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Specific Binding of Peroxidase-Labeled Myelin Basic Protein in Allergic Encephalomyelitis

(antigen/antibody/autoimmune/immunopathology/histochemistry)

ANNE B. JOHNSON*, HENRYK M. WIŚNIEWSKI*, CEDRIC S. RAINE*,
E. H. EYLAR†, AND ROBERT D. TERRY*

* Department of Pathology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461; and † Department of Experimental Biology, Merck Institute, Rahway, New Jersey 07065

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ABSTRACT **A conjugate of horseradish peroxidase and the encephalitogenic basic protein from myelin has been used to study the antigen reactivity of tissue in the autoimmune disease, experimental allergic encephalomyelitis. Control conjugates were also prepared of peroxidase and bovine serum albumin and of peroxidase and lysozyme, another basic protein. The basic protein from myelin conjugate was specifically bound by lymph node cells from rabbits immunized against the basic protein. Some of these cells appeared to be plasma cells. The conjugate was also specifically bound by occasional cells in the spinal-cord infiltrates of animals with early signs of allergic encephalomyelitis. These cells resembled large lymphocytes and plasma cells. There was no difference between the binding of basic protein of bovine and rabbit origin. The findings suggest the possibility that a local release of antibody within the target organ may play a role in the pathogenesis of allergic encephalomyelitis.**

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease believed to be a manifestation of delayed hypersensitivity to the encephalitogenic basic protein of myelin (BP) (1–4). However, the pathogenetic mechanisms involved in EAE, and whether antibody participates in the disease process, are far from clear. We have approached the question of the possible role of tissue- or cell-bound anti-BP antibodies in EAE by using a new technique with labeled antigen (5). The present report describes the successful coupling of BP to horseradish peroxidase (peroxidase). With this conjugate, we have demonstrated cells in the lymph nodes and spinal-cord infiltrates that specifically bind BP in rabbits with EAE. Thus, we have shown that specifically reactive cells are present in the target organ of this autoimmune response.

MATERIALS AND METHODS

A highly purified preparation of BP from bovine brain or spinal cord (A-1 BP) (6) was used for most studies. It was homogeneous on discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate‡. A similar BP preparation from rabbit spinal cord was used in the few instances indicated.

Abbreviations: BP, basic protein; BSA, bovine serum albumin; EAE, experimental allergic encephalomyelitis; PBS, phosphate-buffered saline (pH 7.2).

‡ Kindly performed by Dr. Seymour Greenfield of the Albert Einstein College of Medicine.

BP was coupled to peroxidase with glutaraldehyde, which reacts with the *e*-amino groups of lysine (7), by the procedure developed by Avrameas (5) for other proteins. It was necessary to use small quantities of BP, which has many lysine groups, in order to obtain a soluble product. The molecular ratio of protein to peroxidase of about 1 to 7, however, was similar to that recommended by Avrameas. The conjugate was prepared by dissolving 0.3–0.6 mg of BP and 6 mg of peroxidase (type VI, RZ 3.1; Sigma) in 0.4 ml of 0.1 M phosphate buffer (pH 6.8). Then, 0.1 ml of 0.25% glutaraldehyde, diluted with phosphate buffer from an 8% aqueous preparation (Polysciences), was added dropwise with gentle mixing. After the mixture had stood for 2 hr at room temperature, the unbound glutaraldehyde was removed by dialysis for 24 hr at 4°C against three 2-liter changes of phosphate-buffered saline (PBS; 0.01 M phosphate buffer (pH 7.2)–0.85% NaCl). Centrifugation at 36,000 × *g* for 30 min at 4°C removed any insoluble product that formed. Several modifications of the Avrameas procedure were tried, such as changing the concentration of peroxidase or glutaraldehyde, the pH and the conjugation time, or adding BP to peroxidase previously treated with glutaraldehyde. These modifications often gave active conjugates, but did not offer any advantages. The final BP–peroxidase conjugate was soluble and remained active for more than 2 months when stored frozen in small aliquots to minimize multiple freezings and thawings. As controls, conjugates of peroxidase with bovine serum albumin (BSA) (Sigma) and with lysozyme from egg white (Sigma) were prepared identically, except that 2.5 mg and 0.4 mg, respectively, were used. Lysozyme is a protein similar to BP in molecular weight and isoelectric point. A control conjugate was also prepared from peroxidase and glutaraldehyde without an antigen.

