Prevention of Experimental Allergic Encephalomyelitis with Cobra Venom Factor[1] (35310)

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Experimental allergic encephalomyelitis (EAE) is considered a model of autoimmune disease. The development of cellular immunity of delayed hypersensitivity type in the pathogenesis of this disease seems established (1, 2). Less well defined are the relative contributions of humoral events in this process. Bornstein (3) and Appel and Bornstein (3, 4) demonstrated specific antimyelin antibody activity in this disease in tissue culture, while Sherwin *et al.* (5) showed by immunofluorescence the presence of specific antibody in rabbit central nervous system (CNS) in EAE. More recently, Oldstone and Dixon found that the earliest microscopic change in the CNS of Lewis rats with developing EAE is the appearance of C3, IgG. and fibrinogen in the vessel walls; and IgG and C3 around neurons in the parenchyma itself (6). These deposits seemed to be specific since they were present only in the brain among a number of organs studied and were shown not to reflect more general deposition of serum proteins. Thus, presence of immunoglobulin and complement (C) in the control nervous tissues antedated clinical disease and preceded appearance of the dense cellular infiltrates often seen later in EAE.

From these findings it seemed of interest to analyze the influence of depletion of hemolytic complement on development of EAE. Cobra venom factor (CVF) has been shown to deplete the terminal components of C by activating the complement sequence at C3 (7). C can be decreased *in vivo* to very low levels for 3 to 4 days following a single intraperitoneal injection of CVF. After depression, which is evident within 8 to 24 hr, C concentration rises to normal in 4 to 5 days (8). By using this tool, we have delayed onset and progression of clinical symptoms of EAE in guinea pigs by several weeks without significantly influencing certain histopathological concomitants.

Materials and Methods. The animals sensitized to EAE were outbred Hartley guinea pigs of either sex, weighing 350 to 450 g (Oak Crest Rabbitry, Hanover, Minnesota). EAE was produced by injection of guinea pig spinal cord with incomplete Freund's adjuvant into the two forepaws of each animal. The spinal cord was homogenized with water to yield a 33% suspension and then emulsified with an equal volume of incomplete Freund's adjuvant (Difco, Detroit) to which *Mycobacterium tuberculosis* (H37 RA, Difco, Detroit) was added. The dose of spinal cord was 50 mg of wet weight and *M. tuberculosis*, 2 mg for each animal.

CVF was prepared and purified from lyophilized *Naja haje* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) by DEAE- and carboxymethyl (CM)-cellulose column chromatography as previously described (8). The elutate from the CM column containing 4 to 5 ml in each tube was assayed for C inhibition as follows: 0.1 ml of elute was added to 0.1 ml of normal guinea pig serum and incubated for 1 hr at 37°. The mixture was then assayed for residual hemolytic effect

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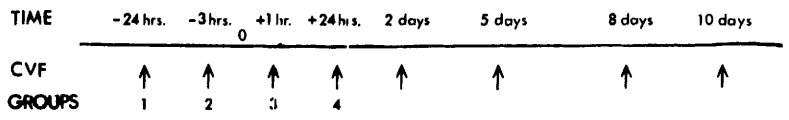

FIG. 1. Cobra venom factor was injected ip (↑) into groups of guinea pigs at various times in relation to a sensitizing injection (0) to induce EAE.

by a method previously described (9), using 37° as incubation temperature for 1 hr. All eluate samples that yielded inhibition to 60 to 90% were pooled and this pool of CVF was used in the experiments. CVF was administered as a single dose ip in varying intervals before or after sensitization of different groups (Fig. 1): 24 hr before (Group 1), 3 hr before (Group 2), 1 hr after (Group 3) and 24 hr after (Group 4). In Expt. A, 2 ml of CVF were given ip/animals; in Expt. B, again 2 ml, but derived from a different preparation of CVF; in Expt. C, 3 ml were given from the same preparation as in Expt. B.

Capillary blood samples for C determinations were taken from all animals prior to and at five daily intervals after CVF injection by the following method. The hindlegs were shaved and a site over the saphenous vein, as it enters the hamstring muscles, was cleaned with alcohol. A sterile needle was used to make a stab wound into the saphenous vein and drops of blood were col-

TABLE I. Cumulative Onset of Frank Paralysis in CVF-Treated and Untreated Guinea Pigs Sensitized to EAE.

