Allergic Encephalomyelitis: Effect of Complement Depletion with Cobra Venom[1] (35880)

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Experimental allergic encephalomyelitis (EAE) has been classified with the delayed (cellular) hypersensitivities because of the mononuclear character of the inflammatory infiltrate and because it can be transferred passively with immunized lymphoid cells but not with serum (1). Furthermore, the disease can be correlated with delayed-type dermal sensitivity to the encephalitogenic protein antigen (1, 2). However, a recent report of passive transfer with plasma from irradiated guinea pigs has thrown the spotlight on humoral factors (3).

Some immunologic reactions mediated by humoral antibody are complement dependent. It is especially in the hyperacute form of EAE that the operation of complement-dependent humoral agents ought to be considered. This is a form of EAE produced in highly susceptible Lewis rats that are immunized with neural antigen and pertussis vaccine with or without Freund's adjuvant (4, 5). It is a disease of exceptional severity, with uniform clinical onset 7 or 8 days after immunization and with progression to paralysis and death within another 1 or 2 days. Although the earliest lesions contain mononuclear cell infiltrates, soon there are heavy perivascular exudates of polymorphonuclear leukocytes and plasma protein including fibrin. In order to detect complement-dependent mechanisms that might be responsible for the neutrophilic exudate, we treated the rats with an anticomplementary factor from cobra venom. This treatment is known to diminish markedly the C3 and terminal components of complement and to inhibit neutrophil accumulation in acute glomerulonephritis and to a lesser extent in Arthus reactions (6). However, the present study has produced no evidence for a significant complement-dependent mechanism of lesion development in EAE, which is in accord with the ineffectiveness of the cobra venom factor on other forms of delayed hypersensitivity (6, 7).

Methods. Three- to 4-month-old male or female Lewis rats from Microbiological Associates, Inc., Bethesda, were fed Purina Laboratory Chow and tap water *ad libitum*. Hyperacute EAE was produced by injection into the right hind foot pad of 0.05 ml of an emulsion made of equal parts of 40% guinea pig spinal cord homogenate and Freund's complete adjuvant, plus injection into the dorsum of the same foot of 0.1 ml concentrated pertussis vaccine (about 20 billion organisms) (4, 5). In experiments 5B to 8, Freund's adjuvant was not used. The immunization was accomplished by intraperitoneal injection of 4 ml of a homogenate containing 200 mg (wet wt) of guinea pig spinal cord and 20 billion pertussis organisms (Expt. 5B); or injection of all four paws with a total of 1 ml of homogenate containing 250 mg of cord and 60 billion organisms (Expts. 6 to 8). Clinical signs of EAE began to ap-

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TABLE I. Effect of La Jolla Cobra Factor on Hyperacute EAE.[a]

Expt.	Days of treatment[b]	C′ at sacrifice[c]	No. of rats	EAE, av day of	
				Onset[b]	Paralysis[b]
1	5 and 6	<5 (all <5)	10	8.6	8.9
	0 and 1	25 (<15 to 30)	5	7.6	7.8
	Saline[d]	20 (<15 to 29)	5	8.1	8.6
2	5 and 6	<5 (<5 to 6)	7	8.5	8.8[e]
	Saline	59 (50 to 73)	5	8.0	8.8[e]
3	6 and 7	<10 (all <10)	7	8.0	8.4
	Saline	59 (31 to >80)	7	8.0	9.0

[a] The rats were immunized with neural antigen, Freund's adjuvant and pertussis vaccine which produced innumerable EAE lesions in all rats.

[b] Day of immunization is day zero. Cobra factor dose 300 units/kg divided equally among four intraperitoneal injections (two on each of the days indicated).

[c] Fifty percent hemolytic units (CH_{50}), average, with range in parentheses (6). The C′ levels were not done on 20% of the rats because they died of EAE before they could be bled. For calculation of averages, the limit of the titration was used when the particular specimen was higher than the upper limit or lower than the lower limit. Immune diffusion assays revealed marked or complete reduction of C3 in each rat treated with cobra factor.

[d] Control groups were given an equal number of saline injections.

[e] This average does not include 4 venom-treated and 2 saline-treated rats killed on day +9 before clinical signs had progressed to paralysis, in order to evaluate nephrotoxic serum glomerulitis.

pear 7 days after any of these immunizing techniques. At first, the tail lost its normal tonus. Weakness ensued and progressed rapidly to paralysis of the hind limbs in another day or two. Paralyzed rats were anesthetized with ether and exsanguinated from the aorta. Spinal cords and hindbrains were fixed in Bouin's solution, embedded in entirety in paraffin and sectioned and stained with hematoxylin–eosin or with phosphotungstic acid–hematoxylin.

Cobra venom factor was prepared and purified in either La Jolla or Boston as described recently (6). The depletion of hemolytic complement activity and C3 protein by the cobra factor is nearly complete within 4 hr but the duration is limited to 4 to 5 days, partly because of catabolism of the factor which is complexed to the plasma C3 proactivator, and, in cases where immunization occurs, by an immune response to it. Therefore, various schedules of administration were employed (see Tables I–III). Complement was assayed on serum obtained and frozen at time of sacrifice, or serially during Expts. 6 to 8 [for methods, see (6, 8)].

