ANTIBODY STUDIES IN RABBIT ENCEPHALOMYELITIS INDUCED BY A WATER-SOLUBLE PROTEIN FRACTION OF RABBIT CORD*, ‡

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Experimental "allergic" encephalomyelitis (EAE) was placed firmly on immunologic grounds by the sensitized lymph cell transfer studies of Paterson (2) and of Stone (3). Since sensitized cells may produce circulating antibodies after transfer (2, 4), as well as participate in hypersensitivity reactions, the pathogenesis of EAE remains unsettled. Previous studies of antibody formation in EAE have sought to show a relationship between the level of circulating antibody and the incidence of disease. In general, consistent correlations have not been found (5–7). However, interpretation of the data presented by such studies is complicated by the fact that multipotential antigens such as whole brain and spinal cord have been used to induce the disease and crude extracts of these same tissues have been employed in the *in vitro* detection and measurement of the antibody. The lack of a positive correlation between the incidence of EAE and antibody titers under such circumstances, therefore, might have been due to the presence of a variety of non-encephalitogenic antibodies. Efforts to identify the agent responsible for EAE have led recently to the isolation of a number of water-soluble protein fractions of nervous tissue with a high degree of encephalitogenic activity in guinea pigs (8). Although these fractions are not homogeneous as yet, they are of greater chemical, and presumably greater immunologic, purity than the whole tissue preparations previously used and, accordingly, less likely to induce as wide a variety of concomitant, non-encephalitogenic antibody. Preliminary studies have shown that of the various techniques explored, the ammonium sulfate precipitation method of Farr (9) was most suitable for the quantitative measurement of antibody to the water-soluble fraction of nervous tissue employed in these studies.

In the present study, therefore, the ammonium sulfate precipitation method was adapted to measure the antibody induced in rabbits by the injection of a

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water-soluble, encephalitogenic fraction of rabbit cord. Since many of these rabbits developed EAE, the relationships between certain immunologic and clinical aspects of the disease are examined and reported.

Materials and Methods

Preparation of the Water-Soluble Fraction of Rabbit Cord.—A water-soluble fraction of rabbit spinal cord (WSF) was prepared by a modification of "Method III" described by Roboz and Henderson (10).

The spinal cords from freshly killed, adult, albino rabbits were stripped of their meninges, homogenized in distilled water, and lyophilized. Approximately 30 gm of the dry cord was extracted twice with 300 ml of acetone and once with 300 ml of ether for 24 hours at −7°C with constant stirring. The residue from the ether extraction was lyophilized for 10 minutes to remove the excess ether and then extracted with 300 ml of 0.5 M sodium acetate for 24 hours at 4°C with constant stirring. This extract was discarded and 300 ml of 0.1 M sodium citrate added to the residue. This mixture was adjusted to pH 4.3 and stirred constantly for 48 hours at 4°C. After centrifugation the supernate still retained some material in colloidal suspension which precipitated out upon dialysis against distilled water for 6 to 12 hours and could then be removed by centrifugation. Dialysis of the supernate was continued for a total of 72 hours and the volume of the extract reduced to 50 ml by evaporation at 4°C. Lyophilization yielded approximately 150 mg of a white powder.

Radioiodination of the Rabbit Cord WSF.—Two mg of the WSF was dissolved in 0.5 ml of distilled water and labeled with 5 mc of I^{131} according to the method of Talmage *et al.* (11). Since 10 to 25 mg of protein are usually iodinated by this technique, the concentration of the carrier KI was changed from 0.01 M to 0.001 M to prevent excessive iodination and subsequent denaturation of the protein. The solution of I^{131}-labeled water-soluble fraction (I*WSF) was first passed through an amberlite resin column[1] and then dialyzed against a continuous flow of 24 liters of 9:1 normal saline borate buffer and stored at 4°C. I*WSF with low specific activity was prepared by labeling 2 mg of antigen with 25 μc of I^{131} by the same technique. This material was dialyzed in distilled water, lyophilized, and used to determine the effect of iodination on the electrophoretic pattern and on the encephalitogenic activity of the antigen.

Characterization of the Rabbit Cord WSF.—Paper[2] electrophoresis of the unlabeled WSF was carried out in veronal buffer, pH 8.6 at 2.5 milliamps for 17 hours. The strips were dried, stained with brom-phenol blue, and analyzed in the analytrol.[3] The WSF labeled with 25 μc of I^{131} was studied in a similar manner and, in addition, the stained strips were then cut transversely into 0.5 cm segments and each segment counted individually in a well-type scintillation counter.[4]

The solubility of the water-soluble fraction in 10 per cent trichloroacetic acid (TCA) was tested by the addition of 1.0 ml of 20 per cent TCA to a mixture of 0.5 ml portions of the I*WSF and 1:10 normal rabbit serum borate buffer. The whole was incubated for 30 minutes at 4°C, centrifuged, decanted, and radiocounted. The radioactive counts in the precipitate were expressed as a per cent of the total counts added.

A nitrogen determination was performed on the antigen by the microanalytical colorimetric (Nessler) method described by Lanni *et al.* (12).

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[1] Rezikit, E. R. Squibb & Sons, Cleveland, Ohio.

[2] Filter paper strips, Beckman No. 320046.

[3] Spinco, Model RB analytrol.

[4] Radioactive counting was accomplished in a well-type, gamma scintillation counter (Hamner Electronics Co. Princeton).

