Immunofluorescent Study of Circulating Antibody in Experimental Allergic Encephalomyelitis[1] (34653)

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The study of experimental allergic encephalymyelitis (EAE) induced by injection of either whole CNS tissue or its encephalitogenic fraction (EF) with Freund's complete adjuvant (FCA) has suggested the existence of circulating antibodies in this disease. It is not known whether the role of the antibodies associated with this disease is pathogenic or protective, nor is it agreed which serum fraction contains these antibodies.

Some of the difficulties in the analysis of circulating antibodies in EAE derive from technical factors. Thus, experiments employing different animal species have given different results. For instance, IgA has been shown to posses myelinolytic properties in rats but not in rabbits (1, 2).

In tissue culture experiments, various reports have implicated IgG, IgA, and IgM as the circulating demyelinating factor (1–4). Further, the specificity of action of these antibodies in tissue culture has been questioned.

Also, experiments using EF as antigen have not confirmed the results of those using whole CNS. For example, precipitating antibody can be demonstrated using immunodiffusion when whole CNS is used as antigen but not if EF is used (5). Similarly, complement fixing antibodies cannot be demonstrated in the sera of animals with EAE when EF is used as antigen (6). They can be demonstrated using ethanol extracts of whole brain as antigen but such extracts do not contain encephalitogenic material (7).

Sherwin, using immunofluorescent methods, demonstrated myelin binding antibodies in the sera of rabbits with EAE (8). Also, Rauch and Raffel localized the encephalitogenic fraction to the myelin sheath in the guinea pig employing the fluorescent antibody technique (9).

This paper describes the results obtained using purified encephalitogenic protein as the immunogen to establish which serum fraction contains antimyelin antibody in the acute phase of the disease in the guinea pig.

Methods. Animals. Hartley albino guinea pigs, weighing 400 to 600 g, were obtained from West Jersey Biological Supply.

Disease production. Encephalitogenic protein was prepared from guinea pig brain as described by Kies (10). Ten guinea pigs were injected in the foot pad with 0.05 mg of protein in 0.1 ml of FCA. Animals were sacrificed by exsanguination at the onset of severe paralysis usually occurring between 16 and 18 days after injection. Control serum was obtained from 10 untreated animals and from 10 guinea pigs injected with FCA alone. The serum from each animal was tested individually.

Protein fractionation. The globulin fraction of the serum was precipitated volume to volume with 36% sodium sulfate giving a final concentration of 18% sodium sulfate. The precipitate was washed twice with 18% sodium sulfate and then dialyzed against 0.03 M Tris, pH 8.5, or phosphate buffered saline (PBS), pH 7.2, when this fraction was to be used directly for immunofluorescent studies. The globulin fraction was separated on a DEAE-cellulose column 1 × 22 cm using both pH and salt gradient. The starting buffer was 0.03 M Tris, pH 8.5, and the final buffer was 0.4 M Tris, pH 4.0. The pooled tubes

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constituting one fraction were dialyzed against 0.15 M sodium chloride and lypholyzed. For use, the serum fractions were reconstituted at a concentration of 2.0 mg of protein/ml.

Immunoelectrophoresis. IEP was performed on microscope slides using the micromethod of Scheidegger (11) in 1% agar with barbital buffer 0.05 M, pH 8.6. The slides were run for 50 min at 8 V/cm. Rabbit antiguinea pig serum was obtained from Hyland Laboratories and was found to bind equally well with each globulin fraction.

Compelment fixation. Complement fixation tests were carried out by the 50% hemolysis assay (12). Samples of antibodies containing 0.1 mg of protein were incubated for 45 min at 37° with 1 mg of brain homogenate and four 50% hemolytic units of guinea pig complement obtained from Hyland Laboratories. The amount of complement fixation was tested by incubating the suspension for 40 min with sheep erythrocytes sensitized with homologous rabbit antibody. The degree of hemolysis was measured spectrophotometrically at 540 mμ.

Passive cutaneous anaphylaxis assay. PCA assay was performed using adult Hartley guinea pigs. The globulin fractions were adjusted to a concentration of 1 mg/ml. 0.1 ml of each antibody fraction was injected into the shaved back of the test animals. 2.5 mg of Evans blue and 0.1 mg of EF in a total column of 1 ml was injected intravenously 6 hr following the intradermal injections. The diameter of the blue area of each test site was measured 15 min and 1 hr following injection.

TABLE I. Fluorescent Antimyelin Antibody Activity in Normal, FCA-Treated, and EAE Guinea Pig Serum.