The tissues to be tested for binding of antigen were obtained from New Zealand albino rabbits, previously injected in all four footpads with 0.4 ml of an emulsion containing antigen dissolved in one part isotonic saline and two parts complete Freund's adjuvant with *Mycobacterium tuberculosis* H37 RA (Difco). Nine rabbits received 0.4–1.25 mg (generally 1.0 mg) bovine BP as an antigen, and three others received 0.4 mg of rabbit BP. Two rabbits were immunized with adjuvant plus 133 mg of a crude homogenate of bovine brain white-matter. Popliteal or axillary lymph nodes were removed from anesthetized animals on days 4, 6, 8, or 11 after immunization. During the first 24 hr of signs of EAE

(day 10–29), another node and lumbar spinal cord were obtained. In addition, lymph nodes were removed from a normal rabbit and, on days 8 and 11, from two rabbits treated only with adjuvant and from four rabbits treated with antigen plus adjuvant, but with either BSA (4 mg), lysozyme (3 mg), or calf-thymus histone (Sigma) (1 or 3 mg) as the antigen. Calf-thymus histone is another low molecular weight, basic protein. In a few instances, nodes were removed between 30 and 60 days after immunization. The tissues were fresh-frozen immediately after removal, and remained reactive during several months of storage at −70°C.

For assay, the tissues were cut at 8 μm in a cryostat, mounted on coverslips, and fixed for 30 min at 4°C in 4% formaldehyde in Sorenson's phosphate buffer, (pH 7.4). Ethanol–ether 3:2 for 45 min at room temperature could also be used as fixative, but buffered 1% glutaraldehyde for 5 min at 4°C destroyed all reactivity. Tissue blocks remained reactive when fixed in cold 4% formaldehyde for 4, but not for 20, hr. After room temperature washes in PBS, the sections were covered with a dilution of the conjugate and incubated horizontally for 90 min at room temperature. Then, they were washed again in PBS, incubated 10–30 min at room temperature in a peroxidase medium containing diaminobenzidine (8), counterstained with methyl green, and mounted in glycerogel or Permount. The optimal dilution of conjugate was determined empirically for each batch of conjugate. First the conjugate was diluted with PBS until its color visually matched a stock preparation of 0.5 mg peroxidase/ml of PBS. Then it was further diluted, generally 2- or 3-fold, until it produced a specific tissue reaction with little nonspecific or background staining. The final concentration of BP in the conjugate, as incubated with tissue, was estimated to be around 0.02 mg/ml. The specificity of conjugate binding was evaluated by treatment of the fixed and washed sections for 60 min with a solution of antigen (0.2 mg of BP, 8 mg of BSA, 0.7 mg of lysozyme, or 0.2 mg of histone per ml of BPS) before the incubation with conjugate.

RESULTS

Lymph nodes from rabbits immunized against bovine BP, BSA, or lysozyme, when tested with a conjugate of the immunogen and peroxidase, contained antigen-binding cells in the perifollicular, paracortical, and medullary regions [Fig. 1*A*,*B*]. The staining patterns in the three types of tissue were similar, but reactivity was also seen within germinal centers in the lysozyme-treated node. Staining occurred throughout the cytoplasm of reactive cells, often appeared somewhat granular, and varied considerably in intensity from cell to cell. Some of the stained cells appeared to be plasma cells [Fig. 1*C*], but most could not be positively

