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Effects of Experimental Allergic Encephalomyelitis on Thymus and Adrenal: Relation to Remission and Relapse (40961)[1]

SEYMOUR LEVINE, RICHARD SOWINSKI, AND BERNARD STEINETZ

Pathology Department, New York Medical College, and Westchester County Medical Center, Valhalla, New York 10595 and Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, New York 10502

Abstract. Experimental allergic encephalomyelitis (EAE) was induced by active immunization with neural tissue and adjuvants and by passive transfer of living lymphoid cells. Severe thymolysis occurred along with severe clinical signs and lesions. Thymolysis was preceded and accompanied by a striking rise in serum corticosterone. Clinical signs then remitted, followed by a decline in serum corticosterone and beginning regeneration of the thymus, but histologic EAE lesions persisted. Adrenalectomy performed early in the remission of EAE was followed promptly by disappearance of serum corticosterone and relapses of clinical signs. Relapses occurred in rats of either sex but not until thymic regeneration had begun. All these data support the theory that nonspecific stress and the state of adrenal hyperactivity in response to neurological disability determine patterns of remission and relapse in EAE by way of immunosuppressive effects on lymphoid tissue.

Experimental allergic encephalomyelitis (EAE) is a disseminated autoimmune inflammatory disease of the central nervous system (CNS) induced by immunization with neural antigen and adjuvants. After an incubation period of 1 to 2 weeks, weakness develops and progresses to paralysis with urinary incontinence, inability to eat, loss of weight, and sometimes a fatal outcome. When EAE is induced in the conventional manner by injection of antigen and Freund's adjuvant into the foot, there is also a swollen, probably painful, inflamed extremity, which interferes with locomotion. It is obvious that animals with EAE are under severe nonspecific stress (1). Presumably, stress contributes to the high mortality of EAE in adrenalectomized rats (2–4), whereas intact rats usually survive. Objective evidence of adrenal cortical reaction to stress of EAE consists of slightly elevated levels of 11-hydroxycorticosteroids at certain times in serum of rats and dogs (5). The thymus is particularly sensitive to elevated levels of corticosteroids, and thymolysis with karyorrhexis of thymocytes (3), as well as decreased thymic weight (6), have been observed in EAE.

In addition to the effect of EAE on the adrenal, the effect of the adrenal and its cortical hormones on EAE has been studied. Exogenous corticosteroids inhibit the development of EAE (7–11), which is in accord with their well-known immunosuppressive property. Nonspecific stress has the same effect (12), presumably due to liberation of endogenous corticosteroids. Exogenous and endogenous steroids probably inhibit both efferent and afferent arms of the immune response, inasmuch as they are effective on EAE produced by passive transfer of living, fully immunized lymphoid cells (13) as well as on EAE produced by active immunization.

The bidirectional relationship between EAE and the adrenal cortex assumed greater importance when it was used to explain spontaneous relapses in EAE (14). According to this hypothesis, the stress of EAE induces hypersecretion of corticosteroids which are responsible for spontaneous amelioration of the disease (remission). The improvement reduces the stress and shuts down the overproduction of steroids, so that the immune response is resumed and a second attack (relapse) ensues. In accord with this theory, adrenalectomy soon after the first attack greatly increased the frequency of relapses by removing the source of immunosuppressive steroids that were responsible for the remission (14).

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In the present work, the secretion and effects of corticosteroids have been measured. The data substantiate the important role of the adrenal in remissions and relapses of EAE.

Materials and Methods. Lewis rats, 150–200 g, from M.A. Bioproducts, Inc., Bethesda, Maryland, were acclimatized at least 1 week before use. Five rats were kept in each hanging wire and metal cage and they were fed Purina laboratory chow and tap water *ad libitum*. Female rats were usually employed because they are less susceptible than males to hemorrhagic and gangrenous cystitis when EAE produces neurogenic paralysis of the urinary bladder.

EAE was produced with frozen guinea pig spinal cord tissue, thawed, and homogenized in saline by cycling between two syringes connected by a double-hubbed needle. Either complete Freund's adjuvant (CFA) or carbonyl iron SF (GAF Corporation, Linden, N.J.) was used as adjuvant. CFA (85 parts Bayol F mineral oil, 15 parts Arlacel A emulsifying agent, 4 mg/ml killed human tubercle bacilli) was emulsified (water in oil) with an equal volume of 40% cord homogenate; a dose of 0.05 ml was injected into one of the right hind foot pads. Alternatively, a 10% (dry wt/vol) suspension of carbonyl iron in saline was mixed with an equal volume of a 10% (wet wt/vol) homogenate of spinal cord; a dose of 2 ml was injected intraperitoneally (ip) (15). It contained 100 mg cord (wet wt) and 100 mg iron. Passive transfer of EAE was accomplished with lymph node cells from male 250- to 300-g donor Lewis rats injected intravenously (iv) into recipients of the same sex and strain. Male rats were used because injections into the penile vein are very simple and rapid. The donors had developed EAE signs 7 days after injection with guinea pig cord–CFA emulsion plus 0.1 ml pertussis vaccine concentrate (20 billion organisms) in the dorsum of the same foot as an ancillary adjuvant. Lymph nodes draining the site of inoculation were harvested, processed into a cell suspension and washed in the cold, and injected into the dorsal penile vein of the recipients (16). Some recipients were given 2 mg glucan suspended in saline iv to intensify the EAE signs (17). EAE signs were checked daily and were scored 1+ for a weak, flaccid tail; 2+ for limb weakness or ataxia; 3+ for paralysis.

