of the proliferative response of lymph node (LN), spleen (SPL) or thymus (THY) cells to PPD (*A*, *D*), G-BP (*B*, *E*) or R-BP (*C*, *F*), after injection into the hind footpads of encephalitogenic G-BP-CFA (*A*, *B*, *C*) or nonencephalitogenic G-BP/ICFA (*D*, *E*, *F*). These cells of three rats were pooled for each time point. Differences of Δ cpm between points of greater than 10^4 were highly significant ($p < 0.01$).

TABLE III

Suppression of the proliferative response to R-BP and G-BP in EAE-recovered rats

Accessory Cells		Cell Line	Antigen Specificity	Proliferative Response[a] to Antigens (cpm × 10^{-3})				% Inhibition[b] of Response to		
Status of donor rat	Organ of origin			None	R-BP	G-BP	OA	R-BP	G-BP	OA
Normal	Lymph nodes	Z1a	BP	1.9	26.7	67.1	1.3			
	Spleen			1.3	19.4	37.2				
	Thymus			2.4	24.6	59.8				
	Spleen	C1a	OA	1.2	1.1	1.2	51.4			
Recovered from EAE	Lymph nodes	Z1a	BP	1.4	21.4	52.9		19	21[c]	
	Spleen			1.2	4.6	18.0		81[d]	53[d]	
	Thymus			2.3	7.8	38.4		75[d]	37[d]	
	Spleen	C1a	OA	1.1			49.2			4

[a] ^{3}H-thymidine incorporation of anti-BP (Z1a) and anti-OA (C1a) cell lines stimulated *in vitro* with antigens in the presence of irradiated accessory cells obtained from either normal rats or rats that had recovered from EAE induced 35 days earlier by inoculation of G-BP/CFA. The cells of two rats were pooled for each point.

[b] Percent of inhibition was calculated by considering as 100% the responses to antigens in the presence of normal accessory cells.

[c] $P < 0.05$.

[d] $P < 0.001$.

much less inhibitory and did not distinguish between responses to R-BP (19% inhibition) and to G-BP (21% inhibition). Essentially similar results were obtained in each of 4 repeat experiments. Therefore, it appeared that immunospecific suppressor cells were present in the spleens and thymuses of rats that had recovered from EAE. These suppressor cells were resistant to irradiation and were more inhibitory of the response to specific R-BP determinants than they were to the response to G-BP determinants.

Suppressor cells specifically inhibit anti-BP line cells in the absence of BP. The above results indicated that rats recovered from EAE have suppressor cells that inhibit the proliferative response of the Z1a anti-BP line to BP. A fundamental question relates to the specificity of these suppressor cells. Were they activated to inhibit the Z1a line cells because they recognized suppressor determinants (11) on the BP antigen, or did they specifically recognize the anti-BP T lymphocytes (19)? To approach this question we designed an experiment to test the effect of suppressor cells on the behavior of the Z1a and C1a lines in the absence of specific antigen. We activated each of these lines by incubating them with the T cell mitogen Con A, to which they respond (2) in the presence of irradiated thymus cells obtained from uninoculated control rats or from rats that had been injected 40 days earlier with CFA or with BP/CFA, the latter having developed and then recovered from EAE. The results are shown in Table IV. We found that the response of the C1a line to Con A was not appreciably inhibited by the presence of irradiated thymus cells from any of the donor rats. In contrast the response of the Z1a line was significantly inhibited ($p>0.001$) in the presence of thymus cells from the donor that had recovered from EAE. In 6 repeat experiments using thymus cells obtained from different EAE-recovered rats, the degree of inhibition of the Z1a line to Con A varied from 22% to 85%. It is noteworthy that in the experiment in Table IV, thymus cells from the rat that had been inoculated with CFA alone showed a 20% suppression of the response of the Z1a and BP (17, 18) illustrated above (Table II). In any case, the suppressor cells in the thymuses of EAE-recovered rats were able to distinguish between the Z1a and C1a lines even in the absence of the BP and OA antigens. This suggests that the Z1a line itself, rather than suppressogenic determinants on BP, was recognized by the suppressor cells. Thus, acquired resistance to EAE may involve a mechanism that can distinguish between EAE effector cells and other T cells.