Immunization of Rabbits with the Rabbit Cord WSF.—Adult male, albino rabbits which weighed between 2 and 3 kilos (9 to 14 weeks old) were obtained from a single commercial breeder. Spontaneous paresis or paralysis has not been observed in any of several hundred rabbits received from the same source. The animals were caged in pairs and fed Rockland Farms ration.[5] They were observed for at least 10 days prior to injection.

The water-soluble fraction was administered in Freund's complete adjuvant. The complete adjuvant was prepared by the addition of 6.4 mg of *Mycobacterium butyricum* (MB), ground to a fine powder with a mortar and pestle, to each milliliter of Freund's incomplete adjuvant.[6] Equal volumes of the complete adjuvant and distilled water containing varied amounts of the WSF were emulsified in a Servall omni-mix at high speed for several 1 minute periods. Forty-nine rabbits received approximately 0.1 ml of this antigen adjuvant emulsion intradermally into each of the four foot-pads. Twelve rabbits received a similar amount of the complete adjuvant only and three rabbits each received 1.0 mg of the I*WSF with low specific activity. All animals received a total of 1.6 mg of MB.

Blood was obtained from an ear vein prior to injection, the 7th day after injection and every 3 to 4 days thereafter for the 1st month, and every 5 to 7 days for the 2nd month. The blood was allowed to clot at room temperature and after the clot had retracted the serum was removed and stored at −20°C.

During the postinjection period, the strength and coordination of each rabbit were determined from the manner in which the animal moved about his cage or the floor of the animal room. The day that limb weakness first became apparent was designated as the day of onset of the disease. The extent of the clinical neurological involvement was graded solely on the basis of the degree of limb weakness; *i.e*, complete paralysis of two or more limbs within 3 days of onset, ++++; complete paralysis of two or more limbs in more than 3 days, +++; moderate paralysis of two or more limbs, ++; and mild weakness of one or more limbs, +. Once either paresis or paralysis had become well established the animal was sacrificed. This was usually accomplished within 6 days of the onset of the disease. The surviving rabbits were killed 60 days after injection. All animals were exsanguinated by cardiac puncture and the brain and spinal cord of the sick animals were removed and fixed in 10 per cent formalin. After fixation, 0.5 cm blocks of tissue from both cerebral hemispheres, midbrain, pons (including the cerebellum), medulla, upper and midcervical cord, upper, mid, and lower thoracic cord, and mid and lower lumbar cord were embedded in paraffin. Sections were stained with hematoxylin and eosin and for myelin by the method of Weil. The severity and distribution of the microscopic lesions were made the basis of a rough and entirely subjective method of grading the histologic changes. A designation of ++++ indicated the greatest and +, the least degree of involvement that was observed.

Measurement of Anti-WSF by 40 Per Cent Saturated Ammonium Sulfate Precipitation.—Approximately 0.1 ml of the I*WSF was diluted in 100 ml of 1:100 pooled normal rabbit serum borate buffer to provide about 50,000 counts/0.5 ml on the day after iodination. When the I*WSF was diluted for testing at longer time intervals after iodination, the same concentration of antigen was maintained by countwise adjustment with the use of an I^{131} decay chart.

The sera to be tested were diluted 1:10 with borate buffer while subsequent dilutions were made with 1:10 pooled normal rabbit serum borate buffer. An 0.5 ml aliquot each of the diluted serum and the I*WSF were mixed in 13 × 100 mm pyrex test tubes and incubated for 2 hours at 4°C. One ml of 80 per cent saturated ammonium sulfate (SAS) was added to each tube so that a final SAS concentration of 40 per cent was achieved. Since immediate and complete mixing was critical, the 80 per cent SAS was added as rapidly as possible with a Brewer automatic pipette to a rack of forty-eight tubes and the rack immediately shaken vigorously for 3

[5] A. E. Staley's Manufacturing Company, Decatur, Illinois.

[6] Difco Laboratories, Inc., Detroit.

minutes. The tubes were kept at 4°C for 30 minutes and then centrifuged at 2,000 RPM for 30 minutes at 4°C. The clear supernates were decanted, the tubes drained for 30 minutes, and the inside surface of the tubes above the precipitates swabbed to dryness. The tubes were each counted in the well-type scintillation counter and the number of counts expressed as a percentage of the total counts added. All samples were tested in duplicate and agreement within 5 per cent of the average was required. The per cent I*WSF bound by serial dilutions of a given serum was plotted on semilog paper. The anti-WSF titers expressed as binding units were calculated from this data (see Results).

Immunologic Reactivity of the Rabbit Anti-WSF.—The specificity of the interaction between the rabbit anti-WSF and I*WSF (I*WSF–anti-WSF system) was shown by the capacity of whole rabbit spinal cord to adsorb anti-WSF and by the capacity of unlabeled WSF to inhibit the I*WSF–anti-WSF system. Three gm of rabbit spinal cord, kidney, and liver were individually homogenized in 12 ml of borate buffer and centrifuged at 2,500 RPM for 30 minutes at

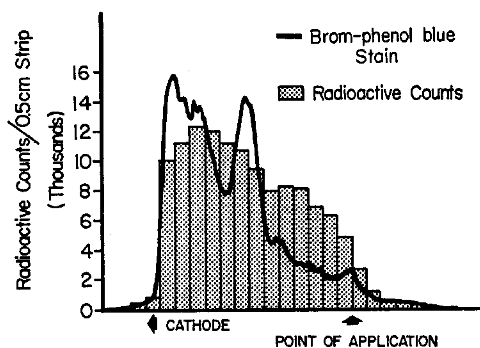

FIG. 1. Paper electrophoretic analysis of the I^{131}-labeled WSF

4°C. The homogenates were washed three times, serially diluted in 2 ml borate buffer, and mixed with equal volumes of a 1:10 dilution of an anti-WSF serum of known titer. These mixtures of tissue homogenate and serum were incubated with frequent shaking for 24 hours at 4°C and then centrifuged at 14,000 RPM for 30 minutes. The capacity of the supernates to bind the I*WSF was measured as previously described and compared to the per cent I*WSF bound by a 1:20 dilution of the unadsorbed anti-WSF serum. In the inhibition studies, unlabeled WSF, bovine serum albumin, and human serum globulin were added separately to the I*WSF solution in concentrations ranging from 1 to 100 gamma/0.5 ml and these mixtures used in the I*WSF–anti-WSF system with an anti-WSF serum of known titer.

