Functional Studies of Cultured Brain Tissues as Related to "Demyelinative Disorders"

Abstract. *The serums from animals with experimental allergic encephalomyelitis and humans with multiple sclerosis produce, in addition to demyelination, rapid, reversible alterations in complex, evoked bioelectric (synaptic) responses of cultured cerebral cortex and spinal cord tissues of the mouse. The active factors are dependent on complement and are not present in serums from normal animals and humans.*

The demyelinative process in animals affected with experimental allergic encephalomyelitis (EAE) has been widely studied, mainly because many of its structural details resemble those observed in the nervous system of patients with multiple sclerosis (MS) and allied diseases. In fact, the hypothesis of an auto-immune pathogenesis for the naturally occurring diseases has been inferred from the analogous EAE processes (*1*). Additional support for such a mechanism has been derived from the observations that serums from animals affected with EAE and from the majority of patients experiencing an active stage of MS produce identical patterns of demyelination in cultured tissue fragments of mammalian central nervous system (CNS) (*2*). In addition, the demyelinating factors in the animal serums have been demonstrated to be complement-dependent antibodies (*3*), and those in human serums have, thus far, also been found to be dependent on complement (*4*). Yet, the process of demyelination alone has not been considered an adequate explanation for all the clinical phenomena in either MS or EAE. In the former, the rapidity of onset and recession of early symptoms, quite unrelated to the severity of their expression, has always challenged the thoughtful clinician and pathologist to seek beyond the striking and dramatic fact of demyelination (*5*). In these experimental models, entire species such as the hamster may demonstrate few if any demyelinating lesions in spite of obvious clinical involvement; even in groups of animals which do exhibit demyelination, a serial selection of animals at intervals after they have been inoculated with CNS antigens has revealed individuals with clinically apparent disease without any discernible lesions in the CNS (*6*).

In the experiments described here, cultured fragments (about 1 mm^3) of mammalian (mouse) CNS tissues were used for investigation of the "demyelinative disorders." The results demonstrate that drastic alterations occur in bioelectric functions of the explanted tissues when they are exposed to the serums from rabbits with EAE or patients with MS.

Cerebral neocortex and spinal cord tissues were cultured for long periods in vitro under conditions which led to the formation of myelin sheaths and synaptic junctions (*7*), as well as to the development of a remarkable degree of physiological organization resembling complex networks of the CNS in vivo (*8*). The characteristic response patterns of these CNS explants to electrical stimulation (*8*) consist of two components: (i) simple spike potentials, of brief duration, representing the propagation of impulses along axons (Figs. 1*B* and 2B_3); and (ii) complex potentials, of much longer duration and latency (Figs. 1*A* and 2*A*), indicating the generation of synaptic potentials in circuitous multineuronal pathways. Technical details of the experimental arrangement used for the electrophysiologic studies were described previously (*8, 9*).

Exposure of the cultured cerebral and spinal cord explants to serum from animals with EAE (at concentrations of 10 to 25 percent of the medium) led not only to a characteristic, specific pattern of demyelination within a few hours (*10*) but, also, to extensive alterations in the bioelectric properties of the tissues long before any morphological changes had been detected. In fact, preliminary electron-microscopic studies of the cultured spinal cord tissue (*11*, see also *10*) revealed no alteration in the typical synaptic ultrastructure after 1 hour of exposure to an EAE serum which produced functional block (as described below) within a few minutes in a simultaneously exposed sister culture (that is, one prepared from the same spinal cord). The complex responses characteristic of synaptic transmission often disappeared within a few minutes after application of EAE serum, but simple axon spikes could still generally be evoked (see Fig. 1, *A* and *B*). In some cases, the threshold for direct excitation of simple spikes also rose markedly, especially after prolonged exposure to the serum. Serums from six rabbits with EAE were tested on 13 cultures of mouse cerebrum and spinal cord. Partial or complete block of complex bio-

