STUDIES ON ACUTE DISSEMINATED ENCEPHALOMYELITIS PRODUCED EXPERIMENTALLY IN RHESUS MONKEYS*

V. Complement-Fixing Antibodies

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Disseminated encephalomyelitis has now been produced in several animal species by injection of emulsions of normal brain or spinal cord tissue with certain adjuvants (paraffin oil, aquaphor, and killed tubercle bacilli) (1–12). Attempts to produce the disease with emulsions of other tissues have been uniformly negative, indicating the etiological specificity of nervous tissue. The mechanism by which the disease is produced is still obscure. However, the regularity with which the condition occurs when Freund's adjuvant technic is used, in contrast to the difficulties in its production before adjuvants were employed (13–15), has been assumed to imply the operation of some immunological process. Adjuvants have been shown to enhance antibody production to a variety of antigens (*cf.* reference 16) and indeed complement-fixing antibodies to alcoholic extracts of brain tissue were obtained in the monkey by this procedure (17). The production of complement-fixing antibodies to brain and to numerous other tissues and organs has long been known (18–23; for a review *cf.* 24) although most of these antibodies have not been produced with adjuvants.

Experiments in this laboratory on the isolation of the encephalitogenic factor have permitted the accumulation of materials for a serological investigation of the complement-fixing antibodies produced by the injection of the brain emulsion and an evaluation of their possible relationship to the disease. Most of the tests have been made with suspensions of lyophilized tissue, standardized by dry weight, as "antigens" in preference to the alcoholic and watery extracts employed by previous workers (17, 19, 23). The use of such

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antigens has demonstrated a complex pattern of antibody response. Some of the antibodies were organ-specific, some were not. In particular, it was found that a large proportion of sera from monkeys injected with brain extracts, as well as with whole brain tissue, contained antibodies which reacted strongly, in complement fixation tests, with placental tissue. Quantitatively, these reactions with placenta much exceeded those obtained with corpus luteum, kidney, and testis (*cf.* also reference 17). While these studies have indeed confirmed the existence of antibodies specific for nervous tissue, the use of placenta has shown that certain "antibodies to brain" may be antibodies to some element of the brain other than specifically nervous tissue, possibly vascular endothelium. Caution is therefore needed in interpreting results obtained with crude brain tissue or tissue extracts as due to antibodies specific for brain tissue. For instance, sera were examined from three persons, who had received prophylactic antirabic treatment and were reported to us (25) as having elaborated anti-brain antibodies. With the technics employed, reactions with placental antigen only were observed with two of the sera while the third serum reacted with both brain tissue and placenta—the latter reaction being the stronger. Other recent reports (26) on brain antibodies in human beings after antirabic vaccination might, therefore, require reevaluation. No correlation between the serum titer of the various complement-fixing antibodies and the development of encephalomyelitis in the monkey was obtained; a similar lack of correlation between complement-fixing antibodies to brain tissue and encephalomyelitis has been reported in dogs (27) and in mice (28).

EXPERIMENTAL

Sera.—Sera were studied from 82 normal control monkeys and from 89 monkeys that received three weekly intramuscular injections of sterile emulsions of brain or brain extracts with adjuvants which had been found by assay in monkeys to have encephalitogenic power. Thirty-two sera were available from monkeys injected with emulsions, which in this laboratory have proven non-encephalitogenic, such as peripheral nerve, testis, and lung, with chemically treated brain emulsions with adjuvants, or with brain emulsions without tubercle bacilli, and with suspensions of lymphoid tissue or with monkey and rabbit sera, and served as controls for the complement fixation reaction. The experimental and pathological data for most of these have already been reported elsewhere (2–5) and in the case of monkeys from more recent series the same standardized experimental regime has been followed. When brain fractions (with the usual adjuvants) have been used for encephalitogenic assay their general nature has been indicated in the tables. More detailed chemical data may be presented later.

All sera contained 0.01 per cent merthiolate; they were handled with aseptic technic and stored at 2° to 4°C. The days of bleeding and of onset of signs of encephalomyelitis are recorded as from the 1st day of the first injection course. For each day's tests, requisite portions were removed aseptically from stock and inactivated for 30 minutes at 56°C. Preliminary studies confirmed the importance of this precaution and showed that inactivation will reduce appreciably anticomplementary properties of older sera. Sera which had become strongly anticomplementary were rejected; these were usually sera which had been standing for some time. In the 121 experimental and 82 normal monkey sera studied the greatest anticomplementary activity has been minimal fixation at 1:10 dilution. At most 20 sera of the 203 used

have shown this degree of anticomplementary activity while the majority of the monkey sera, especially the more recent, have shown none at all. Especial care has been taken in every set-up to have the serum controls for anticomplementary activity in the full range of dilutions used for the actual test.