Complement fixation (13), a modified quantitative precipitin (P-80) (14), and Ouchterlony tests (15) were carried out with the unlabeled WSF and randomly selected sera, including several with high 40 per cent saturated ammonium sulfate titers of anti-WSF antibodies. The immunofluorescent technique was used in an attempt to show a specific interaction between anti-WSF serum and whole nervous tissue. Thin, frozen sections of normal rabbit cord were exposed to fluorescein isothiocyanate–labeled globulin from a high titered anti-WSF serum, washed with normal rabbit serum, and observed with an ultraviolet microscope.

RESULTS

Chemical Characterization of the Rabbit Cord WSF.—Fig. 1 shows a typical analytrol pattern of the brom-phenol blue–stained water-soluble fraction

after paper electrophoresis in veronal buffer at pH 8.6. Although this figure illustrates the pattern of the I*WSF, it is equally representative of the unlabeled WSF. Practically all of the protein-stained material moved toward the cathode as a broad, double peaked band. Since the nitrogen content of the WSF was 16 per cent, it would appear that the WSF consists almost entirely of a mixture of electrophoretically similar, basic proteins.

*Chemical Characterization and Biological Activity of the I*WSF.*—As indicated above the brom–phenol blue–stained patterns of the unlabeled WSF and I*WSF were similar. The ninhydrin-stained patterns of the two materials were also similar by visual comparison and it would appear, therefore, that the iodination procedure did not cause any significant denaturation of the proteins. As shown in Fig. 1 the distribution of radioactive counts on the I*WSF corresponded roughly to the intensity of the brom–phenol blue stain. It is apparent that the slower moving components were proportionately more heavily iodinated than the rapidly moving elements.

The addition of 10 per cent TCA to the I*WSF precipitated 55 per cent of the total radioactive counts. Although dialysis of the I*WSF for an additional 3 to 4 days increased the percent precipitated by 10 per cent TCA, repassage of the I*WSF through the amberlite resin column did not. The per cent of the total I*WSF counts precipitated by 10 per cent TCA fell gradually to 40 per cent by the 5th week. Again this per cent was not increased by passage of the I*WSF through the ion exchange column. These facts suggest that a major proportion of the I*WSF solution consists of polypeptides or small molecular weight proteins and that the proportion of these constituents is increased with time.

Two of the three rabbits injected with I*WSF of low specific activity displayed paralysis 12 and 20 days, respectively, after injection. Thus iodination of the WSF did not alter its encephalitogenic activity when injected into rabbits.

Induction of EAE in Rabbits Injected with the Rabbit Cord WSF.—

The forty-nine rabbits injected with the WSF–adjuvant emulsion were divided into five experimental groups: group I consisted of fourteen rabbits that received 0.5 mg of WSF per rabbit; group II, thirteen rabbits, 2.0 mg each; group III, nine rabbits, 5.0 mg each; group IV, nine rabbits, 8.0 mg each; and group V, four rabbits, 12.0 mg each. All groups received 1.6 mg of mycobacteria per rabbit.

Almost without exception the first sign of EAE was the dragging of one or both hind legs or failure of the rabbit to bring the legs securely up under his haunches. Failure to eat and weight loss frequently accompanied or preceded these findings. Rabbits that became sick before the end of the 3rd week frequently progressed to complete paralysis of two or more extremities over a 24 to 48 hour period. In others, particularly those that developed EAE after

TABLE I

Results in the Five Experimental Groups of Rabbits Injected with WSF

Group	Rabbit No	Weight	Onset EAE	Rabbit killed	Clinical illness	Histologic changes	Binding units Day of death
		kg	*day*	*day*			
I	1	2.4	—	60	None	N.E.*	0
	2	2.6	—	60	"	"	0
	3	2.7	—	60	"	"	0
	4	2.6	—	60	"	"	0
	5	2.2	—	60	"	"	13
	6	2.4	—	60	"	"	81
	7	2.7	—	60	"	"	160
	8	2.1	18	18	4+	3+	0
	9	2.1	18	20	4+	2+	0
	10	2.4	25	28	4+	1+	0
	11	2.0	27	28	4+	2+	0
	12	2.3	31	32	4+	2+	0
	13	2.0	33	44	2+	1+	0
	14	2.6	45	50	2+	2+	0
II	1	2.8	—	60	None	N.E.	0
	2	2.2	—	60	"	"	10
	3	2.4	—	60	"	"	10
	4	2.6	—	60	"	"	44
	5	2.7	—	60	"	"	96
	6	2.5	15	16	4+	4+	0
	7	2.4	18	20	4+	2+	80
	8	3.0	18	18	4+	3+	0
	9	2.4	18	18	4+	2+	0
	10	2.2	18	18	4+	4+	80
	11	3.0	18	18	4+	4+	320
	12	2.7	22	24	4+	2+	0
	13	2.3	25	28	4+	2+	0
III	1	2.4	—	60	None	N.E.	0
	2	2.5	—	60	"	"	40
	3	2.3	—	60	"	"	35
	4	2.3	—	60	"	"	60
	5	2.6	18	18	4+	4+	0
	6	2.3	18	18	4+	3+	21
	7	2.7	20	20	4+	3+	0
	8	2.5	25	32	3+	2+	0
	9	2.4	26	32	3+	2+	0

* N.E., not examined.