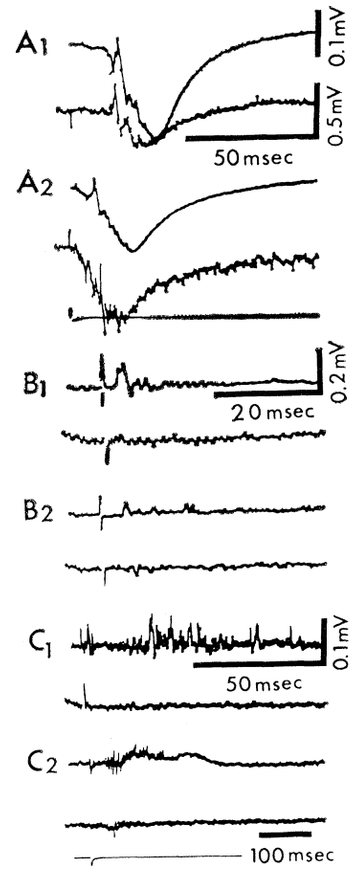

Fig. 1. Effects of serum from a rabbit with EAE on bioelectric activity of cultured fragment (1 mm^3) of cerebral tissue (35 days after explantation from newborn mouse). (*A*) Simultaneous control records of characteristic evoked responses in two cortical regions of explant (100 μ apart) after application (with a 10-μ saline-filled pipette) of a single electric stimulus (0.1 msec duration) to a subcortical zone (A_1) and to a cortical zone (A_2). Stimulus-recording distances were 1.5 and 0.5 mm in A_1 and A_2, respectively. Large, long-lasting, positive potentials follow brief spikes at both recording sites (upper sweep in each record obtained with 25-μ Ag electrode, and lower, with 5-μ saline-filled pipette). Responses to subcortical stimulus (A_1) have much longer latency. Lowest sweep in A_2 shows stimulus signal. (*B*) Ten minutes after exposure to EAE serum (25 percent). All slow-wave components of responses with cortical (B_1) and subcortical (B_2) stimuli have disappeared. Only simple spike potentials occur even with high stimulus-intensity. (C_1) Thirty minutes after replacement of EAE serum with normal serum (25 percent). Long-lasting barrage of spike responses indicates partial recovery of complex activity. (C_2) One hour after further exposure to control medium. Slow-wave components appear with superimposed spike bursts (elicited at much lower stimulus intensity than required for simple responses in *B*). Time and amplitude calibrations apply to all succeeding records, unless otherwise noted; upward deflection indicates negativity at active recording electrode.

electric responses occurred in all cultures after exposure to the serum for less than 1 hour. When the effective serum was removed and replaced with normal nutrient solution or balanced salt solution, the cultured tissue slowly regained its usual bioelectric functions (Fig. 1*C*). Repeated or prolonged exposure to the effective serums decreased the rate and extent of functional recovery of explants returned to the control medium. At least partial recovery occurred, in most experiments, within 5 to 60 minutes. Fresh serums from normal rabbits and guinea pigs (10 to 20 percent) did not produce these changes. Fresh guinea pig serum was therefore, often added to the serum obtained from animals with EAE (or humans with MS) to ensure an adequate concentration of complement during these experiments. Rabbit serum containing antibodies to kidney tissue (*12*), that is, capable of producing severe kidney lesions when injected into mice, did not block the bioelectric responses of cultured mouse cerebral cortex when applied in a concentration of 25 percent for over an hour. Subsequent introduction of serum (25 percent) from a rabbit with EAE produced the characteristic blocking effect within 5 minutes.

The serums of two patients, obtained during acute exacerbations of MS, blocked interneuronal functions of three cerebral explants in exactly the same manner as when serum from animals with EAE was used (Fig. 2, *A* and *B*). The human serums appeared to be less potent than some of the rabbit serums since a 50-percent concentration of human serum was needed to effect the same change as was observed when 10 percent of the rabbit serum was used; moreover, to block complex bioelectric responses completely, 20 to 50 minutes was required as compared with 5 to 10 minutes for some of the animal serums. Serum from normal humans had no effect on the cultures. In fact, human placental serum was present in concentrations of up to 40 percent in the nutrient medium used for long-term maintenance of the cultures as well as for the electrophysiological experiments.