Cerebrospinal fluids from 22 monkeys were also examined; 6 of these came from the 89 monkeys already referred to while the remainder were from animals whose sera were not available. Human sera from 45 cases of multiple sclerosis, from 6 cases of other demyelinating diseases, and from 3 patients following antirabic vaccination were investigated. For these last mentioned specimens we are indebted to Dr. H. Koprowski, New York, and for all the other specimens to Dr. T. J. C. von Storch and Dr. Tiffany Lawyer of Montefiore Hospital and to Dr. L. V. Chiavacci of the Neurological Institute, New York.

Antigens.—These were all prepared from lyophilized tissue. The following were used: Brain from adult monkey, human being, steer, adult rabbit, 3 day old rabbit. With fetal rabbit brain two separate batches had to be used; the first consisted of the brains from a litter of six 18 day, the second from a litter of 9 approximately 20 to 23 day fetuses. Other normal monkey organs and tissues were: Heart, lung, liver, kidney, spleen, adrenal, testis and skeletal muscle, and peripheral nerves. Except for the last named, these were removed aseptically, cut up, stripped of any obvious fibrous stroma and large vessels, homogenized in a blendor, and lyophilized. Monkey peripheral nerves (lumbar and brachial plexuses, sciatic and brachial nerves) were cut into short segments with scissors, pulped in a mortar, lyophilized, and then ground further. Even then the final material, suspended in saline, assumed a stringy, gelatinous character and gross particles were removed after milling with a glass rod against the side of the container. For this reason, peripheral nerve suspension was used, arbitrarily, at twice the concentration of the other antigens; *i.e.*, at 4 mg. per ml. With monkey skin it was found expedient to cut portions—approximately 15 mm. long by 3 or 4 mm. broad—edgewise on the freezing microtome; a mass of 15 micron sections was run off on to the blade of the knife, transferred to a dry bottle, and smeared round the walls, before lyophilizing. The lyophilized material was then ground further and yielded a light homogeneous, flaky powder which suspended well in saline. "Corpus luteum" and placenta were taken from two pregnant rabbits. The former was prepared by first cutting the ovaries on the microtome as for the skin. Sample sections from each were seen microscopically to consist largely of luteal tissue. The pooled placentas were drained as far as possible of blood, homogenized in a blendor, and lyophilized. Steer pituitaries were separated into anterior and posterior lobes and lyophilized as above. Except for the instance quoted of fetal brain, the same lyophilized powders were used uniformly throughout all the tests.

Complement Fixation Technic.—The analytical technics employed for quantitative and kinetic studies on immune hemolysis (29, 30) have been used throughout. The diluent was a barbiturate-buffered NaCl solution with the addition of 1.0 gm. of $MgCl_2 \cdot 6\ H_2O$ and 0.2 gm. of $CaCl_2 \cdot 2H_2O$ to the 2 liter stock solution. This stock concentrate, sterilized by autoclaving, was diluted 1:5 with distilled water for each day's use to make an isotonic solution of pH 7.3 to 7.4. Sheep cells, collected aseptically and preserved in Alsever's solution for periods up to a month, were washed 5 times with buffered saline immediately before use, packed by centrifugation, and a 5 per cent suspension was made in buffered saline. To standardize, 1.0 ml. of the carefully mixed suspension was lysed by the addition of 14.0 ml. of distilled water and the resulting Hb solution was read on the Beckman spectrophotometer at 5400 Å. This wâve length was adopted as optimal for this purpose on the basis of preliminary assays on lysed cell suspensions primarily standardized by repeated hemocytometric counts. At this wave length, suspensions of one million erythrocytes per $mm.^3$ were found to yield, on lysis, a hemoglobin solution with an average optical density of 0.715 in the regular 10 mm. cuvette. The standard cell suspensions for each day's use, after preliminary reading on the Beckman, were adjusted accordingly.

