Myelination Inhibiting and Neuroelectric Blocking Factors in Experimental Allergic Encephalomyelitis

Abstract. *Sensitization of Lewis rats with whole central nervous system tissue or with purified myelin induced both experimental allergic encephalomyelitis (EAE) and a serum factor which inhibited myelin formation in vitro. Sensitization with the encephalitogenic factor, myelin basic protein, induced EAE, but not the myelination inhibition factor. Sensitization with cerebroside induced neither EAE nor myelination inhibition factor. The serums from control animals without EAE as well as from animals sensitized with all of the above antigens blocked evoked electrical responses in vitro.*

Serums from animals with experimental allergic encephalomyelitis (EAE) induced by inoculation of whole central nervous system (CNS) tissue combined with Freund's complete adjuvant (FCA) have been reported to demyelinate already myelinated CNS tissue cultures (*1*) and to inhibit myelin formation if applied to cultures before myelination has begun (*2*). Such studies have been regarded as evidence of the role of circulating factors in EAE induction (*3*). Other studies, however, have shown that most serums from animals with EAE induced by the encephalitogenic factor, a basic protein (BP) component of myelin (*4*), neither demyelinate (*5*) nor inhibit myelin formation (*6*) in CNS cultures, in spite of the presence of detectable levels of antibodies to BP. In studies with rabbits as test animals, evidence has been presented that antibodies to cerebroside may be the serum factors which cause demyelination and myelination inhibition in vitro (*7*). Cerebroside does not induce EAE (*7*), and the demonstration that cerebroside is the component of whole CNS which induces demyelinating and myelination inhibiting factors lends support to the concept that these factors are epiphenomena, not related to the pathogenesis of EAE (*5, 6*).

Also regarded as evidence for the role of circulating factors in EAE induction (*3, 8*) has been the demonstration that serums from animals with EAE produce a reversible blocking of evoked electrical activity in CNS cultures (*9*). Moreover, EAE serums that did not demyelinate tissue cultures also blocked evoked polysynaptic potentials (*10*). It was suggested that this neuroelectric blocking factor might explain some neurological deficit phenomena in animals with EAE and might account for some discrepancies relative to the severity of clinical disease and the paucity of histological lesions (*9*).

The purpose of our study was to investigate further the relation of serum myelination inhibiting and neuroelectric blocking factors to the pathogenesis and clinical state of EAE.

Young male Lewis rats (175 and 250 g) were divided into six groups. One group of 13 animals was inoculated in the hind-foot pads with 0.5 ml of commercially prepared FCA (Difco) to which had been added 3 mg of *Mycobacterium tuberculosis* (H37Ra) per milliliter. The other five groups received an equivalent amount of FCA and *M. tuberculosis* combined with one of the following antigens: 50 mg (wet weight) of guinea pig spinal cord (eight animals); 1 mg (dry weight) of purified guinea pig myelin (*11*) (eight animals); 100 μg (dry weight) of guinea pig BP (nine animals); 100 μg of BP plus 1 mg of cerebroside (eight animals); and 1 mg of cerebroside plus 5 mg of bovine serum albumin (BSA) (three animals). The Lewis rats were evaluated for clinical EAE as manifested by hind-leg paralysis and weight loss. Serums were collected, and animals were killed at either 10, 11, 12, 13, 19, 21 days after inoculation. The rats that developed EAE were in an acute stage of the disease 10 to 13 days after inoculation, while those animals observed for 19 and 21 days had recovered from their paralysis and were gaining weight.