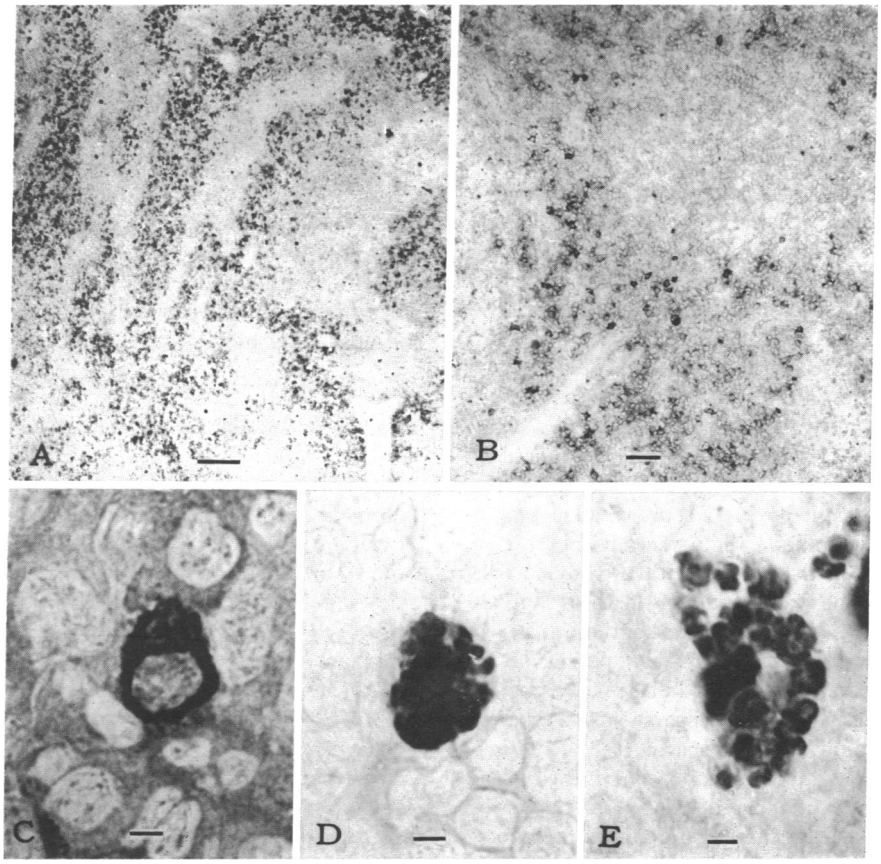

FIG. 1. Lymph nodes from BP-treated rabbits. (*A*) A large number of specifically-reactive cells are present in clusters extending through the paracortical and medullary regions. Day 11; BP–peroxidase conjugate. Scale = 150 μm. (*B*) Specifically-reactive cells surrounding a follicular area. Day 11: BP–peroxidase conjugate. Scale = 50 μm. (*C*) Specifically-reactive cell that resembles a plasma cell. Day 11; BP–peroxidase conjugate. Scale = 3 μm. (*D*) Endogenous peroxidatic activity in an eosinophil. Day 11; conjugate incubation omitted. Scale = 3 μm. (*E*) Nonspecifically-stained macrophage. Day 54; peroxidase control conjugate without antigen. Scale = 3 μm.

identified. The number of reactive cells was much less than the number that took up pyronin in adjacent sections stained with methyl green–pyronin or Wright's stain (9).

The reactivity with conjugate was interpreted as specific because in each case it only occurred with the conjugate prepared from the immunizing antigen. Furthermore, this binding could be specifically blocked (or, in the case of BSA, markedly diminished) by treating the sections with the immunizing antigen before the incubation with conjugate. In each case, substitution of the other proteins for the immunizing antigen in the pretreatment step did not block binding. For the BP conjugate in particular, the specificity of the binding was demonstrated by the observation that it was blocked by pretreatment with BP, but not by pretreatment with either of the other basic proteins—lysozyme or calf-thymus histone—although sometimes the histone treatment diminished the intensity of staining. Conjugate-incubated sections of lymph nodes from normal rabbits and from those immunized with complete Freund's adjuvant only, or adjuvant plus histone, never contained stained cells that resembled the specifically reactive cells.

The specifically reactive cells were first detected in lymph nodes on the eighth day after immunization with bovine BP. Their numbers had increased considerably by the eleventh day, and positive cells were present in moderate numbers at the time of onset of signs of EAE. Specifically-reactive cells were present in each of the seven bovine BP-treated animals that developed EAE and in one of the two similarly treated rabbits that were still without neurological signs 54 days after immunization. The number of reactive cells differed greatly from animal to animal, and often reactive cells were present in only a portion of the node. Immunization against BSA, lysozyme, or histone did not produce clinical symptoms. With BSA and lysozyme, reactive node cells may have appeared earlier than with BP, as many more cells exhibited specific staining on day 8, as well as on day 11.