Kinetics of development of suppressor cells in EAE recovered rats. The appearance and the persistence of the activity of suppressor cells were investigated as a function of time after encephalitogenic immunization. Lewis rats were immunized with G-BP/CFA or with CFA alone. Lymph node, spleen, and thymus cells were obtained at different times after immunization and were compared with cells from normal rats as a source of accessory cells in the response of the Z1a cell line against R-BP. The results are shown in Figure 2.

The proliferative response of the Z1a cell line to R-BP in the presence of normal accessory cells was considered as 100%, and the stimulation of the Z1a cell line against R-BP in the presence of test cells was expressed as the percent of normal. Figure 2 demonstrates that rats that had recovered from EAE (after day 20) had cells that specifically suppressed the response to R-BP and that this suppressive activity resided mainly in the spleen (Fig. 2*B*) and thymus (Fig. 2*A*) but not in the lymph nodes (Fig. 2*C*). Furthermore, it can be seen (Fig. 2*B*) that the suppression of tbe response to R-BP-specific determinants was expressed by cells in the spleen at the onset of EAE (day 10).

The influence of lymph node cells on the anti-R-BP response was noteworthy (Fig. 2*C*). Around the onset of disease, day 10, accessory cell function of lymph node cells from rats injected with BP/CFA was about 50% greater than that of control rats injected with CFA alone. This augmented anti-BP accessory cell function disappeared and became mildly inhibitory after day 20, the period of recovery from EAE. A second peak of augmented function was noted on day 25, which coincided with the second peak of increased responsiveness to R-BP described above (Fig. 1*C*). Thereafter, accessory function was slightly decreased compared to control values. These fluctuations, observed consistently in each of 5 separate experiments, indicate that suppression of the response to R-BP in the spleen and thymus was associated with persistent

TABLE IV

Thymus cells in rats recovered from EAE suppress response of Zla line cells to Con A[a]

Donors of Thymus Accessory Cells, Inoculated 40 Days Earlier with:	T Cell Line	Proliferative Response to Con A (Δ cpm $\times 10^{-3}$)	% Suppression
Not inoculated	Z1a	32.7 ± 2.3	
	C1a	43.5 ± 2.2	
CFA	Z1a	26.3 ± 1.7	20
	C1a	41.7 ± 3.6	4
BP/CFA (post EAE)	Z1a	16.4 ± 1.2[b]	50
	C1a	42.9 ± 3.9	2

[a] Cells of the Z1a and Z1c lines were stimulated by Con A in the presence of irradiated thymus cells from rats that had been injected with CFA alone, or with BP/CFA, and had recovered from EAE. Each group was composed of cells pooled from two rats.

[b] $P < 0.001$.

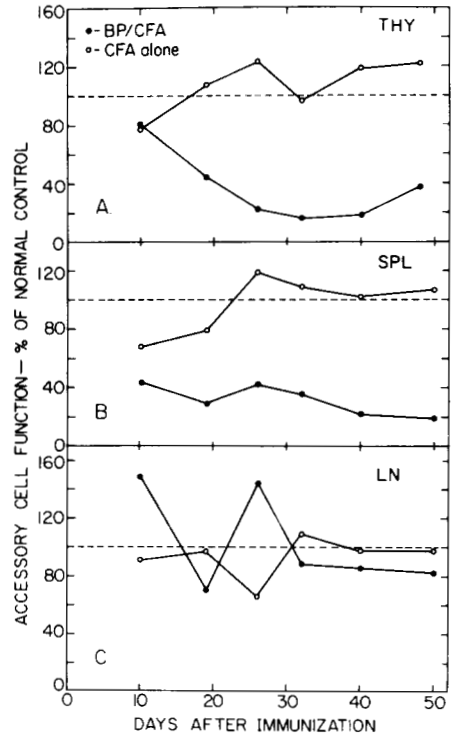

Figure 2. Kinetics of the development of suppression of the response to R-BP in rats recovered from EAE. Cells of the Z1a anti-BP cell line were stimulated *in vitro* with R-BP in the presence of irradiated accessory cells obtained from the thymus (*A*), spleen (*B*), or lymph nodes (*C*) from either normal rats or, at different times after immunization, from groups of two rats each that had been injected with CFA alone or with G-BP-CFA. Results are expressed as percent of the response of Z1a cell line in the presence of normal accessory cells. A percent difference of 30% or greater is significant ($p < 0.01$).

resistance to EAE, whereas augmentation of the response to R-BP in the lymph node was associated with onset of clinical EAE as well as with the second, subclinical, peak of response to R-BP. Hence, the clinical course of EAE might be related to a dynamic equilibrium between cells that helped and cells that suppressed the response of a T cell line to critical antigenic determinants of BP.