On the day of sacrifice, the animal room was entered quietly and for the first time at 8:00 AM. The rats were quickly and gently checked for EAE signs, and ip Nembutal, 40 mg/kg, was administered. As soon as the rats were asleep, they were taken to another room and exsanguinated from the aorta. Thymus and sometimes adrenals were carefully dissected and weighed fresh. Serum was stored frozen and later assayed for corticosterone by a standard fluorescence method (18). This method has a sensitivity of 3 μg/100 ml of serum and replicate analyses fall within $\pm 5\%$. Because the steroid levels were relatively high in normal control rats, the sacrifice procedure was modified for one experiment (Table I): the usual precautions for quiet and speed were observed, but the checking for EAE signs was omitted, and the rats were decapitated within a few seconds after they were removed from their cage. Also, the rats intended for sacrifice on the same day were housed in different cages and racks in order to avoid disturbance to a rat from previous entry into its cage or from entry into a nearby cage. The disadvantage of not knowing the EAE signs on the day of sacrifice was offset by the lower and more uniform corticosterone assays.

Spinal columns were fixed in Bouin's fluid, embedded in paraffin in entirety in longitudinal segments, cut, and stained with hematoxylin–eosin. EAE lesions were scored from 1+ to 4+ according to their number and severity. Rats that received ip inoculations were checked for accidental injection into retroperitoneum or cecum but no errors were found.

Bilateral adrenalectomies were done through a middorsal incision; rats were maintained on saline as sole fluid thereafter. Ether anesthesia was used for surgery, iv, and foot inoculations.

Results. Intact rats immunized with neural tissue and Freund's adjuvant developed clinical signs of EAE after 9 to 13

TABLE I. THYMUS AND ADRENAL DURING THE COURSE OF EAE[a]

Day of sacrifice[b]	Clinical EAE			Histologic EAE[f]	ΔBody weight[g]	Thymus weight[h]	Adrenals weight[h]	Serum corticosterone[i]
	Incidence[c]	Maximum[d]	Day before sacrifice[e]					
Control	0/5	—	0	—	—	0.22 ± 0.02	0.023 ± 0.004	7 ± 1
7	0/5	—	0	0	+7	0.20 ± 0.02	0.024 ± 0.004	5 ± 3
10	0/5	—	0	2.2	+9	0.19 ± 0.04	0.028 ± 0.003	19 ± 5
13	5/5	3.0	3.0	4.0	−13	0.09 ± 0.02	0.038 ± 0.002	20 ± 6
16	5/5	3.0	0.8	4.0	−20	0.06 ± 0.01	0.035 ± 0.005	16 ± 8
21	5/5	2.7	0	4.0	−5	0.14 ± 0.02	0.032 ± 0.002	4 ± 2
35	5/5	2.6	0	3.0	+23	0.15 ± 0.02	0.030 ± 0.003	4 ± 3
49	5/5	2.0	0	1.8	+46	0.13 ± 0.02	0.025 ± 0.002	ND

[a] Thirty-five rats immunized with spinal cord and Freund's adjuvant, and five controls given only a saline inoculation.
[b] Counted from day of immunization as day zero.
[c] Numerator: number of rats that had clinical signs at any time during experiment. Denominator: total number of rats.
[d] Graded daily from 0 to 3+; maximum values were averaged.
[e] Grading omitted on day of sacrifice to avoid effects of handling on serum corticosterone.
[f] EAE lesions scored from 0 to 4+, average.
[g] Difference between initial weight and weight 1 day before sacrifice, average.
[h] Fresh weights as percentage of final body weight, average ± SD. Both adrenals weighed together.
[i] μg/100 ml in serum obtained at sacrifice, average ± SD. ND = not done.