The time after sensitization is divided into 5-day intervals, from days 10 to 30, and a late period beyond 30 days ending with termination of each experiment. Results of three experiments are recorded; Expt. A was terminated on day 120; and Expts. B and C on day 40. Day of onset of late paralysis in brackets.

		No. of animals paralyzed/no. of animals in group				
			CVF treated			
After sensitization (days)	Expt.	No CVF controls	(Hr): −24 (Group): 1	−3 2	+1 3	+24 4
10–15	A	4/7	3/5		2/7	5/7
	B	2/10		1/9	0/10	
	C	3/10			0/10	
16–20	A	6/7	4/5		3/7	6/7
	B	6/10		3/9	1/10	
	C	7/10			2/10	
21–25	A	7/7			3/7	
	B	7/10			4/10	
	C	8/10			4/10	
26–30	A	7/7				
	B	7/10		5/10	6/10	
	C	9/10			5/10	
Late	A		5/5 [72]		4/7 [32] 5/7 [45] 6/7 [60] 7/7 [72]	7/7 [32]
	B			7/10 [33]	7/10 [32]	
	C	10/10 [35]			7/10 [32] 8/10 [38]	

TABLE II. Cumulative Mortality in Guinea Pigs Sensitized to EAE and Treated or Untreated with CVF.

The same time intervals and experiments as in Table I. Day of death in late time interval in brackets.

		No. of animals dead/no. of animals in group				
			CVF treated			
After sensitization (days)	Expt.	No CVF controls	(Hr): —24 (Group): 1	—3 2	+1 3	+24 4
10–15	A	1/7	2/5		1/7	2/7
	B	1/10		0/9	0/10	
	C	1/10			0/10	
16–20	A	6/7	3/5		2/7	4/7
	B	6/10		0/9	1/10	
	C	6/10			2/10	
21–25	A	6/7			3/7	5/7
	B	6/10		2/9	3/10	
	C	8/10			4/10	
26–30	A	7/7	4/5			
	B			4/9		
	C					
Late	A		5/5 [110][a]		4/7 [38] 5/7 [109][a] 7/7 [120][a]	6/7 [35] 7/7 [58]
	B	7/10 [39] 10/10 [40][a]		5/9 [32] 9/9 [40]	5/10 [35] 6/10 [39] 10/10 [40][a]	
	C	9/10 [34] 10/10 [40][a]			10/10 [40][a]	

[a] Number of animals sacrificed at termination of experiment.

lected in a sterile test tube to a total of 0.75 to 1.0 ml of fresh blood. Samples were allowed to clot at room temperature for 1 hr, left to react at 4° overnight, and the serum was harvested after centrifugation. All serum samples were stored at —70° until C determinations were made as previously described (9).

Brain and cord, removed from animals sacrificed at varying periods following sensitization, were fixed in 10% formalin, embedded in paraffin, and stained with hemotoxylin and eosin.

Control animals were of the same breed and of the same weight range as were experimental animals. Clinical evaluation was by daily observations starting day 10 until day 30 after sensitizing injection, and then every second or third day until termination of the experiment. According to motor performance, the animals were graded into five categories: mild, moderate, and severe paresis; early (definite) paralysis; severe paralysis.

Results. 1. Clinical. The influence of CVF on development of EAE is summarized in Tables I and II. In three different experiments, 27 guinea pigs, given only the encephalitogenic emulsion, served as controls. Twenty-two of these were paralyzed by day 25 after sensitization. An equal number of guinea pigs treated with CVF 1 hr after sensitizing had an incidence of paralysis of 11/27 (Table I). Mortality of the control groups was 20/27 (75%) as compared to 11/27 (40%) for the CVF-treated animals (Table II). CVF given 3 hr before sensitization (Group 2) also provided protection. In Group 2, 2/9 (24%) had died by day 25 as