Suppressed EAE effector lymphocytes can be rescued from rats recovered from EAE. The above findings suggested that recovery from and refractoriness to EAE might be related to the suppression of endogenous anti-BP effector cells in the rat. To test this hypothesis, we set out to isolate effector cells whose number and/or activity were suppressed in rats that had recovered spontaneously from EAE. Our approach was to rescue these suppressed cells by culturing them *in vitro* under conditions conducive to their selection and propagation as lines (2).

Figure 3 shows proliferative responses to G-BP, R-BP, EP, and PPD of cells obtained from EAE-recovered rats, before (Fig. 3*A*) and after (Fig. 3*B*) selection and propagation *in vitro*. It can be seen that before selection *in vitro* (Fig. 3*A*) cells in the draining lymph nodes, spleen, and thymus had little or no responsiveness to R-BP or to EP. The cells did respond to G-BP and to PPD. However, after selection and propagation *in vitro* (Fig. 3*B*), cells from these organs manifested the ability to respond to R-BP and to EP. Hence, it was possible to rescue from rats in remission T lymphocytes responsive to the critical determinants displayed on R-BP and EP and to grow them as lines.

To confirm that these rescued T lymphocytes were indeed EAE effector cells, we injected 5×10^6 or 20×10^6 cells of the lines i.v. into Lewis rats. The results tabulated in Table V show that the cells were capable of mediating EAE.

Suppressed lymphocytes in rats inoculated with nonencephalitogenic G-BP/ICFA. Tables I and II and Figure 1 show that rats injected with BP or EP in ICFA failed to develop EAE and had relatively low responses *in vitro* to critical determinants displayed on R-BP or EP. In contrast, rats inoculated with G-BP/ICFA demonstrated a strong proliferative response to determinants displayed on G-BP. It was possible that the critical encephalitogenic determinants present on G-BP were not immunogenic when combined with ICFA. Alternatively, it was conceivable that these critical determinants were immunogenic (20, 21) but that the T cells responding to these determinants were suppressed. Earlier studies by Swierkosz and Swanborg (22, 23) were in favor of the second alternative, and we investigated this possibility. We used the Z1a cell line as an indicator system to uncover possible suppression of the response to R-BP in animals inoculated once with nonencephalitogenic G-BP/ICFA.

Cells of the C1a line responding to OA and the Z1a line responding to BP were stimulated *in vitro* with their corresponding antigen in the presence of irradiated lymph node, spleen, or thymus accessory cells obtained from normal rats or from rats that had been inoculated 35 days earlier with G-BP/ICFA. The results tabulated in Table VI show that lymph node, spleen, or thymus cells from G-BP/ICFA-injected animals, compared to cells from normal rats, suppressed the response of the Z1a cell line to G-BP or to R-BP but did not suppress the response of the C1a line to OA. Furthermore, suppression of the anti-R-BP response was greater than that of the anti-G-BP response. In addition, the spleen and thymus had more suppressive activity than the lymph nodes. Therefore, like rats recovered

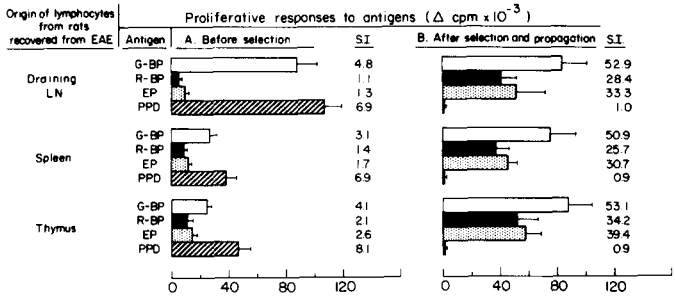

Figure 3. Rescue of suppressed T lymphocytes as cell lines responding to R-BP and EP determinants. Lymph node, spleen, or thymus cells from Lewis rats that had been injected into the hind footpads 30 days earlier with G-BP/CFA were assayed for their proliferative response to antigens immediately upon removal from the rats (*A*), or after selection to G-BP and propagation for 8 weeks, as cell lines (*B*). Results are expressed as Δ cpm and by stimulation index (S.I.).