Strychnine, at concentrations (1 μg/ml of balanced salt solution) which produce only slight stimulating effects in normal cultures (*8*), greatly accelerated the recovery process when explants were returned to control medium (see Fig. 2, *C* and *D*). Pharmacodynamic analysis of these data suggests that the primary bioelectric effects of serums from animals with EAE and humans with MS may be due to selective interference with synaptic processes in these cultured CNS tissues (*13*).

Heating the effective serums at 56°C for 20 to 30 minutes destroyed their ability to alter neural function (Fig. 2*D*), as determined by eight tests. In four experiments the addition of fresh serum from normal guinea pigs (10 to 20 percent) restored the ability of heat-inactivated serum to block bioelectric responses (Fig. 2, *E* and *F*).

The only previous study which appears to demonstrate direct effects of immunologic agents on bioelectric properties of CNS tissue was carried out in vivo in the cat by intraventricular injection of antibodies to caudate nucleus of the cerebrum (*14*). This procedure resulted in gradual alteration and, after several days, abolition of spontaneous bioelectric activity confined mainly to the caudate nucleus. It will be of interest to investigate possible relationships between these phenomena in the living animal and in vitro, bearing in mind the marked differences between the two cases, that is, serum obtained from rabbits affected with EAE following injection of spinal cord antigenic emulsion produce similar functional block in explants of cerebral cortex as well as spinal cord; interference with the bioelectric activities of the cultured tissues may occur within minutes and is, moreover, reversible.