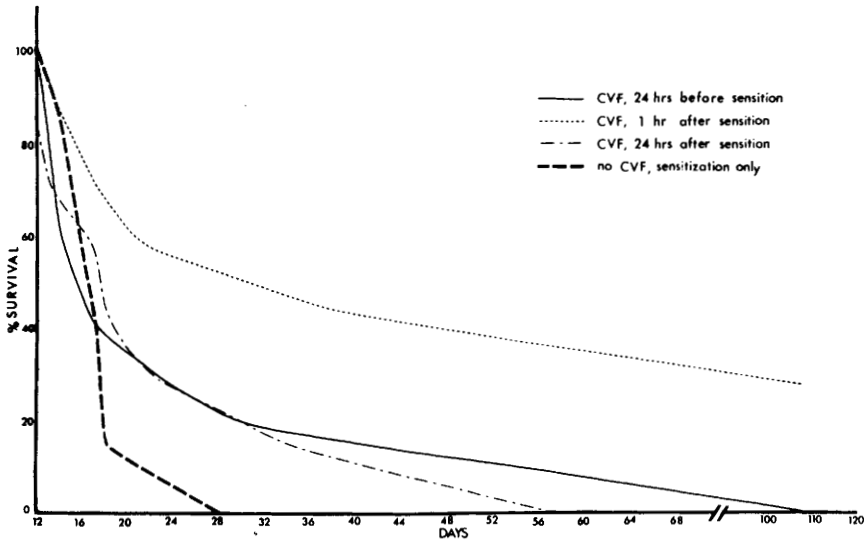

FIG. 2. EAE survival with CVF: Seven control animals (— —) died within 28 days of the sensitizing injection. CVF given to seven animals ip 24 hr after (— -); to five animals 24 hr before (—); and to seven animals 1 hr after (- - -) the sensitizing injection prolonged the lives of 30, 30 and 50% of these animals, respectively, beyond those of the control group.

opposed to 6/10 (60%) of the corresponding control group. Groups 1 and 4, given CVF either 24 hr before or 24 hr after simultaneously injected control groups, did not seem to be protected. In Groups 1 and 4, however, two long-term survivors were observed. Late paralysis (more than 30 days after sensitization) occurred in 12 (25%) of the total of 48 CVF-treated animals listed in Table I and only in 1/27 controls.

Maximal protective effect from death was always produced when CVF had been given 1 hr following the sensitizing injection. Some protection may have been provided when CVF was given within 24 hr prior to (Group 1) the sensitizing injection of spinal cord and adjuvant. The progression of disease as judged by evolution of paresis, paralysis, and death was retarded in the treated group, as can be seen from the curves of Fig. 2 and 3 (Expt. A). Animals of Groups 1, 3, and 4 lived longer after onset of frank paralysis than did the controls. Although delayed sometimes as long as 2 to 3 months, paralysis regularly ensued in the CVF-treated animals (Table I). Such animals then showed gradual progression of paralysis, most clearly seen in Group 3.

2. *Complement.* Twenty-four hr following ip injection of CVF, C levels were depressed to less than 3% in the majority of the animals of Group 3 (23/27) in the three experiments, to less than 10% in another animal, and not at all in the remaining three animals (Table III). Two of these last three were among the long-term survivors.

TABLE III. Complement Levels 24 hr After CVF Injection in Percentage of Value Recorded Before Administration of CVF.

C remaining after CVF (%)	No. of animals in Groups: 1	2	3	4
<3	2	3	23	7
3–10			1	
No change	3	6	3	

By contrast, CVF given prior to sensitization, often did not depress C concentration so effectively. For example, only 2/5 in Group 1 (Expt. A) showed depressed C concentrations to less than 3%, the other did not have demonstrable C depression. In Group 2, only 3/9 experienced pronounced C depression while 6/9 had normal C concentration in their serum 24 hr following injection of CVF. From these data, it seems that the sensitizing injection may strikingly abrogate the com-