Results. Hyperacute EAE. Normal levels of complement were found in serum of rats paralyzed by hyperacute EAE, confirming the results of others in rabbits and guinea pigs with clinical signs of ordinary EAE (9–11). Cobra factor administered on the day of inoculation of encephalitogenic emulsion, and/or 1 day later, did not act long enough to produce profound depletion of complement when the rats were bled and sacrificed 8 to 10 days later (Tables I, II). On the other hand, cobra factor injected 4, 5, 6, or 7 days after encephalitogenic challenge caused a marked reduction of serum complement at the time of sacrifice in almost all instances. In Expt. 4, half of the group that received cobra factor both 1 and 6 days after inoculation probably was depleted of complement for vitually the entire duration of the experiment. The evidence of complement depletion provided by titration of serum complement hemolytic activity was supplemented, in Expts. 1 and 2, by demonstration in agar gel diffusion plates that C3 protein was absent or markedly diminished. Furthermore, a dem-

TABLE II. Effect of Boston Cobra Factor on Hyperacute EAE.[a]

Expt. (route)	Days of treatment[b]	C3 at sacrifice[c]	No. of rats	EAE, av day of	
				Onset	Paralysis
4 (iv)	6	<10 (all <10)	8	7.3	8.1
	1 and 6	Variable[d]	8	8.1	8.8
	1	81 (64–92)	8	7.6	8.0
	Saline	102 (92–106)	20	7.5	8.2
5A (iv)	7	16 (15–18)	5	8.0	8.2
	4	18 (16–22)[e]	6	8.0	8.3
	Saline	136 (124–151)	13	7.3	7.8
5B (iv)	4	53 (15–90)	5	8.8	9.4
	Saline	99 (88–107)	5	7.4	7.8
6 (ip)	5 or 6 or 7	<10 (all <10)	6		9.3
	None	107 (100–116)	6		8.2
7 (ip)	5 or 6 or 7	<10 (all <10)	6		10.3
	None	103 (91–110)	6		9.8
8 (ip)	—1, 0, 1, 2, or 3	106 (53–134)[f]	10		13.4
	None	109 (89–132)	10		11.3[g]

[a] Rats immunized with neural antigen, Freund's adjuvant, and pertussis vaccine (Expts. 4, 5A), or neural antigen and pertussis vaccine only (Expts. 5B, 6, 7, 8).

[b] Cobra factor, 400 units/kg as a single dose on the day indicated except in Expt. 4, 20 units/kg of nonaggregated product were given; this dose was repeated in the second group, as indicated.

[c] Immunochemical determination of C3 protein, expressed as percentage of normal (8).

[d] Half the rats had <10%, presumably due to second injection, but the others were probably resistant and were largely recovered from the first injection (56–81%).

[e] Not including one rat that did not become paralyzed until 10 days and had C3 of 97%.

[f] Serial studies revealed C3 of <10% for 4–5 days after venom injection.

[g] The unusually long incubation period may be due to use of a different batch of pertussis vaccine.

onstration within the animal of the effectiveness of complement depletion was incorporated in Expts. 2 and 3. As soon as paralysis from EAE was noted, or on day +9, nine venom-treated rats and seven saline controls were given an intravenous injection of 1 or 2.2 ml of a potent nephrotoxic serum (rabbit antirat basement membrane serum). They were sacrificed 3 hr later. The saline-treated rats had normal levels of hemolytic complement and C3 protein in the serum and injection of nephrotoxic serum led to accumulation of 26 to 45 polymorphonuclear leukocytes in each glomerular section (av 38, counts of 10 glomeruli). The venom-treated rats had no detectable hemolytic complement or C3 protein and the number of leukocytes in each glomerulus was reduced to 3 to 12 (av 7). The inhibition of complement-dependent neutrophilic accumulation in the glomeruli confirmed the effectiveness of complement depletion, at least at the time the rats were killed.

The effects of complement depletion on EAE were minor and irregular (Tables I, II). Minimal delays in onset or slight decrease in the fibrin in the lesions were noted in some experiments but not in others. In fact, almost every venom-treated rat became paralyzed and all had innumerable histologic lesions. There was no obvious change in the number of polymorphonuclear leukocytes in the EAE lesions, even in the very animals in which accumulation of neutrophils in glomeruli had been inhibited.

A short delay in appearance of clinical signs occurred only in groups immunized without Freund's adjuvant and treated with

TABLE III. Effect of La Jolla Cobra Factor and Basic Protein on Localized EAE Produced by Passive Transfer.

