

Precipitating antibody in multiple sclerosis and experimental allergic encephalomyelitis

Specific binding of radio-iodinated encephalitogenic factor

E. A. CASPARY

From the Medical Research Council, Demyelinating Diseases Research Unit, Newcastle upon Tyne

Numerous studies of circulating antibody to brain and brain derivatives have been made in experimental allergic encephalomyelitis and multiple sclerosis with the intention of demonstrating some correlation between disease and antibody titre (Kies and Alvord, 1959). Kibler and Barnes (1962) have investigated antibody production in experimental allergic encephalomyelitis induced in the rabbit by the injection of a 'water-soluble derivative' of spinal cord, using the quantitative ammonium sulphate precipitation with radio-iodinated antigen method of Farr (1958), and showed that antibody developed early in animals which subsequently developed the disease, though there was no correlation between titre and severity. Since there has been a recent revival of interest in the possible role of circulating antibody in multiple sclerosis and experimental allergic encephalomyelitis the present study was undertaken with an encephalitogen of human origin (Caspary and Field, 1965). To validate the method a group of guinea pigs in which experimental allergic encephalomyelitis had been induced with human brain was also studied to determine their response to this antigen.

METHODS

HUMAN ENCEPHALITOGEN This was prepared by the method described by Caspary and Field (1965), freeze-dried and stored as the dried powder at 4°C.

RADIO-IODINATION OF HUMAN ENCEPHALITOGEN In early experiments 5 mg. of human encephalitogen was labelled with 4 mc. of ¹³¹I following the method of Talmage, Dixon, Bukantz, and Dammin (1951) with the concentration of carrier KI reduced by a factor of 10 to avoid denaturation. Excess reagent was removed by passage through a Sephadex G 25 column followed by dialysis against normal saline until the dialysis fluid counts approximated to background. This procedure resulted in some denaturation, and insoluble material was removed by centrifugation before the final radio-iodinated human encephalitogen was stored at 4°. Protein concentration

was estimated from the extinction at 280 m μ . In later experiments more satisfactory labelling with less denaturation was obtained by the following method. To 3 mg. of human encephalitogen dissolved in 2 ml. of borate buffer (pH 8, 0.1 M) was added 2.5 mc. of ¹³¹I and 0.1 ml. of 0.4% chloramine T at 4°C. After 20 sec. in the dark, 0.1 ml. of 0.2% sodium metabisulphite was added and the preparation dialysed first against N/100 HCl, to neutralize the alkaline buffer, and then against normal saline until radioactivity in the dialysis fluid approximated that of the normal background.

The human encephalitogen-¹³¹I was examined by paper electrophoresis in veronal buffer pH 8.6 for 11 hr. at 1.5 mA and the paper strips analysed in a strip scanner (Nuclear, Chicago).

SERUM Specimens of human serum were stored before use at -60°C. for periods not exceeding eight weeks. Guinea-pigs were bled at the requisite intervals by cardiac puncture and the serum similarly stored.

IMMUNIZATION OF GUINEA-PIGS Eight adult Hartley strain guinea-pigs of both sexes and weighing 300-400 g. were immunized by a single intradermal injection of 0.1 ml. in one foot. The encephalitogenic mixture was 20% (w/v) normal human brain in saline homogenized with Freund's complete adjuvant (Difco) in the proportion of 1:2. Animals were weighed and examined daily and finally sacrificed after 30 days. Diagnosis was confirmed by histology of the brain and cord in each case and disease index scored out of a maximum of 5 according to the method of Kies, Goldstein, Murphy, Roboz, and Alvord (1957).

A further small group of animals was given three injections intradermally at weekly intervals of either human encephalitogen in incomplete adjuvant (five animals) or of complete Freund's adjuvant (Difco) alone (three animals) before challenge with encephalitogenic mixture.

ANTIBODY PRECIPITATION TEST All sera were diluted 1 in 10 in borate buffer pH 8.3, 0.1M before test; dilutions of the antigen were made in the same buffer containing 1% normal rabbit serum. The antigen (human encephalitogen-¹³¹I) was used at a concentration of 10 μ g./ml. with a

count of approximately 500/sec. Subsequent adjustments of concentration were made using the radioactivity in conjunction with a decay curve.

One ml. of the serum dilution was incubated at 4°C. with 0.2 ml. (2 µg.) of human encephalitogen-¹³¹I for 16 hours in a clean plastic disposable tube, and 1.2 ml. of 70% saturated ammonium sulphate added rapidly with a syringe; the tubes were shaken vigorously and then allowed to stand for 30 minutes at 4°C. Thus the final concentration of ammonium sulphate used to precipitate the human encephalitogen-antibody complex was only 35%. This was done in order to reduce precipitation of uncombined human encephalitogen as might well occur at the concentration employed by Kibler and Barnes (1962). The resulting fine precipitate was separated by spinning at 7,500 r.p.m. in a Spinco preparative ultracentrifuge (model L 2) for 15 minutes at 4°C., the clear supernatant being decanted and the tubes allowed to drain for 30 min. over filter paper with tapping at fixed intervals to dislodge drops of fluid adhering to the walls of the tube. Radioactivity was measured in a well-type scintillation counter.