TABLE I—*Concluded*

Group	Rabbit No.	Weight	Onset EAE	Rabbit killed	Clinical illness	Histologic changes	Binding units Day of death
		kg	*day*	*day*			
IV	1	2.2	—	60	None	N.E.	0
	2	2.1	—	60	"	"	36
	3	2.1	15	16	4+	"	28
	4	2.8	16	18	4+	"	110
	5	2.5	22	24	4+	"	320
	6	2.7	25	28	4+	"	35
	7	2.6	25	32	2+	"	100
	8	2.3	28	32	3+	1+	22
	9	2.2	48	50	4+	4+	270
V	1	2.7	—	60	None	N.E.	0
	2	2.0	—	60	"	"	35
	3	2.6	14	14	4+	4+	15
	4	2.3	18	18	4+	3+	90

the 3rd week, paresis was more gradual in onset and in some became stabilized at a point of mild to moderate hind leg weakness. The degree of clinical disease in each rabbit, graded solely on the basis of the rapidity of development and extent of the paralysis, is given in Table I.

The incidence and rate of onset of EAE in experimental groups I to IV, expressed in terms of cumulative per cent rabbits sick at successive 10 day intervals after injection, are plotted in Fig. 2 from the data presented in Table I. Group V was excluded from the chart because of the inadequate size of this group. All groups showed a progressive increase in the total per cent of sick rabbits through the 30th day. A slight, but probably insignificant, difference in the per cent of rabbits sick among the four groups was apparent at the end of 2 months. However, for the first 30 days after injection, group I clearly lags behind the other groups. The average day of onset of EAE in these four groups was 28, 19, 21, and 26, respectively. Rabbit 9, group IV, seems exceptional in view of the development of maximum degree of clinical illness and histologic change with an onset of disease which is later than for any of the other sick rabbits. Exclusion of this rabbit from group IV in calculating the average day of onset of EAE makes the group IV average 22 days rather than 26 days. Since the day of onset of EAE is considered an important quantitative parameter of the disease (16), it would appear then that there was a dose-response relationship up through the 2 mg dose of WSF. The lack of a significant difference in the incidence and rate of onset of EAE among the three groups of rabbits receiving 2 or more milligrams of WSF may indicate that the dose range at which the encephalitogenic response is no longer proportional to dose had been reached. Weight differences among the rabbits at the time of

injection (Table I) did not seem to have any influence on the incidence or severity of the disease among the animals of a single group or of the different groups. The twelve control rabbits which received Freund's complete adjuvant without WSF were observed for 3 months and did not develop signs of paresis or paralysis.

Histological examination of the brain and spinal cord of animals with EAE showed meningeal, subpial, and perivascular collections of mononuclear cells and pleomorphic macrophages. Myelin stains showed accompanying loss of

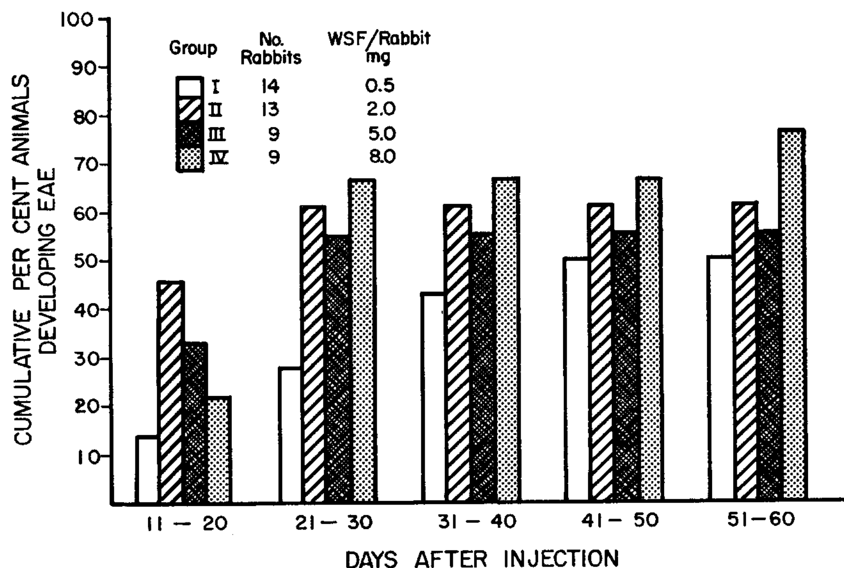

FIG. 2. The cumulative per cent of animals that developed EAE

myelin in those areas infiltrated with macrophages. Where these changes extended into the gray matter of the cord, neurons were well preserved in most instances. Although the spinal cord was predominantly affected, lesions were also observed in some animals in the deep white matter of the cerebral and cerebellar hemispheres and in the optic nerves and tracts. In Table I the changes observed in each rabbit with EAE are graded on the basis of their extent and severity, although some difficulty in grading was occasioned by a discrepancy in these parameters in certain animals. For example, many or most sections of a cord from a rabbit with EAE might show minimal changes while one section might show severe involvement. In addition, selected portions only, and not the entire cord or brain, were sampled.