That the reactivity of rabbit tissue with bovine BP was not merely a reflection of species difference was shown by comparing the bovine and rabbit BP preparations. When nodes from rabbits immunized with bovine BP were tested with a conjugate prepared from rabbit BP, the reactivity was equal to that with the usual bovine BP conjugate. The binding of each of these BP conjugates was completely blocked by pretreatment with either rabbit or bovine BP. Other studies showed that our bovine BP preparation was a stronger encephalitogen in rabbits than was our rabbit BP preparation. Nevertheless, cells reactive with the bovine BP conjugate were present in the lymph nodes of the three rabbits immunized with rabbit BP, although only one of these animals developed clinical EAE during the 54-day period after immunization. (A second rabbit was not killed; it became ill after 5 months.) The positive cells were sparse, however, and not detected before day 11. Similar sparse reactivity was also present in the nodes of the two rabbits immunized with the brain homogenate, both of which developed EAE.

Staining not due to specific binding of antigen also occurred in lymph nodes, but was easily differentiated. The high endogenous peroxidatic activity of the granules of eosinophilic leukocytes (10) caused dark staining of these granules, even when no conjugate incubation was performed [Fig. 1*D*]. Sometimes cells that resembled macrophages, both in the medullary region and in germinal centers, exhibited only slight endogenous activity, but stained strongly after all conjugate incubations [Fig. 1*E*]. Since this staining was present in sharply demarcated, round bodies, and mimicked the lysosomes of adjacent sections stained by the Gomori acid-phosphatase technique, it was interpreted as nonspecific binding of glutaraldehyde-treated peroxidase to some lysosomal constituent of the macrophages. Similar nonspecific, but nongranular, staining was sometimes also present in vascular smooth-muscle cells. In general, nodes with many specifically-reactive cells contained fewer eosinophils than were usually seen and only a few nonspecifically-stained macrophages. The macrophages had become much larger and more numerous, and stained more prominently, some 30–60 days after immunization.

Since EAE is generally believed to be a cell-mediated disease, reactive cells were also sought elsewhere. Smears of buffy coat from two bovine BP-treated rabbits on day 8 did not seem to contain any circulating reactive cells. However, in similarly-treated animals with early signs of EAE, positive cells were found in the spinal cord, and they were reactive with both the bovine and rabbit BP conjugates. These cells resembled large lymphocytes and plasma cells, and were present in the perivascular infiltrates of both the lepto-meningeal region [Fig. 2*A*] and the interior of the cord [Fig. 2*B*]. They comprised only a very small percentage of the cellular infiltrates, usually one or at most three reactive cells being identifiable in any cross-section of spinal cord. These reactive cells exhibited all the evidence of specific binding of the BP conjugate demonstrated in the lymph nodes. There was some nonspecific staining of the neuronal Nissl substance and the cytoplasm of fibrous astrocytes, apparently due to affinity for glutaraldehyde-treated peroxidase, but such staining was light with suitably diluted conjugate preparations. An occasional macrophage exhibited the same nonspecific, granular staining seen in the lymph nodes. Comparison with adjacent sections of spinal cord stained with methyl green–pyronin or Wright's stain revealed that many more cells were present with the characteristics of plasma cells or large lymphocytes than exhibited specific binding of BP.

DISCUSSION

The relationship of the specifically BP-reactive cells, demonstrated in this study of EAE, to the immunization with BP seems clear. The purity of the BP preparations and the presence of reactivity in rabbits immunized with either bovine or rabbit BP, as well as with a crude homogenate of bovine brain, make it unlikely that the specific staining represented reactivity to a contaminant. The immunizing dose of bovine BP, as used with adjuvant, was not large for these rabbits, and regularly produced EAE within 30 days, even when lymph nodes were not removed, in some 75% of the treated animals.

The exact nature of the BP-reactive cells and their possible role in pathogenesis, however, remain to be established. It is not clear whether the present method demonstrates only antibody-producing cells (11), or also the cells with specific membrane receptors that are believed to be thymus-derived (12). Possibly, experiments currently underway to demonstrate the ultrastructure of the BP-reactive cells will help elucidate the cell types demonstrated. However, antibody-producing cells have generally been described as plasma cells or large lymphocytes, and the thymus-derived cells as small lymphocytes.