The amboceptor used was a rabbit anti-sheep cell hemolysin with a minimum hemolytic

titer of 1:1600. For the titration of complement, and for use in the tests, four 100 per cent hemolytic units were used. Pooled guinea pig serum, distributed in 2 to 3 ml. amounts and kept frozen on solid CO_2, was the source of complement. An initial dilution of 1:25 was prepared in a volumetric flask from the freshly thawed complement stock and from this an arithmetic series of dilutions was made in buffered saline, separate pipettes being used for each dilution and all reagents being kept in ice water until the time of final incubation. For the titration, 1 ml. of C′ dilution, 3 ml. of buffered saline (4 ml. for the control tube), and 1 ml. of sensitized cells were measured into acid-washed 50 ml. centrifuge tubes. These were capped and then incubated in the water bath at 37°C. for 45 minutes with frequent shaking. Then 2.5 ml. of buffered saline was added to each tube, the contents well mixed, and transferred to 15 ml. tubes for centrifugation. The decanted supernatants were then read in the Beckman spectrophotometer at 5400 Å and the lysis curve plotted accordingly. The 50 per cent lytic unit determined from the curve was the basic unit of measurement of C′ throughout this work. Earlier, numerous assays were made with 5 such units but as occasional spurious results occurred with partially anticomplementary sera, 6 units were finally adopted as the standard dosage. All results in this paper were obtained with this dosage.

Reading and Interpretation of Results.—Sera were set up in the standard dilutions of 1:2, 1:5, 1:10, 1:20, 1:40, and 1:80, sometimes being run up further to 1:320 or more. Serum controls have been discussed. Appropriate antigen controls were likewise set up. Two-tenths ml. each of serum, complement dilution, and antigen was used in a total volume of 0.8 ml. and 0.2 ml. sensitized sheep erythrocytes added after 45 minutes' incubation at 37°C. The degree of lysis was recorded after an additional 30 minutes at 37°C. as follows: Complete fixation of complement—0; trace of lysis—tr; slight lysis—sl; moderate lysis—m; strong lysis—str; almost complete lysis—ac; and complete lysis (*i.e* no fixation of complement) —c. This method has been used in Table I; but in subsequent tables, results have been condensed as follows: − means no fixation of C′ beyond the limit (if any) of natural anticomplementary effect; ± means fixation of C′ to the next dilution beyond the limit of anticomplementary effect; + means fixation of C′ to two dilutions; ++, fixation of C′ to three and four dilutions; +++, fixation of C′ to five dilutions and more beyond the limits of anticomplementary effect. By adoption of this scheme, it will be seen that plus signs have some quantitative significance, that a single + is a genuine positive representing as it does a degree of C′ fixation beyond the doubtful zone, while the adoption of the ± sign prevents the arbitrary rejection of a possibly significant borderline result.

RESULTS

Table I summarizes complement fixation results with 10 representative sera from monkeys injected with various brain emulsions. All these animals developed clinical and pathological evidence of encephalomyelitis. The diversity of antibody patterns in the various sera is evident. Four of the sera (Nos. 7-4, 1-65, 5-55, 2-21) reacted best with monkey brain and posterior pituitary. This antibody appears to be organ-specific since it occurred in animals which had received rabbit, steer, or human brain. Three of these 4 reacted also with rabbit placenta, 1 (No. 7-4) almost as strongly as with brain, the other 2 (No. 1-65, 5-55) to a lesser extent; the fourth serum (No. 2-21) reacted as strongly with peripheral nerve as with brain and pituitary. Five of the remaining 6 sera fixed complement best with monkey brain, the material which had been injected, and failed to react or reacted but slightly with posterior pituitary

TABLE I

Reaction of Sera from Monkeys with Encephalomyelitis in Complement Fixation Tests with Various Lyophilized Tissue Suspensions

Monkey No.	7-4						1-65						5-55						2-24						2-21					
Material injected	Brain from 1 mo. old rabbit						Brain from 2 mo. old rabbit						Steer brain						Monkey brain formalinized						Human brain					
Dilutions of Serum	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80
Control (no antigen)	0	tr	ac	c	c	c	tr	c	c	c	c	c	ac	c	c	c	c	c	str	c	c	c	c	c	0	c	c	c	c	c
Monkey brain	0	0	0	0	sl	ac	0	0	0	0	0	0	0	0	0	0	0	0	0	tr	ac	c	c	c	0	0	0	0	0	0
Rabbit placenta	0	0	0	0	sl	str	0	0	0	m	c	c	0	0	0	0	sl	c	0	0	0	0	0	m	0	c	c	c	c	c
Monkey peripheral nerve	0	0	str	c	c	c	0	m	c	c	c	c	0	0	0	m	c	c	sl	c	c	c	c	c	0	0	0	0	0	0
Steer pituitary, anterior lobe	0	tr	ac	c	c	c	0	0	str	c	c	c	0	0	0	0	ac	c	str	c	c	c	c	c	0	c	c	c	c	c
Steer pituitary, posterior lobe	0	0	0	0	str	ac	0	0	0	0	0	0	0	0	0	0	0	0	0	m	c	c	c	c	0	0	0	0	0	0