*Per cent I*WSF Bound by Serum from Adjuvant-Injected- and WSF-Injected Rabbits.*—Table II shows the per cent I*WSF bound by sera from the

twelve control rabbits before and after injection of Freund's complete adjuvant. The per cent I*WSF bound by the serum from each rabbit prior to injection is the average of three sera obtained at weekly intervals prior to injection. The preinjection values of the twelve control rabbits ranged between 2.1 and 6.6 per cent. While eleven of the twelve rabbits fell within a more narrow range (2.1 to 3.4 per cent), the high value of 6.6% for rabbit 10 shows that wide variation may occur. Similar variation among preinjection sera has been observed in other groups of animals. It is clear from inspection of the prein-

TABLE II

*Per Cent I*WSF Bound by 1:10 Dilutions of Adjuvant Control Rabbit Sera*

Rabbit No.	Days preinjection				Days postinjection						
	7	4	0	Average	10	14	22	28	36	44	50
1	2.9	2.5	2.5	2.6	+0.2*	+0.9	+1.6	+1.2	+2.0	+2.7	+1.5
2	2.4	4.0	3.8	3.4	+0.3	+1.2	+0.4	+0.4	+2.4	+3.1	+2.9
3	2.6	2.4	3.4	2.8	+1.0	+1.6	+1.2	+1.2	+3.2	+3.6	+3.8
4	2.2	2.2	1.9	2.1	+0.5	+1.0	+1.4	+1.0	+1.7	+2.2	+1.9
5	2.4	2.5	3.5	2.8	+1.0	−0.2	+0.9	+1.1	+2.5	+2.9	+2.3
6	2.5	2.4	2.4	2.4	+0.9	+1.4	+2.0	+1.9	+1.8	+1.9	+1.8
7	3.0	2.6	3.4	3.0	+1.5	+1.4	+1.1	+1.3	+1.6	+2.0	+2.6
8	2.1	2.7	3.2	2.7	+1.1	+1.1	+0.6	+1.1	+2.3	+3.3	+3.3
9	2.8	3.2	2.6	2.9	+1.6	+1.2	+1.1	+1.0	+1.9	+2.8	+2.3
10	6.5	6.4	6.8	6.6	+0.5	+0.2	+0.3	+0.4	+0.4	+1.4	+1.7
11	2.6	3.1	2.7	2.8	+0.2	+0.3	+1.0	+1.3	+1.3	+1.4	+1.4
12	3.0	2.5	2.6	2.7	+0.4	+0.3	+0.1	−0.2	0.0	−0.3	−0.4
Mean					+0.8	+0.9	+1.0	+1.0	+1.8	+2.2	+2.3
SD					±0.5	±0.6	±0.6	±0.5	±0.8	±1.0	±1.1

* Difference between preinjection and postinjection per cent I*WSF bound.

jection data in Table II that such variations were not the result of experimental error, but were a consistent quality of the serum from an individual rabbit. It is apparent, therefore, that each rabbit's own preinjection serum must be used as a control for subsequent observations rather than the mean of the control group.

Following injection of Freund's complete adjuvant eleven of the twelve showed a gradually progressive increase in per cent I*WSF bound above the preinjection value. In Table II the per cent I*WSF bound after injection has been expressed for each rabbit as the difference between the per cent I*WSF bound on the postinjection day observed and the average preinjection value. The average of these differences for the group as a whole is recorded for each postinjection day at the bottom of each column. From the 10th through the

50th day there is a gradual rise in these differences from +0.8 ± 0.5 per cent to +2.1 ± 1.1 per cent.

The reason for the variation in solubility of the I*WSF in individual, control rabbit sera and the reason for the slight increase in the per cent I*WSF precipitated in the presence of sera from rabbits injected with Freund's complete adjuvant are not entirely clear. However, preliminary studies suggest that these features may be correlated in a non-specific manner with the amount of globulin present in the sera. For example, a slight but definite increase in β and γ-globulin was observed in the serum electrophoretic patterns of those rabbits which received Freund's complete adjuvant only (17).

It can now be appreciated that the per cent I*WSF bound by the serum from a rabbit injected with WSF is influenced by factors other than the absence or presence of anti-WSF. These factors are: (*a*) the solubility of the I*WSF in control rabbit serum as reflected by the per cent I*WSF bound by the rabbit's own preinjection serum, (*b*) the effect of the adjuvant on this solubility as estimated from the mean increase in per cent I*WSF bound by the adjuvant control group at the same time period after injection, and (*c*) experimental variation. The influence of the first two factors was determined from the experimental data and allowance for experimental error was made by the addition of four standard deviations to the mean of the adjuvant control group. These factors were made the basis of an arbitrarily defined "lowest significant level" of I*WSF bound. Below this level the three factors listed were considered important influences and above this level binding was considered due to the presence of anti-WSF. For example, given a serum obtained 22 days after injection which bound 10 per cent I*WSF, a preinjection serum from the same rabbit which bound 4.0 per cent, and an average increase in per cent I*WSF bound by the adjuvant control group on the 22nd day after injection of 1.0 ± 0.6 per cent; the lowest significant level of binding by this serum would be 4.0 per cent (preinjection serum) plus 1.0 per cent (adjuvant control group) plus 4 × 0.6 per cent (four standard deviations) or 7.4 per cent. The per cent I*WSF bound related to the presence of anti-WSF would then be 10 per cent minus 7.4 per cent which equals 2.6 per cent.