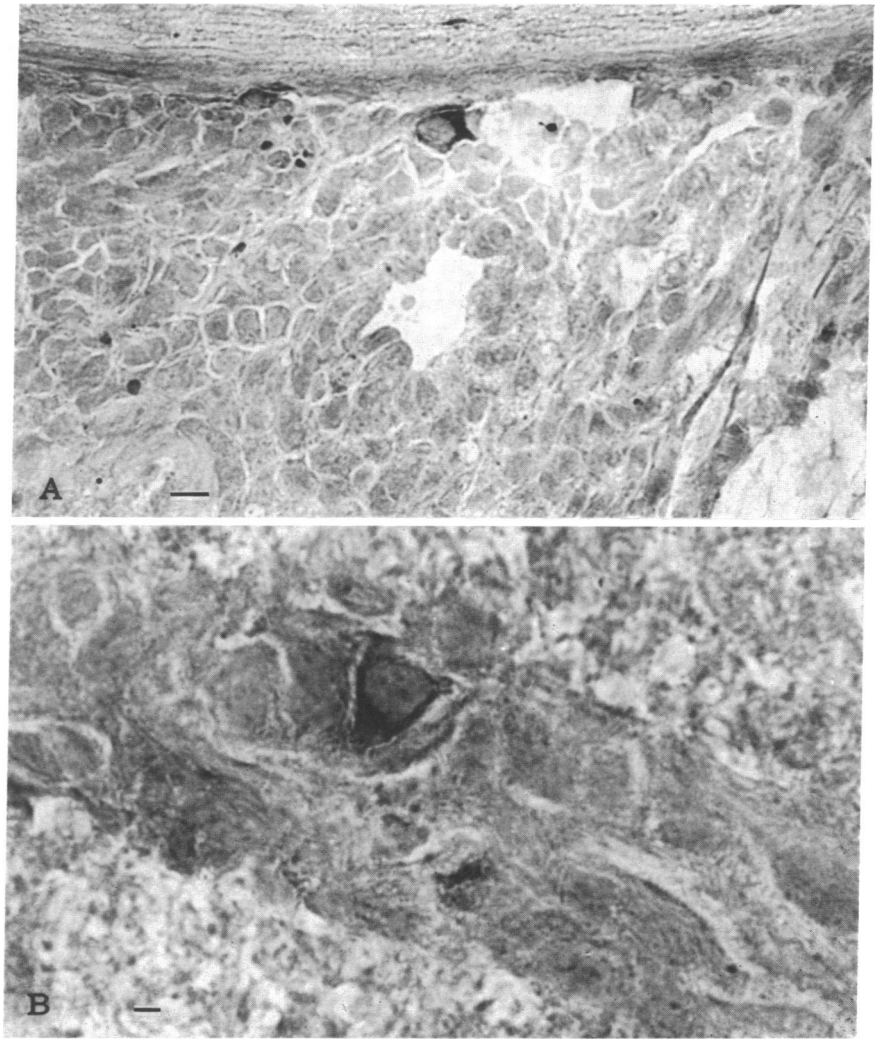

FIG. 2. Spinal cord from a BP-treated rabbit with early signs of EAE. (*A*) Leptomeningeal, perivenous infiltrate containing a single specifically-reactive cell. Day 14; BP–peroxidase conjugate. Scale = 10 μm. (*B*) Specifically-reactive cell in the wall of a vein in the dorsal horn. Day 14; BP–peroxidase conjugate. Scale = 3 μm.

In the lymph nodes, a number of the reactive cells resembled plasma cells, and these cells are most likely the source of humoral anti-BP antibody (13). The role of circulating antibody in the development of the neurological symptoms, however, is uncertain (1–4). In an earlier study by others (14), using immunofluorescent techniques, specific binding of BP to lymph-node cells was also found both in animals that developed EAE and in those that remained asymptomatic. Recent studies (13, 15) have suggested that different sites on the BP molecule may elicit humoral antibody and encephalitogenic activity. Whether the cells reactive with the encephalitogenic site are also demonstrable with the present method is not yet clear. The unidentified reactive node cells may have been such cells, or may have been plasma cell precursors.

In the spinal cord, the BP-reactive cells usually resembled large lymphocytes and plasma cells, an observation that favors the possibility that they were antibody-producing cells. As in the node, however, staining may have represented membrane receptors. Reactive cells were found in the nervous system during the first 24 hr of symptoms, but whether these cells played a role in pathogenesis or were passive participants in the inflammatory response is not certain. This may be resolved by immunization of animals against BP and a second antigen, if the frequency of antigen-binding cells of both types can be determined in the exudates.