— = absent; + = present; 10 animals were used in each group. The serum from each animal in all groups was tested individually.

Serum	FITC antiserum	Myelin fluorescence
Normal GP serum	Anti-GP serum	—
FCA-treated GP serum	Anti-GP serum	—
EAE GP serum	Anti-GP serum	+
	Antirabbit serum	—
	PBS	—
PBS	Anti-GP serum	—
Na sulfate ppt EAE serum	Anti-GP serum	+

Immunofluorescence. Immunofluorescence was studied on sections of fresh normal guinea pig cord which had been quick frozen and cut at 5 μ on a freezing cryostat. The sections were air dried for 30 min at room temperature, incubated for 30 min at room

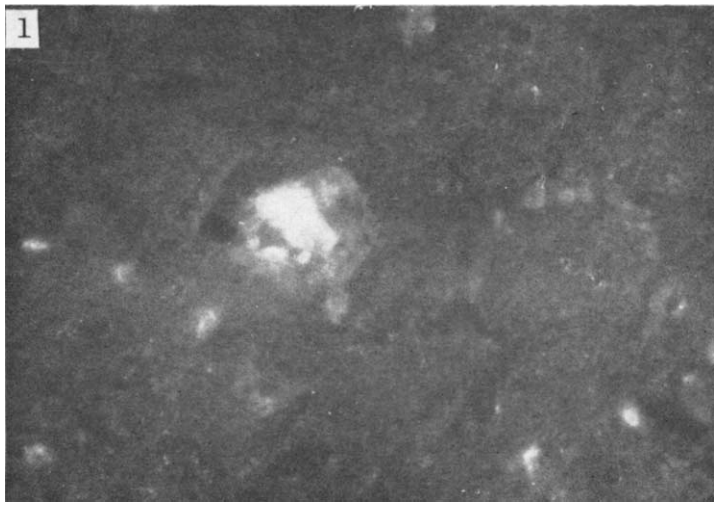

FIG. 1. Photomicrograph of section of guinea pig cord treated with normal guinea pig serum and FITC conjugated antiguinea pig serum showing nonspecific fluorescence of vessel; ×300.

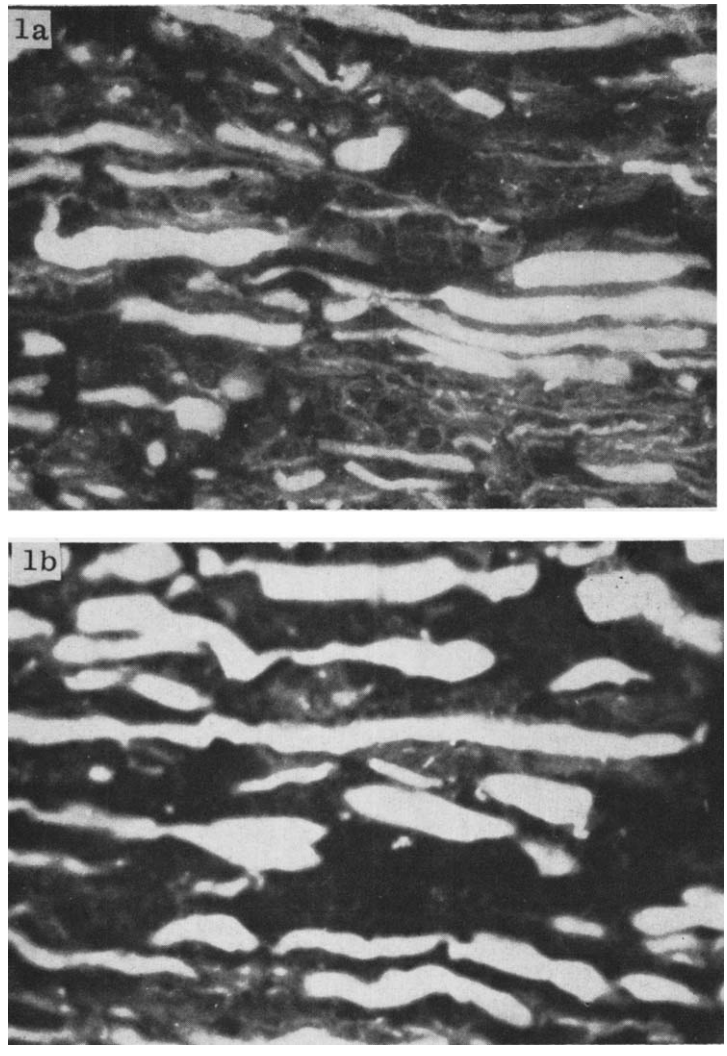

FIG. 1a. Section treated with EAE serum and FITC conjugated antiguinea pig serum showing specific myelin fluorescence; ×300. 1b. Section treated with fraction A of EAE serum and FITC conjugated antiguinea pig serum showing specific myelin fluorescence; ×300.