TABLE V

EAE produced by T cell lines selected from lymphoid organs of rats recovered from EAE[a]

T Cell Line	Origin of T Cell Line	No. of Cells Injected	Incidence of EAE	
			Paralysis	Pathology
ZLa	Draining lymph node	20×10^6	7/10	6/10
		5×10^6	5/10	6/10
ZSa	Spleen	20×10^6	8/10	8/10
		5×10^6	8/10	7/10
ZTa	Thymus	20×10^6	6/10	6/10
		5×10^6	4/7	4/7

[a] T lymphocytes from lymphoid organs of Lewis rats that had recovered from EAE induced 35 days earlier with G-BP/CFA were selected by culture for 3 days with G-BP. The selected lymphocytes were then propagated as cell lines for 8 weeks in the absence of antigen. Before injection i.v. into normal syngeneic recipients they were restimulated *in vitro* with G-BP in the presence of irradiated syngeneic accessory cells for 72 hr.

TABLE VI

Suppression mediated by cells obtained from rats inoculated with G-BP/ICFA of the response of the Zla line to BP

Accessory Cells		Cell Line	Antigen Specificity	Proliferative Response[a] to Antigens (cpm × 10^{-3})				% Inhibition[b] of Response to:		
Status of donor rat	Organ of origin			None	R-BP	G-BP	OA	R-BP	G-BP	OA
None	Lymph nodes	Z1a	BP	1.9	26.7	67.1	1.3			
	Spleen			1.3	19.4	37.2				
	Thymus			2.4	24.6	59.8				
	Spleen	C1a	OA	1.2	1.2	1.2	51.4			
Inoculated with G-BP/ICFA	Lymph nodes	Z1a	BP	1.4	19.7	54.2	1.4	26[c]	19[c]	
	Spleen			1.1	11.6	27.3		42[d]	27[d]	
	Thymus			2.0	18.1	41.0		41[d]	31[d]	
	Spleen	C1a	OA	1.6	1.5	1.6	52.2			0

[a] ^{3}H-thymidine incorporation of anti-BP (Z1a) and anti-OA (C1a) cell lines stimulated *in vitro* with antigens in the presence of irradiated accessory cells obtained from either normal animals or animals that had been injected 35 days earlier with nonencephalitogenic BP/ICFA. Each group is composed of cells pooled from two rats.

[b] Percent of inhibition of response was calculated as described in Table III.

[c] $P < 0.01$.

[d] $P < 0.001$.

from EAE, rats injected with BP/ICFA had cells in their lymphoid organs that specifically suppressed lymphocytes that recognized BP. Figure 4 shows the kinetics of the development of these suppressor cells in the lymph nodes, spleen, and thymus. In general, suppression after injection of BP/ICFA was significant but appeared to be somewhat weaker than that following recovery from active EAE (comapre Tables III and IV and Figs. 2 and 4).

Suppressed EAE effector cells can be rescued from rats after inoculation with BP/ICFA. To confirm the existence of suppressed EAE effector cells in rats that had been injected with BP/ICFA, we sought to rescue these putatively suppressed T lymphocytes, using the same *in vitro* culture technique described above. Lymph node lymphocytes of animals that had been injected 9 days earlier with G-BP, R-BP, or EP in CFA or in ICFA were submitted to *in vitro* selection to G-BP and propagation as cell lines (2). Figure 5 shows the responses to G-BP, R-BP, EP, and PPD before (Fig. 5*A*) and after (Fig. 5*B*) development of T cell lines selected to respond to BP. The results were similar to those obtained with cells from rats recovered from EAE (Fig. 3). Before selection, the response to R-BP and EP was stronger in lymph node cells from rats that had been injected with antigen in CFA than it was in cells from rats that had been injected with antigen in ICFA (Fig. 5*A*). However, the selected cell lines from ICFA rats gained strong reactivity to R-BP and EP (Fig. 5*B*). This reactivity apparently was not generated *de novo* by contact with G-BP *in vitro*, because cells from rats that had been injected with CFA alone failed to show reactivity to BP or EP after selection and culture *in vitro* with G-BP.

Table VII shows that the rescued cells were indeed EAE effector cells and could mediate disease in recipient rats.