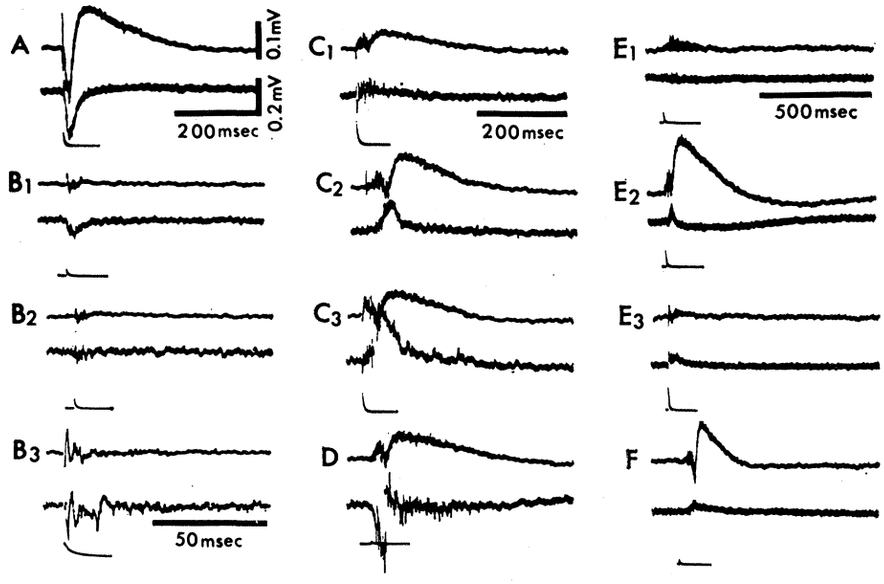

Fig. 2. Effects of serum from a human with MS on bioelectric activity of cultured cerebral tissue (12 days after explantation from newborn mouse). (*A*) Simultaneous control records of characteristic responses evoked in two cortical regions (100 μ apart) with single stimulus, after 15 minutes exposure to 10 percent guinea pig (GP) serum. Diphasic potential with long-duration negativity occurs at one site (upper sweep; recorded with 25-μ Ag electrode) and monophasic positive response at other site (recorded with 8-μ saline-filled pipette). (B_1) Seven minutes after exposure to MS (50 percent) and GP (10 percent) serums. Amplitude and duration of evoked potentials have decreased markedly in both cortical regions. (B_2) Three minutes later—slow-wave components have disappeared almost completely (stimulus threshold much higher now). (B_3) Faster sweep reveals that early-latency spike potentials can still be elicited (at high stimulus intensities) after 20 minutes exposure to MS serum. (C_1) Twenty minutes after replacement of MS and GP serums by balanced salt solution (BSS). Slow-wave responses have returned, but with small amplitude (stimulus threshold still high). (C_2 and C_3) Within 10 minutes after strychnine (1 μg/ml BSS); about 1 hour after removal of serums. Original evoked response has been almost completely restored at one cortical site (upper sweep) and large, negative component has developed at the other site (stimulus threshold down to normal range). (*D*) Thirty-five minutes after replacement of "strychnine-BSS" by 50 percent MS and 10 percent GP serums which had previously been heated to 56°C for 20 minutes. Evoked responses have not been significantly affected except for return of strychnine-inverted component to its original polarity (lower sweep). (E_1) Within a few minutes after addition of unheated GP serum (20 percent) to the heated MS-plus-GP serums. Evoked responses are almost completely blocked (stimulus of higher intensity than used in *D*). (E_2) Negative evoked response with unusually large amplitude still occurs at higher stimulus intensity (upper sweep). (E_3) About 1 hour later. Evoked responses have been almost completely blocked (even with much higher stimulus intensity than in E_2). (*F*) Thirty minutes after replacement of serums by BSS and then addition of strychnine (1 μg/ml). Evoked response has been restored at one site (upper sweep), even with weak stimulus.

This demonstration of circulating factors, dependent upon the presence of complement and capable of producing a reversible synaptic blocking effect when applied directly to CNS tissue, may be significant in explaining the pathogenesis and clinical course of EAE and MS. However, the specificity of the demonstrated reaction in terms of the "demyelinative disorders" cannot be fully evaluated until the serums of patients with destructive but not essentially demyelinative lesions are examined.

MURRAY B. BORNSTEIN
Laboratory of Cellular Neurophysiology, Department of Neurology, Mt. Sinai Hospital, New York, New York

STANLEY M. CRAIN
Departments of Anatomy and *Neurology, College of Physicians and Surgeons, Columbia University, New York, New York*

References and Notes

1. M. W. Kies and E. C. Alvord, Jr., Eds., *"Allergic" Encephalomyelitis* (Thomas, Springfield, Ill., 1959).
2. M. B. Bornstein and S. H. Appel, *J. Neuropathol. Exptl. Neurol.* **20**, 141 (1961).
3. S. H. Appel and M. B. Bornstein, *J. Exptl. Med.* **119**, 303 (1964).
4. M. B. Bornstein, *Natl. Cancer Inst. Monograph 11*, 197 (1963).
5. R. D. Adams, in *"Allergic" Encephalomyelitis*, M. W. Kies and E. C. Alvord, Jr., Eds. (Thomas, Springfield, Ill., 1959), pp. 183–209; A. S. Rose, in *Mechanisms of Demyelination*, A. S. Rose and C. M. Pearson, Eds. (McGraw-Hill, New York, 1963), pp. 215–216; D. McAlpine, in *Multiple Sclerosis—a Reappraisal*, D. McAlpine, C. E. Lumsden, D. Hughes, Eds. (Livingston, Edinburgh, rev. ed., 1965), pp. 142–178.
6. E. C. Alvord, Jr., in *"Allergic" Encephalomyelitis*, M. W. Kies and E. C. Alvord, Jr., Eds. (Thomas, Springfield, Ill., 1959), p. 166.
7. M. B. Bornstein, in *Neurological and Electroencephalographic Correlative Studies in Infancy*, P. Kellaway and I. Petersén, Eds. (Grune and Stratton, New York, 1964), pp. 1–11; M. B. Bornstein, S. M. Crain, G. D. Pappas, in preparation.
8. S. M. Crain, in *Neurological and Electroencephalographic Correlative Studies in Infancy*, P. Kellaway and I. Petersén, Eds. (Grune and Stratton, New York, 1964), pp. 12–26; S. M. Crain and M. B. Bornstein, *Exptl. Neurol.* **10**, 425 (1964); S. M. Crain and E. R. Peterson, *Science* **141**, 427 (1963); ———, *J. Cellular Comp. Physiol.* **64**, 1 (1964).
9. S. M. Crain, *J. Comp. Neurol.* **104**, 285 (1956).
10. M. B. Bornstein, in *4th International Symposium on Immunological Pathology* (Council for International Organizations of Medical Sciences, Monte Carlo, in press).
11. L. L. Ross, in preparation.
12. The serum containing antibodies to kidney tissue was kindly supplied by Dr. Beatrice Seegal, Columbia University.
13. S. M. Crain, in preparation.
14. Lj. Mihailović and B. D. Janković, *Nature* **192**, 665 (1961); ———, B. Belislin, K. Mitrović, Lj. Krzalić, *ibid.* **203**, 763 (1964).
15. Supported in part by PHS grants NB-01913 and NB-03184 from the National Institutes of Neurological Diseases and Blindness and grant 246 from the National Multiple Sclerosis Society. Dr. Crain is a PHS Research Career Development Fellow (award NB-K3-2904 from NINDB).