Monkey No.	5-71						5-07						5-83						5-84						5-88					
Material injected	Monkey brain						Monkey brain*						Monkey brain‡						Monkey brain‡						Monkey brain§					
Dilutions of serum	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80	1/2	1/5	1/10	1/20	1/40	1/80
Control (no antigen)	c	c	c	c	c	c	str	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Monkey brain	0	0	0	0	tr	m	0	0	0	0	0	0	0	0	m	c	c	c	0	0	0	0	str	c	0	0	0	str	c	c
Rabbit placenta	0	0	0	0	sl	ac	str	c	c	c	c	c	c	c	c	c	c	c	m	c	c	c	c	c	c	c	c	c	c	c
Monkey peripheral nerve	tr	m	c	c	c	c	m	c	c	c	c	c	c	c	c	c	c	c	0	ac	c	c	c	c	0	0	str	c	c	c
Steer pituitary, anterior lobe	c	c	c	c	c	c	str	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Steer pituitary, posterior lobe	c	c	c	c	c	c	tr	ac	c	c	c	c	0	ac	c	c	c	c	str	c	c	c	c	c	c	c	c	c	c	c

* Calcium acetate extract of lyophilized, benzene-extracted brain.

‡ Benzene-extracted, lyophilized monkey brain.

§ Residue from calcium acetate extract of lyophilized, benzene-extracted brain.

TABLE II

Reactions of Sera from Monkeys Injected with Brain Tissue from Several Species in Complement Fixation Tests with Lyophilized Brain and Placental Suspensions

Monkey No.	Brain material injected		Clinical signs of encephalomyelitis	Histologic signs of encephalomyelitis	Suspension used as antigen in complement fixation				
	Source	Nature			Monkey brain	Steer brain	Brain from 3 day old rabbit	Fetal rabbit brain	Rabbit placenta
2-69	Monkey	Fresh	+++	+	++	++	++		
5-18	"	Lyophilized	++	+	++	++	−		−
5-07	"	Benzene-extracted residue, calcium acetate-extracted	++	+	+++	+++	±	±	−
2-81	"	Fresh (1/27)	+++	+	+	−	−	−	−
4-62	"	" (1/18)	++	+	+			−	−
4-69	"	Lyophilized	++	+	+++		++	−	−
2-24	"	Formalinized	+++	+	+		++	+++	+++
2-77	"	Fresh (1/9)	+++	+	++		±	−	−
4-03	"	Sediment from saline-extracted brain (1/6)	++	+	−		+	±	±
1-46	Rabbit	Cerebrum from 6 day old animal	−	−	++	++	+++	+++	++
1-50	"	Spinal cord from 6 day old animal	+++	+	+++	+++	+++	+++	+++
1-65	"	Cerebrum from 2 mo. old animal	+++	+	+++		+++	+++	++
1-83	"	Spinal cord from 3 day old animal	+++	+	++		++	+++	+++
1-85	"	" "	++	+	+++		++	++	+
1-86	"	Cerebrum from 3 day old animal	−	−	−		++	+++	+++
1-87	"	" "	−	−	−		++	+	−
1-89	"	" "	−	−	±		++	+++	−
7	"	Fresh	−	−	−		−	+++	−
5-55	Steer	Lyophilized	++	+	+++	+++	+++	+++	++
2-21	Human	Fresh	++	+	+++	+++	+++	+++	−

In Tables II to VII; [1]clinical signs are recorded as follows: +++ means severe symptoms of encephalomyelitis; ++ moderate and + slight. Histological features of encephalomyelitis are recorded as present (+), or absent (−). The fraction signs in parentheses after "material injected" indicate the dilutions of brain used.

and with monkey peripheral nerve; 1 of these, however, showed as strong an effect with rabbit placenta as with brain. The tenth animal (No. 2-24) although injected with monkey brain produced significant amounts of complement-fixing antibody only to rabbit placenta.

Table II provides further data on the organ specificity of the antibodies for brain tissue in many of the sera. Nine sera from monkeys injected with monkey brain are given; all these animals developed encephalomyelitis. Of the first 5 which failed to react with rabbit placenta, the 3 which reacted best with monkey brain fixed equally well with steer brain and 1 of these reacted with rabbit brain. The single animal injected with steer brain reacted better with the brains of all 3 species than with placenta; monkey 2-21 injected with human brain fixed complement strongly with monkey, steer, and rabbit brain, but not with placenta. Other patterns of antibody response are also apparent. Two sera from monkeys injected with rabbit brain produced antibodies to 3 day old and fetal rabbit brain but none to rabbit placenta or monkey brain; 1 animal reacted with fetal brain only.