So that the anti-WSF content of different sera could be more accurately compared, the amount of anti-WSF in each 0.5 ml of serum was expressed in arbitrary terms of a "binding unit." The binding unit was defined as the reciprocal of the serum dilution at which the per cent I*WSF bound fell below the lowest significant level as defined above. Fig. 3 shows how these quantitative values were calculated in the case of rabbit 9, group IV. Serum dilutions are expressed along the ordinate of the semilog paper. The total per cent I*WSF bound by each serum dilution minus the lowest significant level of I*WSF bound is scaled along the abscissa. Accordingly, the reciprocal of the dilution at which the anti-WSF serum dilution curve crosses the base line becomes

the binding unit per 0.5 ml of the undiluted serum. This method for determination of anti-WSF binding units disregards the fact that 1:10 pooled normal rabbit serum was used as the diluent at antiserum dilutions above 1:10. It is apparent that with progressive dilution of the anti-WSF serum, the lowest

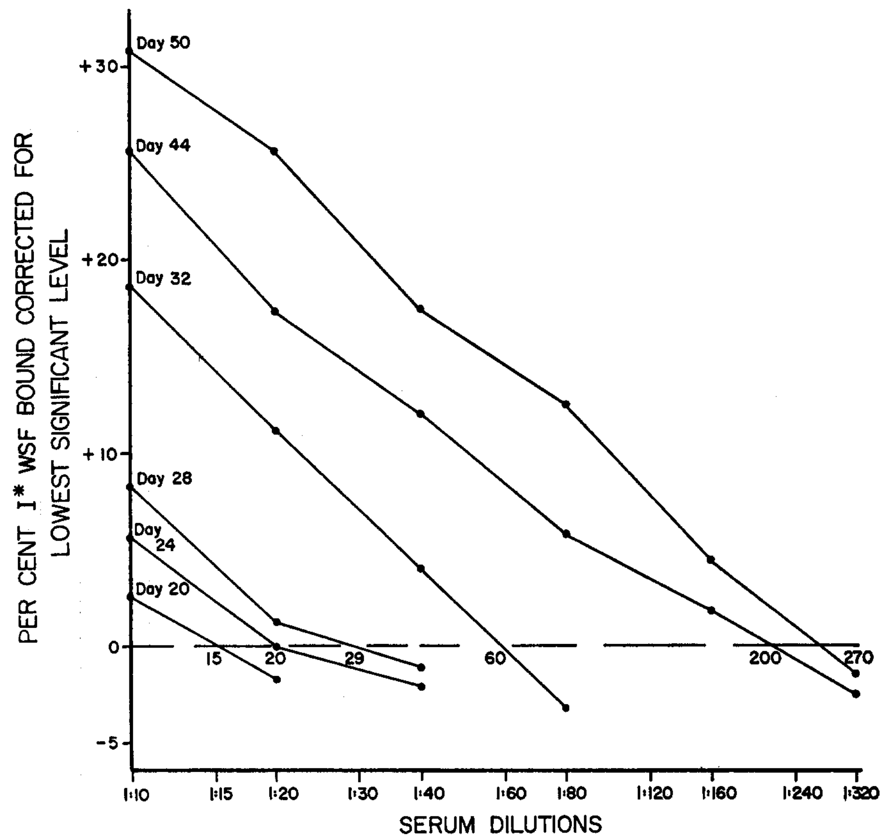

FIG. 3. Method of calculating binding units. The figures at the point of interception of the antibody dilution curves with the base line represent binding units.

significant level of binding would be determined more and more by the non-specific binding properties of 1:10 normal rabbit serum diluent and less and less by the non-specific binding properties of the anti-WSF serum. However, since the per cent I*WSF bound by pooled normal rabbit serum never exceeded 4.0 per cent, the use of a lowest significant level of binding based on the calculations described above for all serum dilutions tended to provide a larger rather than a smaller margin for significance.

The results obtained in the five groups of rabbits injected with the water-

TABLE III

Serial Anti-WSF Titers of the Five Groups of WSF-Injected Rabbits

Group	Rabbit No.	Binding units Days after injection									
		14	16	18	20	24	28	32	38	44	50
I	1	0			0	0		0	0	0	0
	2	0			0	0		0	0		0
	3	0			0	0		0	0		0
	4	0			0	0		0	0		0
	5	0			0	0		0	12	0	13
	6	0			0	40		40	60		81
	7	0			0	12		15	70		160
	8	0		(0)*							
	9	0			(0)						
	10	0			0	0	(0)				
	11	0			0	0	(0)				
	12	0			0	0		(0)			
	13	0			0	0		0	0	(0)	
	14	0			0	0		0	0	0	(0)
II	1	0			0	0		0	0		0
	2	0			0	0		0	0		10
	3	0			0	0			0		10
	4	0			0	13		10	12		44
	5	0			0	15		22	40		96
	6	0	(0)								
	7	0			(80)						
	8	0		(80)							
	9	0		(0)							
	10	0		(0)							
	11	51		(320)							
	12	0			0	(0)					
	13	0			0	0	(0)				
III	1	0			0	0	0	0	0		0
	2	10			15		17		20		40
	3	0			10	12	14		30		35
	4	0			0		0		60		60
	5	0		(0)							
	6	0		(21)							
	7	0			(0)						
	8	0			0	0	0	(0)			
	9	0			0	0		(0)			

* Parentheses indicate day rabbits with EAE were sacrificed.