In the comparison of groups of normal and multiple sclerotic patients or of normal and experimental animals, results have been expressed as the difference between the amount of antigen bound by the normal and by the pathological sera expressed in micrograms of human encephalitogen. Serial estimations in guinea-pigs are shown in arbitrary units.

RESULTS

The electrophoretic pattern of the radio-iodinated

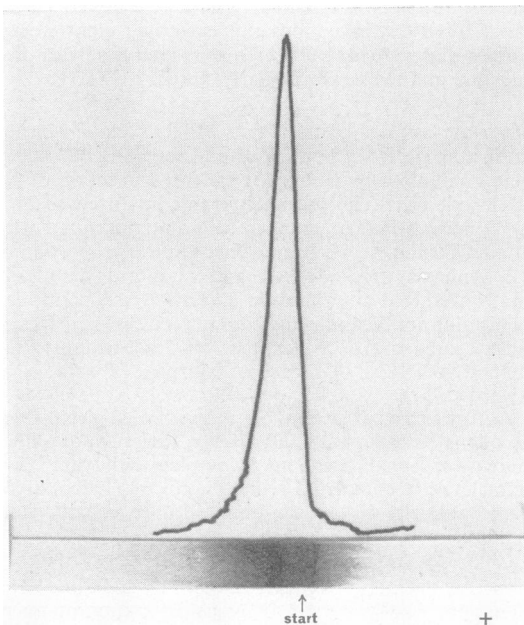


FIG. 1. Paper electrophoresis and radioactivity scan of radio-iodinated encephalitogenic factor (EF-¹³¹I).

human encephalitogen used in these tests is shown in Fig. 1. Protein staining and radioactivity scan were coincident showing a single component moving towards the cathode with a mobility approximately half that of the unmodified human encephalitogen. That the preparation had not been extensively hydrolysed into polypeptides or other small protein fragments was shown by the fact that 80% of the total radioactivity could be precipitated by trichloroacetic acid.

From Fig. 2a it is apparent that when a pronounced antibody response occurs it does so around the time of onset of the disease (11-17 days), though the response was slow in one case and in another the titre rose slightly and then fell. This last animal died of experimental allergic encephalitis before completion of the experiment and this, in conjunction with the severity of disease in the others, suggests that antibody response does not necessarily correlate with occurrence or severity. Figures 2b and 2c show that five animals pre-immunized with human encephalitogen gave the expected marked response 18 days after challenge, whilst three receiving Freund's complete adjuvant alone gave little or none, though the initial bound antigen level was raised above normal in both pre-immunized groups. The method may, therefore, be accepted as offering a measure of antibody response to the inoculated brain.

Antigen specifically bound by serum from a group of patients with active multiple sclerosis (12) together with controls (14) and also from a group of guinea-pigs with experimental allergic encephalitis (8) with controls (11) is shown in Table I. Since all sera bind human encephalitogen to some extent (Kibler and Barnes, 1962), possibly due to the highly charged nature of the basic protein, it is necessary to

TABLE I

HUMAN ENCEPHALITOGEN-¹³¹I BOUND BY MULTIPLE SCLEROSIS SERUM AND SERUM FROM GUINEA-PIGS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

	Human Encephalitogen- ¹³¹ I (µg.)	Significance
Multiple sclerosis vs. controls	(15) (14) 6.4 (S.D. 14.5) ¹	0.3 > P > 0.2
Experimental allergic encephalomyelitis vs. controls	(8) (11) 32.1 (S.D. 13.6) ¹	P < 0.001

¹Pooled standard deviations

compute specific binding from the statistical means of the results found in patients and controls. The high values of the standard deviation indicates the relative variability of this test. In multiple sclerosis