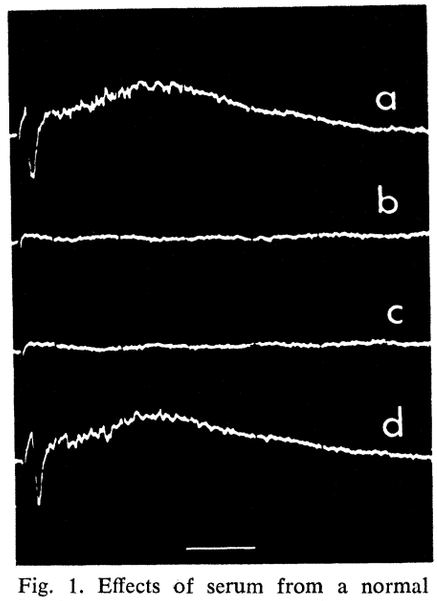

Fig. 1. Effects of serum from a normal Lewis rat on a mouse cerebral neocortex culture which had been 15 days in vitro. The time base marker at the bottom of the figure represents 10 msec. (a) Baseline extracellularly recorded response to electrical stimulation. Recording and stimulating electrodes were etched tungsten microelectrodes with tip diameters of less than 1 μm. (b) Thirty seconds after application of normal rat serum in a 25 percent concentration. The evoked activity has been abolished. (c) Two minutes after application of normal rat serum, the block of electrical activity persists. (d) Return of evoked electrical potentials after removal of the rat serum and replacement with normal recording medium (buffered Simms X-7 balanced salt solution).

Cerebellar tissue cultures derived from newborn mice and cerebral neocortex cultures derived from 2- to 3-day-old mice were prepared and maintained as described (*5, 6, 12*). Cerebellar cultures were exposed from the time of explantation to test or control rat serums incorporated into the nutrient medium in a 23 percent concentration, along with pooled guinea pig serum (10 percent) as a source of complement. Each serum was initially applied to seven explants, but some cultures were lost, because of contamination or for other reasons, during feeding manipulations to which they were subjected twice weekly. The explants were observed for 15 to 16 days for signs of myelination, well beyond the 9- to 12-day period when myelin usually appears in these cultures (*6*). A single myelinated fiber was considered sufficient to regard a culture as myelinated (*2*), and a serum was considered as negative for myelination inhibition factor if half or more of the cultures exposed to it contained myelinated fibers (*6*).

Cerebral neocortex cultures ranging in age from 11 to 27 days were selected for electrophysiological recordings. Methods for recording extracellular evoked responses were identical with those described (*13*). The medium that bathed the cultures during electrophysiological procedures consisted of Simms X-7 balanced salt solution (BSS) buffered with 0.015*M* Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). After baseline evoked electrical responses were established (Fig. 1a), test serums were applied directly to the cultures, either as part of a fully constituted medium as used in the tests for myelination inhibition, in which case the medium replaced the

BSS bathing the cultures, or by adding the serums to the recording medium (BSS) to a concentration of 25 percent. Most of the serums were evaluated by the latter method. If a complete blocking of evoked electrical potentials occurred within 30 seconds and was maintained for at least 2 minutes (Fig. 1, b and c), the serum was rated strongly positive (++) for neuroelectric blocking factor. If a concentration of serum greater than 25 percent was required (up to 40 percent), or if addition of complement (pooled guinea pig serum, up to 20 percent) was necessary to achieve a complete blocking, or if the blocking became complete later than 30 seconds after application of test serum, the serum was rated as positive (+) for neuroelectric blocking activity. After electrical responses were blocked, all serums were evaluated for reversibility of this effect by removing the test serum and replacing it with BSS (Fig. 1d).

All of the animals that received either whole CNS antigen or purified myelin displayed clinical manifestations of EAE (Table 1). Serums from five of the eight rats sensitized with whole CNS (animals 1A to 8A) were positive for myelination inhibition factor; these positive serums were collected during acute EAE. Serums from four of the eight rats inoculated with purified myelin (animals 1B to 8B) inhibited myelin formation; two of the four were collected during acute EAE and two were collected during the recovery phase.

Eight of nine rats inoculated with BP (group C) developed EAE. The one animal (1C) that had no demonstrated clinical signs of EAE at the time of serum collection was killed 10 days after inoculation. The serum from this animal was positive for myelination inhibition factor, while the serums from all of the remaining animals in this group failed to inhibit myelination. The addition of cerebroside to BP as part of the antigenic mixture did not alter the failure to induce myelination inhibition factor, as none of the serums from animals so inoculated inhibited myelin formation. All but one of the rats in this latter group exhibited both hind-leg paralysis and weight loss. One animal, rated ±, had developed weight loss only, and this animal was gaining weight at the time it was killed 19 days after sensitization.