11 February 1965

Visually Evoked Electrocortical Responses in Kittens: Development of Specific and Nonspecific Systems

Abstract. *Visually evoked electrocortical responses were differentiated ontogenetically in young kittens by age of onset, latency, and polarity. A long-latency negative wave, present by 4 days of age and abolished later by lesions of the superior colliculus and pretectal region of the midbrain, is attributed to the nonspecific sensory system. A short-latency, positive and negative, diphasic wave, developing by 10 to 15 days of age and blocked by lesion of the lateral geniculate body, is identified with the specific visual system.*

Recently there has been heightened interest in the structural, biochemical, and electrophysiological properties of the developing brain and its relationship to the ontogeny of behavior (*1*). Extensive investigations of the electrophysiology of the visual system have been made in adult animals, but there have been few studies of the maturation of electrocortical activity of the visual area in newborn and young animals, apart from electroencephalographic studies in human infants (*2*). Cross-sectional samplings, at different ages, of electrocortical responses to visual stimulation have been made in the rabbit (*3*) and kitten (*4*). These have revealed important changes in visually evoked potentials as a function of age.

Our interest has focused upon the significance of the time sequence of development of visually evoked responses in kittens as revealed in both longitudinal and cross-sectional studies from the time the kittens are a few days old, when the first response component appears, until they are 1 or 2 months old, when the evoked potential has attained essentially the form typical of the mature cat. The separation of early-developing and late-developing components of the evoked response in the natural course of the maturational process has made it possible to study the characteristics of the components separately as a function of age. Additionally, the separation of response components (by time of onset, polarity, latency, amplitude, and cortical distribution) at certain ages has made it possible to study the effect upon these components of selective midbrain and thalamic lesions.

The lesions were made on the basis of the hypothesis that an initially appearing wave of negative polarity, long latency, and widespread distribution is mediated by the nonspecific sensory system, and that a later-developing diphasic wave of positive and negative polarity, short latency, and more limited distribution is mediated by the classical, specific, visual projection system. The hypothesis appears to have been confirmed by our studies, in that lesions of the superior colliculus and pretectal region of the midbrain blocked the long-latency response, and lesions of the lateral geniculate body interfered with the short-latency response.

Forty kittens from 20 litters were studied at various ages; emphasis was placed on serial recordings. In the most extensive longitudinal sequence recordings were made repeatedly on the same kitten approximately every 5 days from the 4th to the 56th day of age. Both acute preparations (temporarily anesthetized for each recording) and chronic preparations (unanesthetized animals with permanently implanted electrodes) were used. In the case of the former the kittens were immobilized by light Nembutal anesthesia each time recordings were made. Brief flashes from a Grass PS-1 photostimulator were collimated and focused by an optical system and delivered monocularly. The eyelids were held open with a retractor, and the pupil was dilated with homatropine. Monopolar recordings (a mouthpiece served as grounded reference lead) and bipolar recordings were obtained bilaterally over two visual-response sites (shown in *I* of Fig. 2). In the chronic preparations screw electrodes were implanted in the skull over the same visual-response sites.

The sequence of development of the visually evoked responses was similar in cross-sectional and longitudinal methods of study.

Figure 1 shows a typical longitudinal sequence recorded at increasing age levels in the same kitten. The first response to appear over the visual cortex on the side opposite the eye stimulated (the contralateral visual cortex) was a single, long-latency wave of negative polarity with a peak latency