Table III summarizes the complement fixation tests on a variety of sera from monkeys which had received emulsions of various tissues, some of which, as established by assay in monkeys, had encephalitogenic potency while others did not. The range of reactivity with the various organs is shown. Individual reaction patterns are readily discernible; some sera show no antibody, others only antibody to monkey brain or to rabbit placenta. Some sera show broader reactivity, reacting with two or three or four of the organ suspensions. These organs comprise a sharply restricted group including only brain, placenta, corpus luteum, and kidney with the first two giving the strongest reactions, corpus luteum was intermediate and kidney gave the weakest reactions. No evidence of the reactivity with testis reported by other investigators (31, 17) was found. Following immunization with non-encephalitogenic materials, antibodies only to placenta and to corpus luteum were found.

Eighty-two control sera from normal healthy untreated monkeys, 28 of which were subsequently injected and developed encephalomyelitis or complement-fixing antibodies or both, were tested with monkey brain. Most of them were tested against fetal rabbit brain and placenta and representative groups were tested against the other antigens. Results were uniformly negative except for 1 apparently healthy female in which a sample of blood serum taken 160 days after delivery of a healthy offspring reacted with rabbit placenta to a titer of 1:80; this result was verified on the same portion of serum on three separate occasions.

Additional data on 81 monkeys are summarized in Tables IV to VII, including 59 of the 89 monkeys injected with encephalitogenic material and 22 of the 32 animals injected with non-encephalitogenic material. The results are arranged to show the various patterns of complement fixation with different organ suspensions. The day of onset of signs and the day the blood sample was obtained are also given. Table IV shows 20 sera which fixed complement with monkey brain but not with rabbit placenta; a few of these sera also reacted with monkey peripheral nerve and with fetal rabbit brain. Table V

TABLE III

Complement Fixation Tests with Various Lyophilized Tissue and Organ Suspensions

Monkey No.	Material injected	Clinical signs of encephalomyelitis	Histological signs of encephalomyelitis	Tissue or organ suspension used as antigen in complement fixation											
				Brain	Placenta	Corpus luteum	Testis	Kidney	Adrenal	Heart	Liver	Spleen	Muscle	Skin	Lung
	Emulsions with encephalitogenic potency														
2-0	Monkey brain	+++	+	−	−	−	−	±							
2-1	" "	+++	+	−	−	−	−	−							
2-2	" "	+++	+	−	−	−	−	±							
4-2	" "	+++	+	+				±							
4-83	" "	++	+	+	+++	++	−	+							
5-71	" "	+++	+	+++	++	−	−	±			−			−	
3-1	White matter from monkey brain	+++	++	−	−	−	−	−							
5-60	Monkey brain, benzene-extracted residue	++	+	+++	−	−	−	−	−	−					
5-61	" " " " "	+++	+	+++	−	−	−	−	−	−	−	−	−	−	−
5-83	" " " " "	++	+	++	−	−	−	−						−	
5-84	" " " " "	++	+	+++	±	−	−	±						−	
5-87	Monkey brain, benzene-extracted residue calcium acetate-extracted	+++	+	+	−	−	−	−						−	
5-88	" "	++	+	++	−	−	−	−						−	
4-58	Monkey brain, residue after desoxycholate extraction	−	−	−	−	−	−				−			−	
1-3	Rabbit brain	++	+	−	++	−	−	−							
7-4	Cerebrum from 1 month old rabbit	+++	+	++	++	++	−	±	±	−	−	−	−	−	−
7-8	" " 12 day " "	−	−	−	++	+	−	−							
1-65	" " 2 month " "	+++	+	+++	+++	+	−	±	−	−	−	−	−	−	−
2-21	Human brain	++	+	+++	−	−	−	+	−	−	−	−	−	−	−
5-55	Steer brain (lyophilized)	++	+	+++	++	±	−				−			−	