Table 1. Serum myelination inhibition and neuroelectric blocking factors induced by sensitization of Lewis rats with Freund's complete adjuvant (FCA) combined with CNS antigens. DPI refers to the number of days after inoculation on which serums were collected. The designation + under EAE refers to acute EAE, while +R designates an animal recovering from EAE. The column designated cultures myelinated indicates the ratio of the number of cultures that developed myelin to the number of cultures tested. MI, myelination inhibition factor; NT, not tested; BP, myelin basic protein; BSA, bovine serum albumin; NB, neuroelectric block.

Animal No.	DPI	EAE	Cultures myelinated	MI	NB
		FCA + whole CNS			
1A	10	+	2/6	+	NT
2A	10	+	0/5	+	+
3A	11	+	0/7	+	++
4A	12	+	0/5	+	++
5A	13	+	5/7	−	++
6A	13	+	1/7	+	+
7A	19	+R	4/5	−	+
8A	21	+R	5/7	−	+
		FCA + purified myelin			
1B	10	+	1/7	+	NT
2B	10	+	5/7	−	++
3B	11	+	2/7	+	++
4B	12	+	3/5	−	+
5B	13	+	5/7	−	+
6B	13	+	4/5	−	+
7B	19	+R	1/5	+	+
8B	21	+R	1/6	+	+
		FCA + BP			
1C	10	−	1/6	+	NT
2C	10	+	3/3	−	+
3C	11	+	4/7	−	++
4C	12	+	6/7	−	+
5C	13	+	6/7	−	++
6C	13	+	5/7	−	+
7C	19	+R	3/5	−	+
8C	21	+R	7/7	−	+
9C	21	+R	7/7	−	NT
		FCA + BP + cerebroside			
1D	10	+	5/7	−	NT
2D	10	+	6/7	−	+
3D	12	+	4/7	−	+
4D	13	+	4/7	−	+
5D	13	+	4/6	−	+
6D	19	±R*	5/6	−	+
7D	21	+R	4/7	−	NT
8D	21	+R	5/7	−	++
		FCA + cerebroside + BSA			
1E	11	−	5/6	−	+
2E	13	−	5/7	−	+
3E	19	−	5/6	−	++
		FCA controls			
1F	10	−	6/6	−	NT
2F	10	−	4/4	−	+
3F	10	−	7/7	−	+
4F	11	−	5/7	−	+
5F	12	−	6/7	−	++
6F	12	−	5/6	−	+
7F	13	−	0/5	†	+
8F	13	−	7/7	−	+
9F	13	−	4/4	−	+
10F	19	−	5/6	−	++
11F	19	−	5/7	−	++
12F	21	−	5/7	−	+
13F	21	−	6/7	−	++

* Animal had developed weight loss only, without hind-leg paralysis, and was gaining weight at the time of serum collection. † Toxic; viable neurons could not be maintained in the presence of this serum.

None of the animals that received cerebroside and BSA or FCA alone developed EAE. The serums from none of these animals inhibited myelin formation in cerebellar cultures. One of the serums from the FCA group had a toxic effect on the explants, and viable neurons could not be maintained in the presence of this serum.

All of the serums from animals in all groups and in all stages of disease, including those who had no disease, reversibly blocked evoked electrical activity in cerebral neocortex cultures. Serums which were strongly positive were distributed among all six groups, including the group of rats that had received FCA alone. The consistent presence of neuroelectric blocking factor in all of the test serums led us to evaluate the serums of three normal rats. These serums were also positive for neuroelectric blocking factor, one of them strongly positive, and the effect of this latter serum is illustrated in Fig. 1. Pooled guinea pig serum tested by itself significantly attenuated evoked polysynaptic potentials after a concentration of 40 percent had been attained.