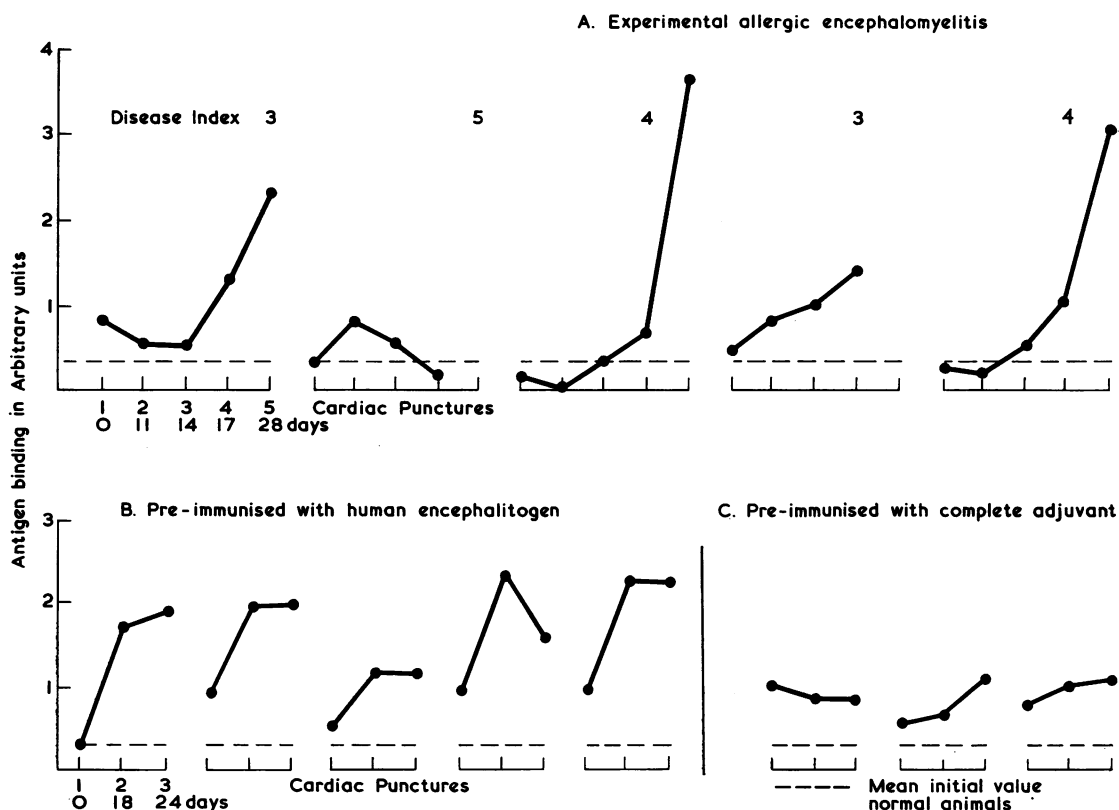


FIG. 2. Serial antibody titres in experimental allergic encephalomyelitis animals (A)¹, animals pre-immunized with human encephalitogen (B) and animals pre-immunized with Freund's complete adjuvant (C). Severity of disease is indicated by the disease index (A) and was less than 2 for the other groups (B) and (C).

¹Cardiac puncture no. 5 not tested in the second and fourth animals.

binding is seen not to be significant. In the animal series, however, the difference is marked.

DISCUSSION

In view of the demonstration of a myelotoxic principle in the serum in experimental allergic encephalitis and in active multiple sclerosis (Bornstein and Appel, 1961, 1965) particular interest attaches to antibodies capable of combining specifically with an active encephalitogen of human origin. These have been demonstrated by tanned red cell agglutination in multiple sclerosis and other degenerative conditions of the central nervous system (Field, Caspary, and Ball, 1963) and also in experimental allergic encephalitis in the guinea-pig (Caspary and Field, 1965). In experimental allergic encephalitis antibody of precipitating type, however, has not been detected either by double diffusion in agar or by classical quantitative precipitation methods (Kibler

and Barnes, 1962; Caspary and Field, 1965), though Alvord (1965) has reported multiple precipitin lines against heterologous encephalitogen and at least one against the homologous protein.

The Farr technique measures antigen combined with antibody and carried down by ammonium sulphate precipitation. It is in effect a precipitation method of high sensitivity. The absolute amount of antigen bound is a function of the 'valency' of the antibody, *i.e.*, the number of antigen molecules it will combine with, but detection by this method is not limited to the insoluble complexes of antigen and antibody in the equivalence zone commonly demonstrated by classical precipitation methods.

Serial antibody estimations in guinea-pigs with experimental allergic encephalitis with human encephalitogenic factor gave results comparable with those obtained in rabbits by Kibler and Barnes (1962) though they used an antigen of lower biological activity extracted from rabbit spinal cord. Their

encephalitogen comprised three basic proteins on electrophoresis and only 55% of the activity of the radio-iodinated preparation was precipitated as protein. The antigen used in the present study was homogeneous on paper electrophoresis and in the ultracentrifuge, but several minor components could be shown by electrophoresis on acrylamide. Of this preparation, 80% of the activity of the radio-iodinated encephalitogen was precipitable as protein. The present work, using the more refined encephalitogen of human origin, supports the suggestion that antibody measured by Farr's (1958) method is directed against the active encephalitogenic basic protein and not against some of the minor impurities.