	Emulsions which showed no encephalitogenic potency														
1-6	Fetal rabbit brain	–	–	–	++	+	–	–							
1-7	" " "	–	–	–	++	–	–	–							
1-48	Cerebrum from 6 day old rabbit	–	–	–	++	+	–	–							
2-11	Monkey testis	–	–	–	–	–	–								
2-12	" "	–	–	–	–	–	–								
1	Rabbit lung	–	–	–	+++						–	–	–	–	–
3	" "	–	–	–	++						–	–	–	–	–
4	" "	–	–	–	++						–	–	–	–	–
2-3	Serum from monkeys with encephalomyelitis intravenously	–	–	–	–	–	–	–							
1-56	Monkey brain emulsion without tubercle bacilli in right side, adjuvant emulsion without brain left side	–	–	–	++		–	–			–	–	–	–	–

gives the complement fixation results with 10 sera from animals injected with encephalitogenic material and with 9 sera from monkeys injected with non-encephalitogenic emulsions. All these sera fixed complement with rabbit placenta but not with monkey brain or, except for 1 animal, with monkey peripheral nerve; reactions with fetal rabbit brain were usually similar to or slightly weaker than those with rabbit placenta. Seven of the 10 animals in-

TABLE IV

Sera from Monkeys with Disseminated Encephalomyelitis Fixing Complement with Suspensions of Monkey Brain but Not Rabbit Placenta

Monkey No.	Material injected	Clinical signs of encephalomyelitis	Histologic signs of encephalomyelitis	Day of onset of signs	Day of blood sample	Complement fixation with			
						Monkey brain	Fetal rabbit brain	Peripheral nerve (monkey)	Placenta (rabbit)
	Emulsions with encephalitogenic potency								
4-69	Monkey brain	++	+	34	233	+++	−	−	−
2-02	" " (1/9)	++	+	36	43	+++	−	+++	−
2-50	" " (1/2)	+++	+	26	28	++	−	−	−
2-68	" "	++	+	51	60	++	−	−	−
2-77	" " (1/9)	+++	+	25	26	++	−		−
1-98	" " (1/3)	+++	+	26	30	+	−	−	−
2-66	" "	+++	+	23	26	+;±	−	−	−
2-81	" " (1/27)	+++	+	26	29	±	−	−	−
4-62	" " (1/18)	++	+	60	259	+	−		−
5-28	Monkey brain, benzene-extracted residue (1/9)	++	+	29	76	+++	−	−	−
5-84	" " (1/3)	++	+	25	25	+++	±	+	±
5-60	" " (1/3)	++	+	19	19	+++	−	±	−
5-61	" " (1/3)	+++	+	16	16	+++	−	−	−
5-32	" "	++	+	52	52	++	−	−	−
5-62	" " (1/3)	+++	+	20	20	++	−	−	−
5-83	" " (1/3)	++	+	24	24	++	+	−	−
5-07	Monkey brain, benzene-extracted residue calcium acetate-extracted	++	+	103	103	+++	±	±	−
5-88	" " (1/3)	++	+	23	23	++±	−	++	−
5-87	" " (1/3)	+++	+	22	22	+	−	−	−
2-21	Human brain	++	+	24	32	+++	+++	+++	−

jected with encephalitogenic brain material failed to react in the complement fixation test with monkey brain although they had received emulsions containing monkey brain. Table VI summarizes data on monkey sera which showed complement fixation with both monkey brain and rabbit placenta; the sera are divided into three groups; (1) those giving stronger reactions with brain than with placenta; (2) those with reactions of comparable intensity to both; (3) those with stronger reactions to placenta than to brain. Table VII presents 3 sera which reacted neither with monkey brain nor with placenta

but which showed complement fixation with fetal rabbit brain or with monkey peripheral nerve; data on 22 sera which showed no complement fixation with any of the 4 antigens are also included; 11 of these animals received encephalitogenic emulsions. Data on the remaining sera have not been included since they showed essentially the same patterns as those in Tables IV to VII. None

TABLE V

Sera from Monkeys Fixing Complement with Rabbit Placenta but Not with Monkey Brain

Monkey No.	Material injected	Clinical signs of encephalomyelitis	Histologic signs of encephalomyelitis	Day of onset of signs	Day of blood sample	Complement fixation with			
						Adult monkey brain	Fetal rabbit brain	Peripheral nerve (monkey)	Placenta (rabbit)
	Emulsions with encephalitogenic potency								
5-69	Monkey brain	++	+	32	29	−	+++		+++
4-03	" " A (1/6)	++	+	23	24	−	±	+	±
2-88	Own brain	+++	+	26	26	−	+	−	+++
2-98	" "	+++	+	19	20	−	±	−	++
3-71	Monkey brain B (1/2)	+++	+	29	261	−	+++		+++
7-8	Cerebrum from 13 day old rabbit	−	−	−	79	−	++	−	++
1-66	" " 3 month " "	−	−	−	138	−	+	−	+
1-3	" " adult rabbit (1 injection only)	++	+	22	46	−	++	−	++
	Emulsions which showed no encephalitogenic potency								
4-88	Monkey brain C	−	−	−	140	−	++	−	+++
4-87	" " "	−	−	−	140	−	+	−	++
1-7	Rabbit brain, fetal	−	−	−	49	−	++	−	++
1-6	" " "	−	−	−	40	−	+	−	++
4-1	" " "	−	−	−	60	−	+	−	++
1-86	Cerebrum from 3 day old rabbit	−	−	−	156	−	+++	−	+++
1-48	" " 6 day " "	−	−	−	165	−	++	−	++
1-47	" " 6 day " "	−	−	−	164	−	+	−	+
1	Rabbit lung	−	−	−	98	−	+	−	++
3	" "	−	−	−	94	−	++	−	++
4	" "	−	−	−	87	−	+	−	++