TABLE III—*Concluded*

Group	Rabbit No.	Binding units Days after injection									
		14	16	18	20	24	28	32	38	44	50
IV	1	0			0	15		15		0	0
	2	0			10	20		38		38	36
	3	18	(28)								
	4	0	40	(110)							
	5	14			52	(320)					
	6	0			11	19	(35)				
	7	0			38	84	(100)				
	8	0			0	13	22	(22)			
	9	0			15	20	29	60		200	(270)
V	1	0			0		0		0		0
	2	0		0	0		20		51		35
	3	(15)									
	4	90		(90)							

soluble fraction can now be considered. Table I gives the antibody titers of these rabbits at the time of sacrifice. Out of the total of twenty-nine rabbits which developed EAE, thirteen had circulating anti-WSF and sixteen did not; while of the twenty animals which survived, twelve demonstrated circulating anti-WSF and eight did not. Inspection of Table III, which records the serial antibody titers in each animal, reveals additional information. Circulating antibodies did not make their appearance in any of the animals prior to the 14th day after injection. However, from the 14th day through the 20th day, fifteen animals developed significant antibody titers and twelve of these were rabbits which had, or subsequently developed, EAE. The initial appearance of circulating antibody, when it occurred after the 3rd week, was observed exclusively in the surviving animals with the exception of rabbit 8, group IV. This relationship between early appearance of antibody and EAE is illustrated in Fig. 4 in which the titers of all rabbits which made anti-WSF are charted. It is also clear that, as a group, the antibody-producing animals which survived had lower titers than the antibody-producing animals with EAE, and of the three surviving rabbits which made antibody before the end of the 3rd week, none had over 10 binding units/0.5 ml undiluted serum. Rabbit 9, group IV, is again of special interest in view of the very high antibody titer and late onset of disease.

Specificity of the Rabbit Anti-WSF.—The results of the tissue adsorption studies in which an anti-WSF serum was exposed to homogenates of rabbit spinal cord, kidney, and liver are shown in Table IV. The anti-WSF serum

bound 16 per cent of the I*WSF both before and after exposure to liver and kidney homogenates, but after adsorption by the rabbit spinal cord homogenate the anti-WSF bound only 8 per cent. These results indicate that at least 50

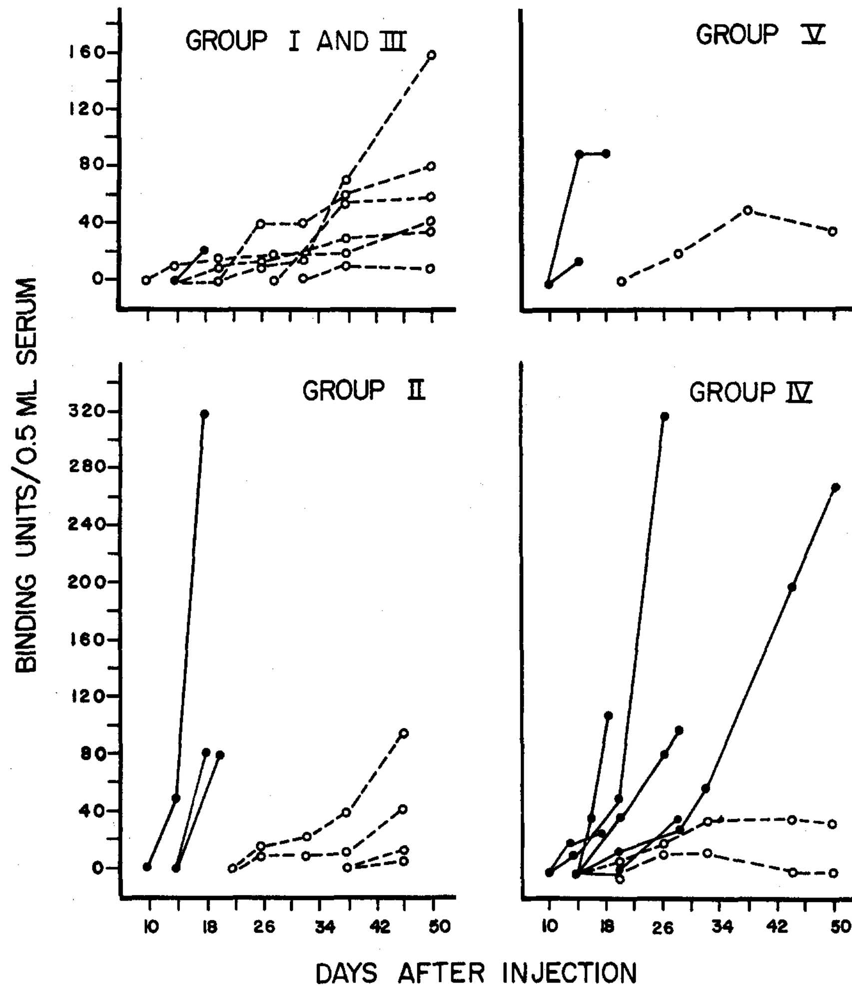

FIG. 4. Anti-WSF titers of sera from all animals with detectable circulating antibodies The solid lines and circles represent the animals with EAE, and the broken lines and open circles the animals that survived.

per cent of the binding capacity of the anti-WSF serum was specific for rabbit spinal cord.

The inhibition of the binding capacity of an anti-WSF serum by unlabeled

WSF is shown in Table V. Significant inhibition was achieved with 0.1 gamma unlabeled WSF/0.5 ml of the I*WSF test solution and complete inhibition of the binding capacity of the anti-WSF serum tested was achieved with a concentration of 10 gamma/0.5 ml. Neither bovine serum albumin nor human gamma globulin showed any effect over the same range of protein concentration.