Characterization of rescued T cell lines and the EAE mediated by them. The effector cell lines rescued from rats that had recovered from EAE and from rats that had been inoculated

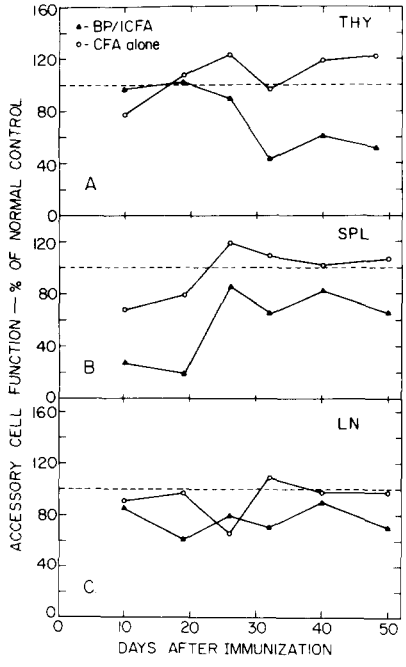

Figure 4. Kinetics of the development of suppression of the response to R-BP in rats that had been inoculated with nonencephalitogenic G-BP/ICFA. The anti-BP Z1a cell line was stimulated *in vitro* with R-BP in the presence of irradiated accessory cells from the thymus (*A*), spleen (*B*), or LN (*C*) obtained at different times after immunization from either normal animals or animals that had been injected with CFA alone or with G-BP/ICFA. Results are expressed as percent of the response of Z1a cell line in the presence of normal accessory cells.

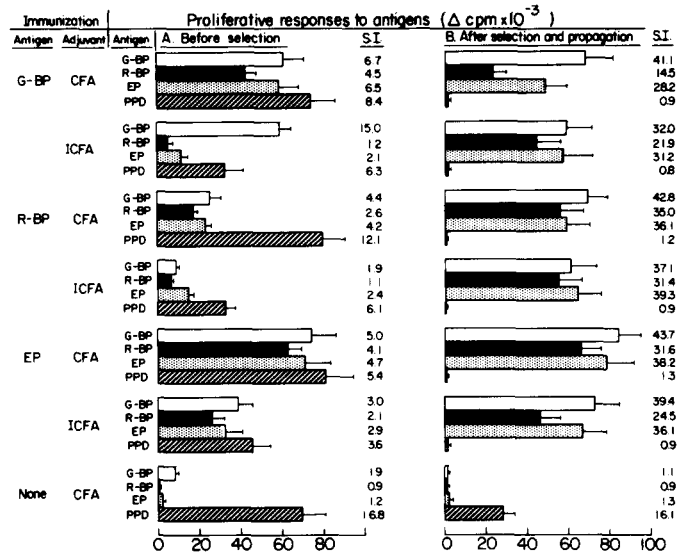

Figure 5. Rescue of suppressed T lymphocytes as cell lines responding to R-BP and EP determinants from rats injected with G-BP/ICFA. Lewis rats were inoculated with encephalitogenic G-BP/CFA, R-BP/CFA or EP/CFA, or with nonencephalitogenic G-BP/ICFA, R-BP/ICFA, or EP/ICFA. Nine days later, the draining LN cells were assayed for their *in vitro* responses to antigens immediately upon removal from the animals (*A*), or (*B*) after selection *in vitro* to G-BP and propagation for 8 weeks as cell lines. Results are expressed by Δ cpm and by stimulation index (S.I.).

TABLE VII

EAE mediated by T cell lines selected from rats that had been inoculated with nonencephalitogenic BP/ICFA or EP/ICFA, or with encephalitogenic BP/CFA or EP/CFA

T Cell Line	Obtained from Rats Immunized 9 days Earlier with:	Incidence of EAE after Inoculation of 10^7 Cells[a]	
		Paralysis	Pathology
Z-9-1a	G-BP/CFA	11/12	5/5
Z-9-2a	G-BP/ICFA	10/12	6/7
Z-9-3a	R-BP/CFA	5/7	5/7
Z-9-4a	R-BP/ICFA	7/7	7/7
Z-9-5a	EP/CFA	6/7	6/7
Z-9-6a	EP/ICFA	6/7	6/7

[a] EAE was induced by intravenous inoculation of T cell lines as indicated in the legend to Table V.

with G-BP/ICFA were found to be composed of T lymphocytes. Cells of these lines responded to the T cell mitogen concanavalin A (data not shown). Furthermore, essentially all the cells were positive for a specific T lymphocyte surface marker detected by monoclonal antibodies (clone W3/13 HLK (24)), using a fluorescence-activated cell sorter, and none of the cells was positive for surface immunoglobulins (data not shown).