Monkey brain A, sediment from saline-extracted brain centrifuged at 19,000 R.P.M.

Monkey brain B, desoxycholate extract of "A."

Monkey brain C, whole monkey brain but with streptococcus toxin instead of killed tubercle bacilli, as adjuvant; none of these animals developed encephalomyelitis.

of the sera reacted with suspensions of lyophilized monkey heart, lung, liver, spleen, muscle, or skin; 45 sera tested with lyophilized monkey testis were also negative. Eight of 48 sera tested with monkey corpus luteum reacted, the highest titer being 1:10; all of these 8 reacted with rabbit placenta, but there appeared to be no quantitative parallelism in the reactions to the two antigens. Some sera which reacted with placenta failed to react with corpus luteum.

Nine of 57 sera tested reacted in low titer with monkey kidney; 3 of these showed no reaction with placenta.

Monkey Cerebrospinal Fluids.—The 22 fluids examined were all taken from monkeys injected with monkey brain tissue and were tested undiluted and diluted 1:3 and 1:9. In no instance were there any reactions with monkey brain. With rabbit placenta, however, 4 of 17 fluids fixed complement when undiluted

TABLE VI

Sera from Monkeys Which Fixed Complement with Monkey Brain and Rabbit Placenta

Monkey No.	Material injected	Clinical signs of encephalomyelitis	Histologic signs of encephalomyelitis	Day of onset of signs	Day of blood sample	Complement fixation with			
						Adult monkey brain	Fetal rabbit brain	Peripheral nerve (monkey)	Placenta (rabbit)
	Brain > placenta								
1-85	Spinal cord from 3 day old rabbit	++	+	81	83	++	++	+	+
1-65	Cerebrum from 2 month old rabbit	+++	+	38	45	+++	+++	+	++
2-06	Monkey brain (1/27)	–	–	–	121	++	±	–	+
5-70	" "	±	+	–	29	++	+		+
5-55	Steer "	++	+	44	45	+++	+++	++	++±
5-71	Monkey "	+++	+	27	27	+++	++±	±	++±
5-59	" " benzene-extracted residue	+++	+	20	24	++	++	–	+±
	Intermediate								
1-50	Spinal cord from 6 day old rabbit	+++	+	64	64	+++	+++	+++	+++
7-4	Cerebrum from 1 month old rabbit	+++	+	29	29	++	++	±	++
1-46	" " 6 day old rabbit*	–	–	–	82	++	+++	–	++
	Placenta > brain								
5-17	Monkey brain, benzene-extracted residue	–	–	–	106	+	+	–	++
1-83	Spinal cord from 3 day old rabbit	+++	+	87	88	++	+++	–	+++
4-82	Monkey brain (1/9)	–	+‡	–	156	++	++±		+++
2-24	" " (formalinized)	+++	+	52	53	+	+++	–	+++
4-83	" " (1/9)	++	+	77	77	+	+++	+	+++
5-58	" " (1/3)	–	+‡	–	44	+	+++	–	+++
5-73	" "	++	+	30	29	+			+++

* Non-encephalitogenic emulsion.

‡ Few mild lesions at necropsy.

and 2 of these showed some fixation at 1:3 dilution; a fifth undiluted fluid showed a trace of fixation. The other 12 were completely negative. In 3 instances it was possible to compare the reactions obtained with serum and cerebrospinal fluid. The serum reacted with placenta to a titer of 1:80 in all 3 instances while 1 cerebrospinal fluid was negative and the others reacted when tested undiluted and in a 1:3 dilution respectively.

Human Sera.—Of the 3 sera from patients treated with antirabic vaccine 1 reacted with monkey brain to a titer of 1:5, and with human and steer

brain to a titer of 1:10. This serum, reacted, however, to a titer of 1:80 with rabbit placenta. The other 2 sera reacted at 1:10 and 1:2 respectively with placenta but showed no reaction with brain.