Three points can be made on the basis of the results of our study. First, the component of whole CNS which induces myelination inhibition factor does appear to be in myelin, as serums from animals inoculated with purified myelin were almost as effective in inhibiting myelin formation in vitro as serums from animals sensitized with whole CNS. Second, cerebroside given either with BSA or in combination with BP did not induce myelin inhibition factor in Lewis rats observed for the same time period as the whole CNS- and purified myelin-sensitized animals whose serums were positive for this factor. Third, the results of the electrophysiological studies suggest that the neuroelectric blocking factor is a nonspecific serum factor found in control animals as well as in animals with EAE, and that it is not related to the clinical state of EAE. The reasons for the difference between our results and the negative results reported by Bornstein and Crain (*9*) for control serums may be related to the small size of their control sample and to their omission of FCA-sensitized controls.

We had previously demonstrated a dissociation between EAE induction and induction of a serum factor which inhibits myelin formation in vitro (*6*), a demonstration confirmed by our

study. This study adds the finding that purified myelin is capable of inducing both EAE and myelination inhibition factor, suggesting that the agent responsible for induction of the latter is a component of myelin. Our results also indicate that the ability of serums to block evoked electrical responses in tissue cultures is not specifically related to the presence of neurological deficit phenomena in EAE. The finding of neuroelectric blocking factor in serums from control animals as well as from animals with EAE argues against a specific role for this factor in the pathogenesis of EAE.

FREDRICK J. SEIL
MARION E. SMITH
Department of Neurology, Veterans Administration Hospital, and Stanford University School of Medicine, Palo Alto, California 94304
ARNOLD L. LEIMAN
Department of Psychology, University of California, Berkeley 94720
JAMES M. KELLY, III
Veterans Administration Hospital, Palo Alto, California 94304

References and Notes

1. M. B. Bornstein and S. H. Appel, *J. Neuropathol. Exp. Neurol.* **20**, 141 (1961); S H. Appel and M. B. Bornstein, *J. Exp. Med.* **119**, 303 (1964).
2. M. B. Bornstein and C. S. Raine, *Lab. Invest.* **23**, 536 (1970).
3. M. B. Bornstein, in *Progress in Neuropathology*, H. M. Zimmerman, Ed. (Grune & Stratton, New York, 1973), vol. 2, p. 69; M. B. Bornstein, *Mt. Sinai J. Med.* **41**, 46 (1974).
4. R. H. Laatsch, M. W. Kies, S. Gordon, E. C. Alvord, Jr., *J. Exp. Med.* **115**, 777 (1962).
5. F. J. Seil, G. A. Falk, M. W. Kies, E. C. Alvord, Jr., *Exp. Neurol.* **22**, 545 (1968).
6. M. W. Kies, B. F. Driscoll, F. J. Seil, E. C. Alvord, Jr., *Science* **179**, 689 (1973); F. J. Seil, H. C. Rauch, E. R. Einstein, A. E. Hamilton, *J. Immunol.* **111**, 96 (1973).
7. M. Dubois-Dalcq, B. Niedieck, M. Buyse, *Pathol. Eur.* **5**, 331 (1970); J. M. Fry, S. Weissbarth, G. M. Lehrer, M. B. Bornstein, *Science* **183**, 540 (1974).
8. P. Y. Patterson, *Annu. Rev. Med.* **20**, 75 (1969); F. J. Seil, *Calif. Med.* **116**, 25 (May 1972).
9. M. B. Bornstein and S. M. Crain, *Science* **148**, 1242 (1965).
10. ———, *J. Neuropathol. Exp. Neurol.* **30**, 129 (1971).
11. M. E. Smith, *Biochim. Biophys. Acta* **164**, 285 (1968).
12. M. B. Bornstein and M. R. Murray, *J. Biophys. Biochem. Cytol.* **4**, 499 (1958); S. M. Crain and M. B. Bornstein, *Exp. Neurol.* **10**, 425 (1964); F. J. Seil, *Brain Res.* **42**, 33 (1972); ———, J. M. Kelly, A. L. Leiman, *Exp. Neurol.* **45**, 435 (1974).
13. A. L. Leiman and F. J. Seil, *Exp. Neurol.* **40**, 748 (1973).
14. We thank R. A. Fisk for technical assistance. J.M.K. holds a Veterans Administration fellowship in neurobiology.