The pathology of the disease produced by i.v. inoculation of these cell lines was characterized by perivascular mononuclear cell infiltration throughout the central nervous system. There was little or no damage to myelin. The lesions were similar to those observed in EAE induced by immunization with G-BP/CFA but were milder (data not shown).

DISCUSSION

The results of the studies presented in this report lead to several conclusions: 1) Onset of EAE, spontaneous recovery from clinical disease, and subsequent resistance to a second induction of EAE are associated with the response of T lymphocytes to specific antigenic determinants of BP. 2) Depression of the response to the critical antigenic determinants in rats recovered from EAE or in those that have received BP/ICFA seems to result from active suppression rather than from clonal deletion of specific effector T lymphocytes. 3) Latent

effector T lymphocytes suppressed in their activity and/or number can be rescued by *in vitro* culture and propagated as cell lines capable of producing EAE in normal recipient rats.

The role of specific antigenic determinants of BP was analyzed in these studies by comparing the responses *in vitro* to G-BP, R-BP, and EP. Dose-response studies were carried out to determine the optimal concentration by weight of each antigenic preparation (not shown), since their molecular weights vary greatly; EP is a relatively small molecule (13), and R-BP contains both small and large BP (25). Therefore, we compared responses at the optimal concentration for each antigen. Since injection of rats with G-BP/CFA produced EAE, we can conclude that G-BP must display antigenic determinants critical for induction of EAE and that it shares these determinants with R-BP. Additional evidence that G-BP displays these critical determinants *in vitro* derives from the fact that we were able to use G-BP to rescue *in vitro* suppressed clones of T lymphocytes that were responsive to R-BP and EP and that were able to mediate EAE (Figs. 3 and 5 and Tables V and VII). Nevertheless, the proliferative responses to R-BP and EP appeared to be better associated with clinical EAE than was the response to G-BP (Table II, Fig. 1). Strong responses to G-BP were obtained from rats that had recovered from EAE or that had been inoculated with nonencephalitogenic BP/ICFA. These results can be explained by the presence of additional antigenic determinants on G-BP that are not relevant to induction of EAE in rats, a likely supposition in view of the fact that G-BP is foreign to the rat and should have foreign antigenic determinants. The encephalitogenic peptide sequence 68–88 in the G-BP and R-BP molecules differs by the substitution of 1 amino acid at position 79, serine in G-BP and threonine in R-BP (26, 27). It is conceivable that this difference contributes to the creation of a foreign determinant in G-BP absent in R-BP. Such a foreign determinant could act as a "carrier" to enhance the immunogenicity of the encephalitogenic self-determinant that must be present in the same peptide (28). An alternative explanation for the greater encephalitogenicity of EP of guinea pig origin could be the presence of a suppressogenic determinant in BP and EP with the rat-specific sequence (29). In this case, the substitution at position 79 in G-BP could be interpreted as abolishing the hypothetical rat-specific suppressor determinant. In any event, we were able to detect the activity of T lymphocytes specifically involved in mediating EAE by contrasting the proliferative responses to R-BP and EP with those to G-BP.

We found a close correlation between the clinical state of EAE and the proliferative response to R-BP lymphocytes obtained from the draining lymph nodes (Figure 1*C*). Especially noteworthy was the finding that remission of EAE was associated with depression of the response specific to R-BP but not to G-BP. This suggested the possibility that recovery might involve active suppression of clones of T lymphocytes responsive to the critical encephalitogenic determinants.