If the reactive spinal-cord cells in EAE are antibody-producing cells, the local release of antibody could play a role in pathogenesis. Immunofluorescent techniques have been used to demonstrate the presence of IgG in cells of EAE infiltrates and in myelinated tracts before the development of symptoms, but the specificity of the IgG was not determined (16). Also, peripheral blood cells are capable of producing an *in vitro* demyelinating factor, possibly antibody, before the onset of EAE symptoms (17). The low number of specifically-reactive cells demonstrated in the target organ in the present study is consistent with isotopic studies on passive transfer in delayed hypersensitivity, where the donor cells were found to comprise less than 4% of the infiltrating cells and to resemble large lymphocytes (18, 19). Over 90% of the infiltrating cells belonged to the bone marrow–macrophage line and originated from the recipient. Possible patho-

genetic mechanisms to consider in a role for locally produced antibody are direct effects on antigen sites, such as the proposed 5-hydroxytryptamine receptor (20), opsonization of nearby myelin so that it is tagged for subsequent attack by macrophages (21, 22), alterations of vascular permeability (16, 21, 22), and synergistic or facilitative actions on other cells that participate in the response (23).

The present work has shown the feasibility and specificity of the peroxidase-labeled BP method in the study of EAE. This method has the advantage over immunofluorescence that it is potentially applicable at the ultrastructural level. Similar techniques should also be helpful in the study of other immune states and diseases. In particular, use of the BP-peroxidase conjugate may prove fruitful in the investigation of other demyelinating diseases, especially multiple sclerosis.

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1. Alvord, E. C., Jr., in *Handbook of Clinical Neurology; Multiple Sclerosis and Other Demyelinating Diseases*, ed. P. J. Vinken and G. W. Bruyn (North-Holland, Amsterdam, 1970), p. 500.
2. Bornstein, M. B., in *Textbook of Immunopathology*, ed. P. A. Miescher and H. J. Muller-Eberhard (Grune and Stratton, New York, 1969), Vol. II, p. 507.
3. Levine, S., E. M. Hoenig, and M. W. Kies, *Science*, **161**, 1155 (1968).
4. Paterson, P. Y., in *Annual Review of Medicine*, ed. A. C. DeGraff and W. P. Creger (Banta, U.S.A., 1969), p. 75.
5. Avrameas, S., *Immunochemistry*, **6**, 43 (1969).
6. Eylar, E. H., J. Salk, G. C. Beveridge, and L. V. Brown, *Arch. Biochem. Biophys.*, **132**, 34 (1969).
7. Avrameas, S., and T. Ternynck, *Immunochemistry*, **6**, 53 (1969).
8. Graham, R. C., Jr., and M. J. Karnovsky, *J. Histochem. Cytochem.*, **14**, 291 (1966).
9. Turk, J. L., *Brit. Med. Bull.*, **23**, 3 (1967).
10. Behnke, O., *J. Histochem. Cytochem.*, **17**, 62 (1969).
11. Avrameas, S., and E. H. Leduc, *J. Exp. Med.*, **131**, 1137 (1970).
12. Modabber, F., S. Morikawa, and A. H. Coons, *Science*, **170**, 1102 (1970).
13. Lennon, V. A., A. V. Wilks, and P. R. Carnegie, *J. Immunol.*, **105**, 1223 (1970).
14. Rauch, H. C., and S. Raffel, *Ann. N.Y. Acad. Sci.*, **122**, 297 (1965).
15. Burnett, P. R., and E. H. Eylar, *J. Biol. Chem.*, **246**, 3425 (1971).
16. Oldstone, M. B. A., and F. J. Dixon, *Amer. J. Pathol.*, **52**, 251 (1968).
17. Dowling, P. C., and S. D. Cook, *Neurology*, **18**, 953 (1968).
18. McCluskey, R. T., B. Benacerraf, and J. W. McCluskey, *J. Immunol.*, **90**, 466 (1963).
19. Najarian. J. S., and J. D. Feldman, *J. Exp. Med.*, **118**, 341 (1963).
20. Carnegie, P. R., *Nature (London)*, **229**, 25 (1971).
21. Lampert, P., *Acta Neuropathol.*, **9**, 99 (1967).
22. Wiśniewski, H., J. Prineas, and C. S. Raine, *Lab. Invest.*, **21**, 105 (1969).
23. MacLennan, I. C. M., and B. Harding: *Immunology*, **18**, 405 (1970).