The experiments in guinea-pigs pre-immunized with human encephalitogen in incomplete adjuvant show a true secondary antibody response, but those pre-injected with Freund's complete adjuvant alone gave little or no secondary response. Both of these groups, however, had slightly raised initial levels of antibody and were also protected against disease.

This absence of secondary response is surprising in view of the findings by Field *et al.* (1963) which suggested some shared antigenic determinants between human encephalitogen and mycobacterial products. Moreover, further (unpublished) studies have shown a well-marked agglutinating antibody response to brain following pre-immunization with either human encephalitogen or Freund's complete adjuvant (*M. butyricum*).

In multiple sclerosis precipitating antibody has been shown against an enzyme digest of white matter (Ross, 1962; Ritzel, Wüthrich, and Rieder, 1963), but this material contains lipid and mucoproteins and is not encephalitogenic. Enzyme digests of brain have been shown to share antigens with a number of other tissues (Caspary and Field, 1963). They were unable to find precipitating brain specific antibodies in the serum of patients with multiple sclerosis claimed by Ritzel *et al.* (1963). The present study again failed to show significant precipitating antibody in multiple sclerosis using as antigen the encephalitogenic basic protein of human origin, *i.e.*, the putative auto-allergen. Experimental allergic encephalomyelitis in the guinea-pig, as in the rabbit, gives a significant antibody response. It is suggested that the weak antibodies detectable only by the most sensitive methods, found in multiple sclerosis and other degenerative disorders of the nervous system, occur as a consequence of breakdown of myelin and release of antigen to the reticulo-endothelial system, and may have little significance for the pathogenesis of these diseases.

SUMMARY

An encephalitogenic basic protein of human origin was labelled with radio-iodine and shown to retain electrophoretic homogeneity. Precipitating antibody to this antigen was demonstrated in guinea-pigs with allergic encephalomyelitis; serial determinations showed the maximum response between two and three weeks after challenge.

A true secondary response was obtained from animals pre-immunized with encephalitogen but not from those pre-injected with Freund's complete adjuvant. Both these groups were protected against challenge with encephalitogenic mixtures.

A group of patients with active multiple sclerosis showed no significantly increased precipitating antibody titre.

The possible significance of these findings is briefly discussed.

I wish to thank Dr. E. J. Field for his constant advice and encouragement in this work.

REFERENCES

- Alvord, E. C. Jr. (1965). Research in demyelinating diseases. (Discussion of paper by Caspary and Field). *Ann. N.Y. Acad. Sci.*, **122**, 195.
- Bornstein, M. B., and Appel, S. H. (1961). The application of tissue culture to the study of experimental 'allergic' encephalomyelitis. 1. Patterns of demyelination. *J. Neuropath. exp. Neurol.*, **20**, 141-157.
- , — (1965). Tissue culture studies of demyelination. *Ann. N.Y. Acad. Sci.*, **122**, 280-286.
- Caspary, E. A., and Field, E. J. (1963). Immunological examination of the soluble antigens of human brain and their modification by proteolytic enzymes. *Dtsch. Z. Nervenheilk.*, **184**, 478-486.
- , — (1965). An encephalitogenic protein of human origin; some chemical and biological properties. *Ann. N.Y. Acad. Sci.*, **122**, 182-198.
- Farr, R. S. (1958). A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J. infect. Dis.*, **103**, 239-262.
- Field, E. J., Caspary, E. A., and Ball, E. J. (1963). Some biological properties of a highly active encephalitogenic factor isolated from human brain. *Lancet*, **2**, 11-13.
- Kibler, R. F., and Barnes, A. E. (1962). Antibody studies in rabbit encephalomyelitis induced by a water-soluble protein fraction of rabbit cord. *J. exp. Med.*, **116**, 807-825.
- Kies, M. W. and Alvord, E. C. Jr. (1959). 'Allergic' Encephalomyelitis. Thomas, Springfield, Illinois.
- , Goldstein, N. P., Murphy, J. B., Roboz, E., and Alvord, E. C. Jr. (1957). Occurrence of lipemia in experimental allergic encephalomyelitis. *Neurology (Minneapolis)*, **7**, 175-188.
- Ritzel, G., Wüthrich, R., and Rieder, H. P. (1963). Zur Frage der Antikörper gegen Nervengewebe in Serum und Liquor von Multiple-Sklerose-Kranken. *Schweiz. med. Wschr.*, **93**, 1336-1339.
- Ross, J. (1962). Über den Nachweis von Autoantikörpern gegen Hirnextrakte im Serum von Kranken mit multipler Sklerose. *Verh. dtsch. Ges. inn. Med.*, **68**, 566-568.
- Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J. (1951). Antigen elimination from the blood as an early manifestation of the immune response. *J. Immunol.*, **67**, 243-255.