temperature with the serum or fraction to be studied and then washed twice for 5 min in PBS. They were then incubated for 30 min with FITC conjugated rabbit antiguinea pig serum (obtained from Hyland Laboratories), washed twice in PBS, and mounted in glycerol–PBS (90–10%).

Microscopy. Microscopy was performed on a Zeiss photomicroscope using excitatory filters BG 38 and BG 12 and barrier filters 44 and 53. Photography was performed using Kodak Tri X panchromatic film.

Results. Fresh tissue sections from normal guinea pigs were treated with serum from normal guinea pigs, FCA-treated guinea pigs and EAE animals and subsequently incubated with FITC antiguinea pig serum. The results are shown in Table I. Nonspecific fluorescence associated with blood vessels was seen in all sections (Fig. 1). As shown, positive fluorescence of the myelin sheath was obtained only with EAE sera and the globulin fraction of this sera (Fig. 1a). Each of the 10 sera tested produced positive fluores-

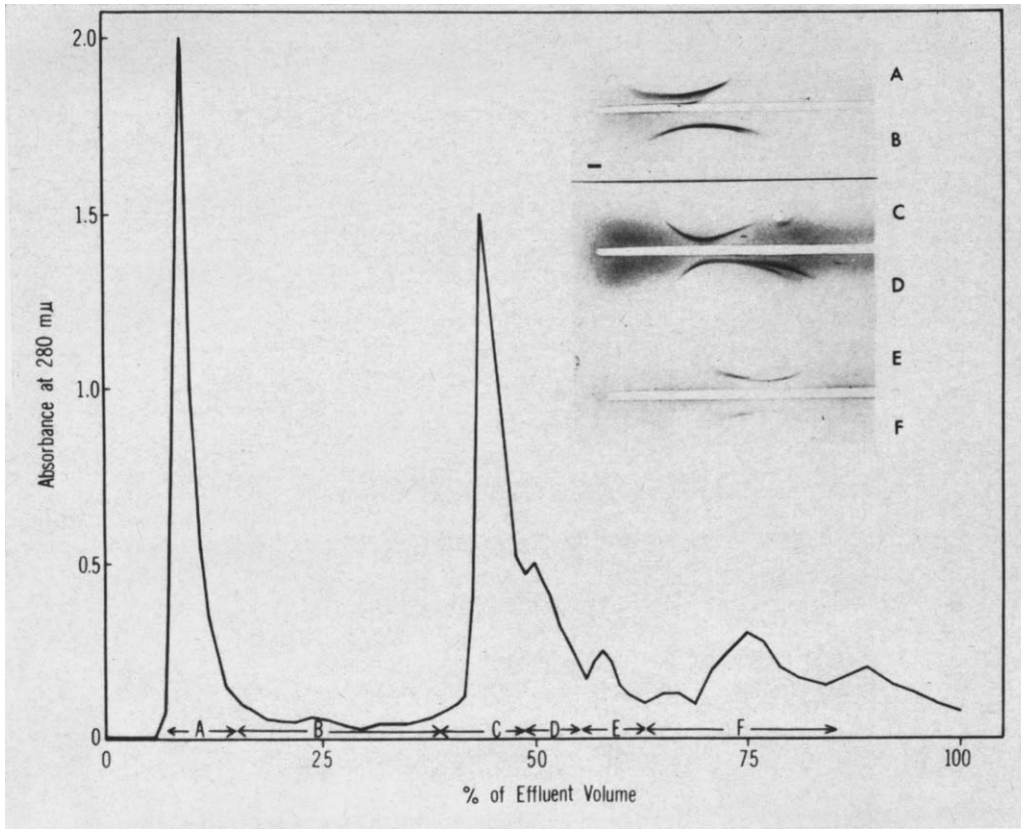

FIG. 2. Column fractionation of guinea pig EAE serum in DEAE-cellulose with pH-salt gradient starting buffer 0.015 M Tris, pH 8.5; final buffer 0.4 Molar Tris, pH 4.0. Immunoelectrophoresis patterns of guinea pig globulin fractions separated on DEAE-cellulose. Troughs contain antiguinea pig serum.

cence. After absorption of the EAE serum with purified encephalitogenic protein only nonspecific fluorescence was observed.