Expt.	Treatment of recipients ip	Treatment of recipients iv	C′ at sacrifice	No. of rats	EAE score[a]
9	Cobra factor[b]	—	<5 (all <5)	6	2.0
	Saline	—	25 (18 to >30)	6	2.0
10	Cobra factor	Saline	<5 (all <5)	5	1.3
	Saline	Saline	53 (50 to 67)	4	2.0
	Cobra factor	BP[c]	<5 (all <5)	5	0
	Saline	BP	65 (55 to 83)	4	0
11	Cobra factor	—	<5 (all <5)	4	2.0
	Saline	—	83 (61 to >100)	4	1.5
	Cobra factor	Cyclo[d]	<5 (all <5)	4	1.3[d]
	Saline	Cyclo	78 (67 to 96)	4	1.5

[a] Average score based in number and intensity of EAE lesions adjacent to zone of thermal coagulation necrosis in brain; scale 0 to 2; slides randomized and read without knowledge of treatment.

[b] Two hundred (Expt. 9) or 300 (Expts. 10, 11) units/kg divided among 3 spaced injections the day before transfer and 1 injection 2 hr before transfer.

[c] Guinea pig brain myelin basic protein, 0.2 mg in saline 1 hr after transfer, a known inhibitory agent (15).

[d] Cyclophosphamide (125 mg/kg) 1 day before transfer in order to produce the neutrophilic form of EAE. The scores for these groups are based on polymorphonuclear neutrophils rather than mononuclear cells.

cobra factor early. In these animals complement was depleted during early immunization but most had normal complements at the time of paralysis (Expts. 5B and 8). This 1.5- to 2-day delay in clinical onset was therefore probably not related to an effect on production of the lesions. Furthermore, all these rats did become paralyzed and had innumerable lesions of hyperacute EAE.

These results are in substantial agreement with those reported recently by Pabst *et al.* (12) for guinea pigs. These authors produced some delay in onset and reduction of mortality when cobra factor was given 1 hr before or 3 hr after encephalitogenic inoculation, but not if it was given 24 hr before or 24 hr after, or if it was timed so as to deplete complement at the onset of lesions or clinical signs. Histologic lesions of EAE were not prevented or modified even in the "protected" guinea pigs. The fact that there was poor correlation of protection with complement depletion seems to detract from the authors' inference that cobra factor interferes with a humoral mechanism in EAE.

Passive transfer of EAE. Although complement depletion had exerted little or no effect on hyperacute EAE, we confirmed these findings in a passive transfer system. EAE produced by passive transfer of living, immunized lymphoid cells does not have many polymorphonuclear leukocytes in the lesions or massive fibrin exudation, but it permits the isolation of events concerned with lesion formation into a brief 24-hr period (13, 14). Donor rats were immunized as for Expts. 1 to 5A. Seven days later, when many had clinical signs, the lymph nodes draining the inoculation sites were harvested, processed into a cell suspension, and injected intravenously into isohistogenic Lewis recipients (13, 14). The donor:recipient ratio was 1:1. The recipients were prepared beforehand with a thermal brain injury that served to accelerate and localize the EAE lesions (14). The rats were killed 24 hr after transfer and EAE lesions adjacent to the zone of thermal coagulation necrosis were scored histologically.

Cobra factor was administered to the recipients the day before and 2 hr before trans-

fer. Scores of localized EAE in brains revealed only minor and inconstant effects in three experiments (Table III). For comparison, Expt. 10 included groups of recipients treated with 0.2 mg of a basic protein from myelin. The basic protein produced *complete* inhibition of EAE, as reported previously (15), and did so regardless of concomitant injections of cobra factor.

It was of particular interest to test the cobra factor in a new form of passive localized EAE in which polymorphonuclear neutrophils may predominate in the lesions (16, 17). Therefore, Expt. 11 included two groups of recipients treated with cyclophosphamide 1 day before passive transfer. As reported, the drug reduced the mononuclear cell component of the EAE lesions and increased the relative and absolute number of neutrophils in brain parenchyma adjacent to the thermal injury. The concomitant administration of cobra factor did not alter this reaction.

Conclusion and Summary. Complement depletion of rats by cobra venom factor had only minor and variable effects on the hyperacute form of EAE. It appears likely that the exudation of polymorphonuclear leukocytes and massive amounts of fibrin in this condition is not dependent on complement. However, it is not possible to eliminate all traces of complement with cobra factor. Therefore, it is theoretically possible that small amounts of complement, too little to be detected by hemolytic activity or gel diffusion, might accumulate in the EAE lesions during the 1- to 3-day period in which they evolved. However, inhibition of the neutrophil accumulation of nephrotoxic serum glomerulitis attested to the efficacy of the cobra factor. This internal control provided evidence against the binding of C3 and terminal components, at least for the last 3 hr of development of EAE lesions. As C3 levels fall within 4 hr of the initial injection, it is most probable that complement depletion spanned the time during which EAE lesions developed.

Passive transfer of EAE can be accomplished in a single day. The rapidity of lesion development makes it highly likely that there was effective depletion of complement during the entire disease. Nevertheless, complement depletion of recipients had little or no effect on the outcome, either in conventional passive EAE in which mononuclear cells predominate or in the new, neutrophilic variety in which polymorphonuclear leukocytes are prominent.

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