days. The serum corticosterone level was normal during the incubation period (7 days after inoculation). Thymus weight was slightly (but not significantly) reduced, possibly due to earlier stress from the swollen, inflamed, inoculated foot (Table I). There was a further reduction in thymus weight on Day 10 but these rats already had histologic lesions of EAE, which were near maximum severity in two of them, and the serum corticosterone values were already elevated. A much greater decrease in thymic weight was found during the phase of acute clinical signs (Day 13), accompanied by continued high serum corticosterone. The thymolysis progressed even after signs had largely resolved (Day 16) and corticosterone levels had begun to fall. All of these rats had severe histologic lesions of EAE. Restitution of the thymus and normalization of serum corticosterone was noted on Day 21 despite the persistence of severe EAE lesions. Changes in body weight were in accord with thymic weights: retardation of normal weight gain during the preclinical stage, loss of weight during acute EAE signs, progression despite diminution of signs, and restitution despite persisting EAE lesions. Adrenal weights exhibited a reciprocal pattern, increasing progressively through preclinical and acute EAE phases followed by return toward normal on Days 16 and 21 and thereafter.

Additional experiments with EAE produced by the same adjuvant (CFA), by carbonyl iron adjuvant, and by passive transfer confirmed these findings (Table II). Rats immunized with neural tissue and carbonyl iron did not have a decrease of thymic weight during the preclinical stage, perhaps because there was no inflamed site of inoculation (carbonyl iron is relatively nonirritating (15)). Clinical signs developed early (8–10 days) and were accompanied by severe thymolysis which progressed despite beginning resolution of signs on Day 13. There were not enough survivors to follow this experiment longer.

After passive transfer, clinical signs started on Days 6–8. Again, there was no decrease of thymic weight in the preclinical stage even though early histologic EAE lesions were present on Day 5; presumably the normal thymus reflected the absence of an inflamed site of inoculation. Thymolysis developed concomitant with the severe clinical signs of EAE in recipient rats given glucan. No regression of signs was noted and rats intended for later observations died before these could be made. Recipient rats not given glucan had only mild signs of EAE and only minimal thymolysis.

Serum corticosterones in these experiments (Table II) had a higher baseline than recorded for Table I because of the different manner of collection, but they revealed a similar pattern of transient elevation during the period of clinical EAE signs.

EAE relapses in adrenalectomized rats. EAE was induced with the aid of Freund's adjuvant, because this is the only form of EAE in which relapses can be induced regularly by adrenalectomy (14). Adrenalectomy was done 15–18 days after immunization, when EAE signs had already resolved but thymus weights were at their nadir. Rats were killed at intervals after surgery, regardless of the occurrence of relapses, in order to study the effect of the adrenals on the thymus in the presence of EAE. The first experiment showed that adrenalectomy accelerated the recovery in size of the thymus in a mere 2 days in rats convalescing from EAE (Table III). The second and third experiments showed that serum corticosterone had dropped to background levels in normal rats killed 1 or 2 days after adrenalectomy, as expected from its short half-life (19). The same was true of rats recovering from EAE, but relapses were absent in 1 day and few after 2 days (Table III). In the fourth experiment, adrenalectomies were done 3 or 4 days before sacrifice. This allowed enough time for most of the rats to relapse, and a greater degree of thymic regeneration was found than in the foregoing. There was no difference in thymic size between rats that relapsed and those that had not.

Keith has found that spontaneous relapses occurred in female but not in male rats (20). All our previous work on relapses induced by adrenalectomy (14) has been done on female rats. Therefore, we adre-

TABLE II. THYMUS AND CORTICOSTERONE IN VARIOUS TYPES AND STAGES OF EAE

Immunization	Day of sacrifice[a]	Clinical EAE			Histologic EAE[a]	Thymus weight[a]	Serum corticosterone[a]
		Incidence[a]	Maximum[a]	At sacrifice			
None	Control	0/3	—	0	—	0.24 ± 0.04	33 ± 8
Cord + CFA	7	0/3	—	0	ND	0.19 ± 0.02	32 ± 4
	10	0/5	—	0	0.5	0.23 ± 0.01	33 ± 8
	13	5/5	2.0	2.0	ND	0.13 ± 0.05	54 ± 6
	16	3/3	3.0	0.3	ND	0.07 ± 0.01	28 ± 9
	21	2/2	3.0	0	ND	0.17 ± 0.00	13 ± 7
Iron only	Control	0/5	—	0	—	0.23 ± 0.02	—
Cord + iron	3	0/5	—	0	ND	0.23 ± 0.02	—
	7	0/5	—	0	1.0	0.23 ± 0.01	—
	10	5/5	2.4	2.4	3.8	0.10 ± 0.03	—
	13	6/6	2.7	1.8	4.0	0.08 ± 0.00	—
None	Control	0/6	—	0	—	0.13 ± 0.01[b]	11 ± 4
Passive transfer	5	0/3	—	0	0.7	0.15 ± 0.00	18 ± 4
	6	3/3	1.0	1.0	3.7	0.13 ± 0.01	28 ± 5
	8	3/3	2.0	1.0	4.0	0.13 ± 0.01	27 ± 3
	12	3/3	1.0	0	1.5	0.12 ± 0.01	17 ± 4
Transfer plus glucan	5	0/3	—	0	0.5	0.12 ± 0.01	24 ± 3
	6	3/3	1.0	1.0	3.0	0.10 ± 0.01	26 ± 3
	7	3/3	3.0	3.0	4.0	0.07 ± 0.01	42 ± 0.5

[a] See Table I.