Control experiments using conjugated antirabbit serum in place of conjugated antiguinea pig serum or omitting the guinea pig serum prior to the application of conjugated antiguinea pig serum showed only nonspecific fluorescence as shown in Fig. 1.

In order to determine which immunoglobulin fraction or class contains antibody to myelin, EAE serum was fractionated on DEAE-cellulose using pH-salt gradient (Fig. 2). This resulted in the recovery of two major and one smaller and less distinct peak. Effluents under these peaks were pooled separately and labeled fractions A, C, and F. The effluent between these peaks were also pooled separately and labeled fractions B, D, and E.

Immunoelectrophoresis of the various fractions against rabbit antiguinea pig serum revealed fractions A and C to consist of slow and fast migrating gamma globulin, respectively. Since it has been shown that 7S gamma$_2$ can be distinguished from the 7S gamma, fraction of guinea pig gamma globulin by its ability to fix complement (13), fractions A and C were studied with complement fixation tests. Only fraction A was found to have significant complement fixing properties (Table II) indicating that this fraction is the 7S gamma$_2$ antibody. The various fractions were further studied by PCA assay. Fractions A through F failed to give a positive PCA reaction. The electrophoretic mobil-

TABLE II. Complement Fixation Employing 4 $C'H_{50}$ Units with 1 mg of Lypholized Guinea Pig Brain as Antigen.

Fraction	Antibody added (mg)	$C'H_{50}$ fixed (%)
A	0.1	88
C	0.1	11

ity and lack of complement fixing properties of fraction C are consistent with the 7S $gamma_1$ antibody. Fraction E was found to consist of some fast migrating gamma globulin and a faster migrating fraction, possibly IgM.

When fraction A was used in immunofluorescence studies the myelin showed the same degree of fluorescence as when using the whole EAE serum or globulin fraction (Fig. 1b). Fractions B through F failed to produce the specific myelin fluorescence. When fraction A was absorbed with the purified EF, it failed to show the fluorescence of the myelin sheaths (Table III). Absorption of the FITC-rabbit antiguinea pig serum with fractions B and C did not reduce the fluorescence while absorption with fraction A eliminated the fluorescence.

Discussion. These results show that, during the acute stage of EAE, guinea pigs produce a circulating antibody against myelin. This antimyelin antibody is present in the globulin fraction prepared by precipitation of the serum with sodium sulfate. It appears to reside in the 7S $gamma_2$ fraction, when fractionated by DEAE-cellulose. The fluorescent reaction is abolished by absorption of the serum or 7A $gamma_2$ fraction with purified EF, indicating the specificity of this antibody for EF. The presence of this antibody in the 7S $gamma_2$ fraction is consistent with the reports of 7S IgG demyelinating antibodies in rabbits (1). In rabbits distinction between $gamma_2$ and $gamma_1$ fractions of IgG has not been made to date.

TABLE III. Fluorescent Antimyelin Antibody Activity in Globulin Fractions of EAE Serum.

Fraction	Amount (mg/ml)	FITC antiserum	Myelin fluorescence
Fraction A	2	Anti-GP serum	+
B	2	Anti-GP serum	—
C	2	Anti-GP serum	—
D	2	Anti-GP serum	—
E	2	Anti-GP serum	—
F	2	Anti-GP serum	—
Fraction A & purified EF	2	Anti-GP serum	—
Fraction A	2	Anti-GP serum abs with fractions B & C	+
Fraction A	2	Anti-GP serum abs with fraction A	—

It is interesting to note that while 7S $gamma_1$ antibody can be demonstrated in the acute EAE sera of guinea pigs by immunoelectrophoresis as the fast migrating fraction this fraction does not give a PCA reaction using EF as antigen. Falk *et al.* (14) have shown that the fast migrating fraction in the sera of animals protected by injection of EF prior to the encephalitogenic dose of EF and FCA does give a PCA reaction in the chronic stage.

Summary. Specific myelin binding antibodies have been demonstrated in the serum and globulin fractions of guinea pigs with EAE. This myelin binding antibody is present only in the 7S $gamma_2$ fraction and appears to be specific for the encephalitogenic fractions of myelin since the immunofluorescence can be inhibited by absorption of the 7S $gamma_2$ fraction with purified EF.

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