28 August 1974; revised 25 November 1974

Disc Shedding in Rodlike and Conelike Photoreceptors of Tree Squirrels

Abstract. *Electron microscopic observations suggest that the rodlike and conelike photoreceptors of diurnal tree squirrels shed outer segment discs. Twenty-four hours after injection of tritiated* L-*leucine, the rodlike photoreceptors show a band of radioactivity at the base of the outer segment. The conelike photoreceptor outer segments show only a pattern of diffuse labeling. These results strongly suggest that disc shedding can occur in photoreceptor outer segments in which proteins are diffusely renewed.*

Autoradiographic investigations of the outer segment renewal process in vertebrate photoreceptors show that a distinct band of radioactive material can be identified at the base of rod outer segments within hours after the administration of radioactive amino acids (*1*). It is thought that these labeled amino acids are incorporated into protein that is used in the assembly of new rod discs at the outer segment base (*2*). The labeled discs are continuously displaced from the base to the apex of the rod outer segment (*1, 3*). At this point the labeled discs detach from the outer segment tip and are withdrawn into the pigment epithelium as phagosomes (*4*). Electron microscopic evidence confirms that groups of rod discs detach intermittently from the outer segment tips, after which a process of degradation occurs within the pigment epithelium (*5, 6*).

The mode of outer segment renewal described in mature vertebrate cones is thought to involve neither the synthesis of new discs nor the disposal of old discs, since radioactive label that is incorporated into cone outer segments does not accumulate in a distinct band as it does in rods (*7*). Rather, the labeled material is seen as diffusely incorporated throughout the cone outer segment (*8*). In this report we present electron microscopic evidence that implicates both the conelike and rodlike photoreceptors of diurnal tree squirrels in a process of disc shedding. We also show that proteins in the conelike outer segments are diffusely renewed like those in all other vertebrate cones examined (*7*).

Cohen (*9*) described the fine structure of two photoreceptor classes in the retina of the Eastern grey squirrel (*Sciurus carolinensis*), an arboreal species whose retina had been thought to contain only cones (*10*). He showed that the double-tiered photoreceptor layer was composed of an outer, more sclerad tier with short cylindrical outer segments whose discs were continuous with the outer cell membrane in the basal third of the outer segment. He also described an inner, more vitread tier with outer segments that were somewhat longer and of reduced diameter. In these receptors, only rare instances of disc-membrane continuity were observed. Cohen designated the inner tier photoreceptors as R or rodlike and the outer tier photoreceptors as C or conelike. This classification was confirmed by West and Dowling (*11*).

Laties and Liebman (*12*) showed that the outer segments of vertebrate cones selectively take up the dye Procion Yellow after it is injected in vivo into the vitreous humor. It is believed that the observed fluorescence in cone outer segments is due to the infiltration of the dye into the spaces between the infolded disc membranes which are open to extracellular space (*12, 13*). Only the conelike outer segments take up Procion Yellow in the retina of the Eastern grey squirrel (*14*). This observation lends support to the receptor designations originally proposed by Cohen (*9*).

We reexamined the fine structure of the photoreceptors in retinas of the Eastern grey squirrel and the closely related Western grey squirrel (*Sciurus griseus*). In all aspects examined thus far, the retinas of these two species are alike. In each case, fixation was accomplished by intracardiac perfusion or by immersion in a solution of 2.5 percent glutaraldehyde buffered with 0.067*M* sodium cacodylate (*p*H 7.4). After the initial fixation, the posterior segments of the eyes were briefly washed and then postfixed in 2 percent osmium tetroxide buffered with veronal acetate (*p*H 7.4). Sections for electron microscopy were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I or Siemens 101 electron microscope.

In both species a process of disc shedding, similar to that previously described only in vertebrate rods (*5, 6*),