[b] These control thymic weights were relatively low because the rats were 13- to 14-week-old males whereas the other experiments utilized 8- to 10-week-old females. Data from normal and glucan-treated control rats were pooled because they were similar.

TABLE III. EFFECTS OF ADRENALECTOMY (ADX) DURING RECOVERY FROM EAE

Expt	Subjects	Interval (days) between adx and sacrifice	Relapses incidence[a]	Thymus weight (%)	Serum corticosterone[b]
1	Intact, recovering from EAE	—	0/6	0.04 ± 0.01	ND
	Adx during recovery from EAE	2	2/6	0.07 ± 0.02	ND
2	Adx during recovery from EAE	1	0/5	0.13 ± 0.02	3.0
	Adx, normals	1	NA/5	0.21 ± 0.02	2.8
3	Adx during recovery from EAE	2	0/6	0.11 ± 0.02	3.2
	Adx, normals	2	NA/4	0.26 ± 0.02	1.0
4	Adx during recovery from EAE	3,4	0/6	0.18 ± 0.03	0.3
	Adx during recovery from EAE	3,4	8/8	0.18 ± 0.03	2.0

[a] Numerator: number of rats with recurrence of EAE signs after clinical recovery. NA = not applicable (controls not given EAE). Denominator: total number of rats.

[b] μg/100 ml in serum obtained at time of sacrifice. All values listed are in range of background. ND = not done.

nalectomized nine male Lewis rats 15 or 16 days after immunization with guinea pig cord emulsified in CFA. All of them had been paralyzed 1 to 3 days before surgery, and all of them had mild, residual clinical signs at the time of surgery. Seven of these nine rats became paralyzed for the second time by 2 to 6 days after adrenalectomy and they died a day or two later. The remaining two rats had a return of weakness a few days after adrenalectomy and they died 1 day later; paralysis may have been missed because they were examined only once daily. These results show that both sexes are susceptible to adrenalectomy-induced relapses.

Discussion. Decreased thymic weight in EAE probably reflects the cumulated effects of excessive adrenal secretory activity, in contrast to elevation of serum corticosterone which probably indicates adrenal hyperactivity at the moment of bleeding. This explains the progressive loss of thymic weight, even after EAE had improved and after corticosterone levels had reverted toward normal. Also, it is likely that the later onset of EAE induced with the aid of Freund's adjuvant, compared to rats given the carbonyl iron adjuvant, was due to the cumulated immunosuppressive effects of stress from the inoculated foot. This conjecture is supported by the fact that adrenalectomy accelerates the onset of EAE (3, 16, 21, 22).

The data presented herein support the previously stated theory that adrenal activity is involved in remissions and relapses of EAE (14). This theory can now be restated as follows. Serum corticosterone rises concomitantly with the development of severe EAE lesions and clinical signs. This is accompanied by thymolysis. The loss of thymic T cells is accompanied by a decrease of peripheral T cells, manifested by shrinkage of draining peripheral lymph nodes (23, 24). This results in a decrease and then complete remission of clinical signs. With the relief from the stress of paralysis, the serum corticosterone falls rapidly despite the persistence of severe histologic lesions. As soon as corticosterone levels are normalized, regeneration of the thymus begins. At this point, reactivation of encephalitogenic mechanisms might induce a spontaneous relapse unless hindered by sequestration of residual inoculum, loss of antigen by metabolic degradation, inaccessibility of the nervous system due to local vascular blockade (25), or suppressor cell activity (26). On the other hand, adrenalectomy accelerates the fall in corticosterone levels and the recovery of lymphoid tissues, so there is less time for these hindrances to develop, and relapses occur within a few days. The rapid disappearance of corticosterone indicates that the 3- to 4-day interval between adrenalectomy and relapses cannot be ascribed to persistence of immunosuppressive steroids in the circulation, but the possibility of persistence in the tissues has not been excluded. It seems more likely that the delay represents the time necessary for recovery of the lymphoid tissues, and the

temporal coincidence between relapses and beginning regeneration of the thymus (Table III) favors this view.

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