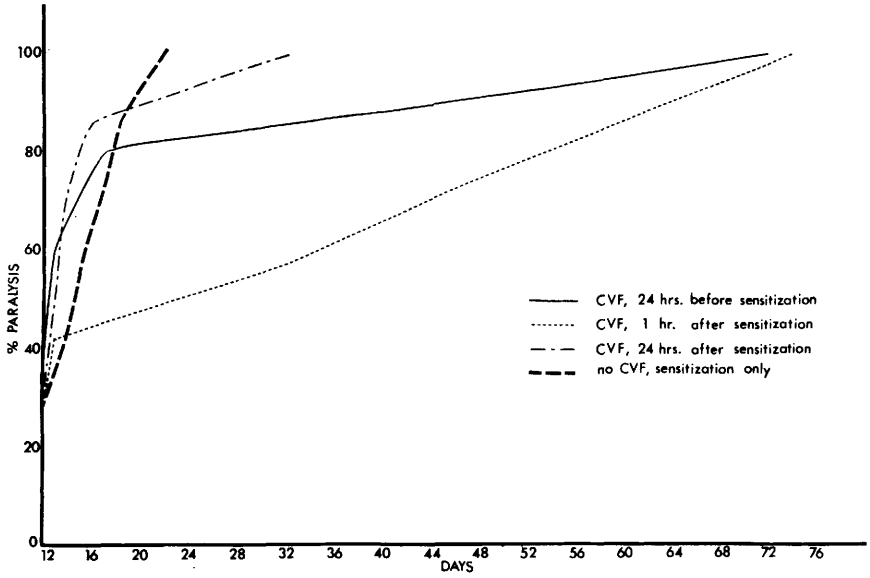

FIG. 3. Onset of paralysis in a typical experiment: 100% of seven control animals (— —) were paralyzed by day 22 after the sensitizing injection. Seven animals given CVF 24 hr after the sensitizing injection (— -); five with CVF 24 hr before (—); and seven with CVF 1 hr after the sensitizing injection (- - -) reached paralysis of the total group considerably later than the controls.

plement-depressing influence of CVF.

3. Histology. Histopathological findings in all animals studied did not vary noticeably from those of unmodified EAE. Animals sacrificed on day 9 after sensitization often showed a few foci of mononuclear and lymphoid cell infiltrations in the cerebrum and brain stem, regardless whether treated with CVF or belonging to control groups. Guinea pigs from Groups 2 and 3, with maximal protection from paralysis and death by CVF, showed an incidence of perivascular foci and infiltrations of choroid plexus and subarachnoid areas throughout the central nervous system equal to that observed in control groups from day 9 until termination of the experiment. There was no correlation of the histology with the clinical protection afforded by CVF.

Discussion. These experiments demonstrate that CVF can decrease paralysis and death when given immediately before or after injection of an encephalitogenic emulsion of spinal cord and Freund's complete adjuvant. By contrast, CVF injected 24 hr or more prior to, or 24 hr or more after, the encephalitogen did not significantly influence development of EAE.

Although it is attractive to relate the influence of CVF on EAE to its capacity to depress C concentrations, this relationship does not seem absolute. A number of animals injected with CVF immediately prior to the encephalitogen (Group 3) did not show depressed C levels when these were studied 24 hr later yet seemed to be well protected from paralysis and death. Indeed, studies of the C levels, although showing a correlation of maximal protection with maximal C depression, could be taken to suggest that CVF may protect against EAE by an action other than its capacity to reduce C. A rather surprising finding, and one deserving further analysis, was the apparent diminution of the CVF effect on C by injection of encephalitogenic emulsion soon after the CVF.

In our experimental Groups 2 and 3, the striking delay of paralysis and death was not accompanied by demonstrable depression of central nervous system lesions. These findings seem to support earlier observations of Stone *et al.* (10), which showed that clinical manifestations of EAE do not correlate well with histopathologic involvement of the central nervous system. Although further study will be necessary to elucidate the precise ba-

sis of the amelioration of EAE by CVF, it seems to us that these observations argue for the importance of humoral events in the clinical manifestations of this experimental disease. A perplexing aspect of our studies is that a single critically timed injection of CVF given at about the same time as the sensitizing encephalitogen can prevent disease which appears some 2 to 3 weeks later. Depression of serum C concentrations lasts for a period of 4 to 5 days following CVF injection, yet CVF does not influence the course of EAE when this period is timed to coincide with first appearance of lesions or of clinical manifestations of the disease. It seems likely that CVF interacts with a very early event in development of EAE. Such an event could, for example, relate to the local reaction to the encephalitogen either in the footpad or the regional lymph nodes draining the site of injection of the encephalitogen. It may be recalled that excision of the regional lymph nodes during the incubation of EAE will completely prevent EAE in rabbits (11).

Actions of CVF, other than its effect on C, must be considered. Even the purified CVF with which we worked contains several proteins. Antigenic competition is a possibility although antigens like hemocyanin and bovine serum albumin, known to be effective in antigenic competion, did not influence development of EAE when their presentation was timed as was the CVF (unpublished data). The important nerve growth factor recently described in cobra venom was prepared by methods very different from those used in preparation of our cobra venom factor and should not present confusion.

The findings presented here, taken with other available data (6, 12) are consonant with the view that development and full expression of EAE requires both humoral and cellular immune mechanisms. Further studies to elucidate the role of CVF in preventing paralysis and death in this model autoimmune disease seem in order.

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