Complement fixation, quantitative precipitin, and Ouchterlony tests failed to detect antibody to the water-soluble fraction in any of the sera tested, including those with high 40 per cent SAS anti-WSF titers. Attempts to show a specific interaction between an anti-WSF serum and frozen tissue sections of normal rabbit cord by the fluorescein technique were unsuccessful because of the marked non-specific staining obtained with control sera. This was presumably due to the high degree of affinity between fluorescein and fatty substances of the cord such as myelin.

Table IV
Tissue Specificity of the Anti-WSF

Tissue dilution	Tissue used to adsorb test antiserum*		
	Spinal cord	Liver	Kidney
1:1	8‡	15	16
1:2	8	15	15
1:4	9		
1:8	9		
1:32	10		

* A 1:20 dilution of the unadsorbed test antiserum bound 16 per cent and a 1:20 dilution of pooled normal rabbit serum bound 4 per cent.
‡ Per cent I*WSF bound.

DISCUSSION

Induction of experimental allergic encephalomyelitis (EAE) with water-soluble protein fractions of nervous tissue has been previously reported in guinea pigs (8). It is of interest, therefore, that one of these fractions is also effective in inducing EAE in rabbits, even when extracts of homologous nervous tissue are employed. The qualitative features of the clinical and histological changes appeared identical to those observed previously in this laboratory (17), and by Morrison (18), in rabbits injected with whole rabbit cord. The quantitative features of the disease also compare favorably with those reported by Waksman and Morrison (19) in whole cord–injected rabbits, with the exception that the average day of onset of EAE in the latter study was 13 days compared to 22 days (groups II to V) in the present report. However, this lag

in time of onset might be related not only to differences in effectiveness of the two encephalitogenic agents but also to differences in the adjuvant mixtures used in the two studies since certain features of the adjuvant, such as its viscosity and the ratio of mycobacteria to antigen, are now known to modify the quantitative parameters of the disease (20–22).

Although complete chemical analysis of the WSF was not carried out, it seems reasonable to assume that the water-soluble fraction of rabbit cord prepared for these studies is principally protein since it contained 16 per cent nitrogen by weight and, as shown by Roboz and Henderson in their preparations from bovine cord, only small amounts of carbohydrates and lipids. The high degree of biological activity demonstrated for certain of these preparations

TABLE V

The Blocking Effect of Unlabeled WSF on the In vitro Binding of Anti-WSF

Concentration of unlabeled WSF	I*WSF bound by a 1:10 dilution of an anti-WSF serum*
*Gamma/0.5 ml I*WSF*	*per cent*
0	23
0.05	22
0.1	18
1.0	13
10.0	7
100.0	6

* Lowest significant level of binding for this serum was 7 per cent.

in guinea pigs by Kies and Alvord (8) suggests that the encephalitogenic agent in such preparations is itself protein in nature. The latter workers showed that a citrate extract of partially defatted bovine cord had 30 per cent of the activity of lyophilized whole cord. Although no such comparison has been attempted in this study, it has been recently found that 50 gamma WSF/rabbit is an effective encephalitogenic dose, EAE having been induced in 3/6 injected rabbits with an average day of onset of 27 days. Thus if the encephalitogenic agent is not protein, it must be some extremely potent lipid or polysaccharide contaminant.

The ammonium sulfate precipitation method of Farr (9) depends upon the precipitation of I* antigen–antibody complexes under conditions selected so that any of the uncomplexed I* antigen remains in solution. This test has been used extensively in the BSA–anti-BSA system and a concentration of 50 per cent saturated ammonium sulfate (SAS) has been employed. A concentration of 40 per cent SAS was used in the present study, however, since the I*WSF was considerably more soluble at this concentration.

Despite the fact that some rabbits developed EAE without detectable circulating anti-WSF and others had anti-WSF without EAE, the occurrence of

EAE in twelve out of the fifteen rabbits which had anti-WSF before the end of the 3rd week suggests a possible relationship between antibody and disease. The difference in the height of the anti-WSF titers between the rabbits with EAE and those which survived is also suggestive. The occurrence of EAE in some rabbits without measurable anti-WSF may, as proposed by Kabat (23), be due to rapid clearing of antibody from the circulation by central nervous system tissue although the binding of anti-brain antibody *in vivo* has yet to be shown. Nevertheless, strong evidence for nervous tissue specificity of the anti-WSF is shown by adsorption of the anti-WSF with fresh rabbit cord but not with kidney or liver tissue homogenates. While the high anti-WSF titers in some of the rabbits with EAE may be eventually attributed to concomitant rather than primary immunologic processes, further investigation of the role of antibody seems warranted, especially so, as more purified encephalitogenic preparations become available.

SUMMARY

A water-soluble protein fraction of nervous tissue was prepared by extraction of rabbit spinal cord with sodium citrate at pH 4.3. Characterization by nitrogen content and paper electrophoresis showed it to be a mixture of basic proteins. This extract demonstrated encephalitogenic activity when injected into rabbits.

The most suitable technique for the measurement of serum antibody to the rabbit cord antigen proved to be the precipitation of antigen-antibody complexes by 40 per cent saturated ammonium sulfate. Antibody could not be demonstrated by the techniques of complement fixation, quantitative precipitation, and Ouchterlony plates.

The early appearance of circulating antibody occurred almost exclusively in rabbits that subsequently developed EAE. Specificity of the antibodies for nervous tissue was demonstrated by appropriate blocking and adsorption experiments.

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