We succeeded in detecting specific suppressor cells by using antigen-specific T lymphocyte lines as indicator cells. We found (Table III) that spleen and thymus cells from rats that had recovered from EAE suppressed the proliferative response of Z1a line cells to R-BP and less to G-BP. These spleen cells had no suppressive influence on the reactivity of the C1a line to the specific antigen OA. Lymph node cells from recovered rats showed less suppressor activity, and did not distinguish between the responses to R-BP and G-BP. Cells obtained from rats at the onset of EAE not only failed to suppress but actually augmented the response of the Z1a line cells against G-BP (Fig. 2). Thus, onset of EAE possibly could be related to "helper" cells in the draining lymph node, whereas recovery from EAE was associated with the appearance of immunospecific suppressor cells detectable primarily in the spleen and thymus. The nature of these regulatory cells remains to be investigated. Some evidence suggests that the thymic suppressor cells may specifically recognize T cells bearing anti-BP receptors rather than suppressor determinants on BP. We found that these suppressor cells inhibited Con A stimulation of the Z1a but not of the C1a line. Hence, the line itself was suppressed in the apparent absence of BP (Table IV). Consistent with an anti-idiotypic mechanism was the finding that the suppressor cells seemed to discriminate, at least partially, between T cells of the Z1a line responding to R-BP and those responding to G-BP (Table III). Moreover, we have found that it is possible to generate resistance to subsequent induction of active EAE by "vaccinating" rats with specifically attenuated cells of the Z1a anti-BP line (30). However, these findings, although suggestive, do not prove that EAE is subject to regulation by spontaneous anti-idiotypic immunity; more direct evidence is needed to resolve this point.

The finding of suppressor cells in rats recovered from EAE prompted us to attempt to rescue their suppressed subjects, T lymphocytes responsive to R-BP and EP, capable of mediating EAE. Such cells were successfully obtained (Table V and Fig. 3) using techniques of *in vitro* culture suitable for selecting and propagating lines of antigen-specific T lymphocytes (2). Moreover, we were able to rescue such suppressed EAE effector T lymphocytes from rats that had been inoculated with antigens in ICFA (Fig. 5 and Table VII), a procedure shown to generate cells capable of suppressing EAE (22, 23). It is unlikely that these effector cells resulted from primary sensitization *in vitro* because cells from control rats that had been injected with CFA alone did not develop responsiveness to GP or EP (Fig. 5). Hence, the *in vitro* culture could select clones of primed lymphocytes but did not generate them *de novo*.

Suppressor cells also have been described in other studies of EAE in rats (22, 23) and mice (31), in which suppression was induced by injecting animals with BP/ICFA. Using systems of passive transfer of cells to naive recipients, other investigators have attempted to detect suppressor cells after injection of encephalitogenic BP/CFA. In one study, suppression was detected in the spleen early in the course of EAE but not after recovery (32). In another study, nylon wool adherent suppressor cells were detected in lymph nodes after recovery (33). However, it appears that our *in vitro* system of measuring suppression of antigen-specific T cell lines may be a more sensitive way to detect immunospecific suppression directed against the autoreactive effector lymphocytes.

The detection of suppressor cells and covert EAE effector T lymphocytes in rats both after inoculation with encephalitogenic BP/CFA and suppressogenic BP/ICFA suggests that the same mechanism of suppression may work in both cases of acquired refractoriness to EAE.

The existence of suppressed effector lymphocytes in rats that have recovered from EAE (34) or have been injected with BP/CFA (20, 21) has been suggested previously. These findings, together with our results, indicate that covert or latent differentiation and survival of autoimmune effector cells may occur in the face of ostensibly suppressogenic immunization. It has been proposed that latent differentiation and selective clonal suppression are processes that may be critical in regulation of immune responses to foreign as well as to self-antigenic determinants (35). An important conclusion from the

results of these experiments is that self-tolerance to BP can be maintained by suppression rather than by clonal deletion (8) of autoreactive lymphocytes.

Earlier studies on the regulation of autosensitization showed that normal unimmunized rats also possess latent lymphocytes potentially capable of developing into autoreactive effector cells. This was first shown in the case of cytotoxic lymphocytes reactive against self-antigens on fibroblasts (36) or thymus reticulum cells (37). It was later found that effector T lymphocytes could also be activated against syngeneic brain antigens (38). The message of these experiments was that self-tolerance was fragile and that autoreactive effector lymphocytes, kept in a latent state *in vivo*, could be unleashed *in vitro*. It was also suggested that regulatory mechanisms, among the self-antigen in a soluble form (39, 40), might function *in vivo* to block self-recognition or to maintain suppression. The results of the experiments reported here extend these earlier observations to the more defined EAE model. They show that the clinical course of an autoimmune disease and restoration of self-tolerance involve reversible suppression of autoreactive effector lymphocytes directed against specific self-antigenic determinants. It remains to define the cells and molecular mechanisms that mediate this suppression.

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