TABLE VII

Sera from Monkeys with Unusual Patterns and with Negative Complement Fixation Tests

Monkey No.	Material injected	Clinical signs of encephalomyelitis	Histologic signs of encephalomyelits	Day of onset of signs	Day of blood sample	Complement fixation with			
						Adult monkey brain	Fetal rabbit brain	Peripheral nerve (monkey)	Placenta (rabbit)
7	Cerebrum from adult rabbit	−	−	−	94	−	+++	−	−
1-89	" " 3 day old rabbit*	−	−	−	160	±	+++	−	−
2-72	Monkey brain (1/3)	+++	+	25	67	−	−	++	−
3-4	Monkey peripheral nerve*	−	−	−	62	−	±	±	±
2-0	Monkey brain	+++	+	15	15	−	−	−	−
2-1	" "	+++	+	32	32	−	−	−	−
2-2	" "	+++	+	30	41	−	−	−	−
9-9	" "	++	+	116	149	−	−	−	−
1-24	" "	+++	+	23	68	−	−	−	−
1-95	" " (1/3)	−	+‡	−	134	−	−	−	−
2-51	" " (1/2)	+++	+	20	20	−	−	−	−
4-05	Monkey brain, residue after desoxycholate extraction	++	+	29	44	−	−	−	−
4-58	" "	−	−	−	291	−	−	−	−
2-26	Monkey brain, formalinized	+++	+	27	27	−	−	−	−
3-1	" " white matter	+++	++	28	28	−	−	−	−
3-8	Rabbit " , fetal*	−	−	−	74	−	−	−	±
2-9	Monkey peripheral nerve*	−	−	−	65	−	−	−	−
3-0	" " "	−	−	−	65	−	−	−	−
1-51	Monkey brain, residue after ethyl alcohol extraction*	−	−	−	165	−	−	−	−
7-1	Adjuvants only intracerebrally*	−	−	−	148	−	−	−	−
2-19	Human brain§	−	−	−	151	−	−	−	−
1-40	Abdominal exudate and lymphoid tissue*	−	−	−	204	−	−	−	−
2-3	Monkey serum intravenously*	−	−	−	86	−	−	−	−
2-4	" " "	−	−	−	82	−	−	−	−
8-7	Rabbit " "	−	−	−	58	−	−	−	−

* Non-encephalitogenic material.

‡ Few mild lesions.

§ Brain emulsion without tubercle bacilli in right side; tubercle bacilli emulsion without brain left side.

The multiple sclerosis sera came from 45 patients with well established cases dating back 1 to 25 years; there were sera from 6 cases of suspected early multiple sclerosis and from one case diagnosed as neuromyelitis optica. All these sera were tested with the lyophilized human brain, monkey brain, and rabbit placenta used in the tests on the monkey sera. The complement fixation tests were completely negative throughout.

DISCUSSION

The data presented make abundantly clear the complexity of the antibody response to the injection of a heterogeneous group of antigens such as whole brain. Individual monkeys produced complement-fixing antibodies to one or more of the constituents of brain tissue and by examination of the various patterns of these sera a number of types of distinct antibodies could be recognized. One of these types is organ-specific brain antibody which reacts with human, monkey, rabbit, and steer brain, and with the posterior lobe of the pituitary but which fails to react with other tissues; this type of antibody was produced only by the injection of brain tissue but the species of brain employed was not of consequence, monkey, rabbit, human, and steer brain stimulating this type of antibody (Tables I, II, IV, and VI) in some instances.

A second type of antibody was detected primarily by its reaction with rabbit placenta. This antibody also appears to be "organ- " rather than species-specific in that it was produced by the injection of monkey, rabbit, or steer tissues. The antigen involved does not appear to be limited exclusively to brain and placenta since the antibody reacting to placenta was produced in the 3 monkeys injected with emulsions of rabbit lung (Tables III and V). This antigen appears to be responsible for most of the complement fixation reactions with fetal rabbit brain tissue in animals which showed no complement fixation with adult monkey brain (Table V) since the reactions to the two antigens are generally similar. With both the fetal brain suspension and the antisera to rabbit lung, the possibility of some species-specific antigen in addition to the placental antigen cannot be ruled out except by further study. It is not known to which constituent of monkey brain or rabbit placenta this reaction is attributable but it is possible that it may be vascular endothelium since both of these tissues are so highly vascular. The reactions with fetal brain would be consistent with this interpretation. Were this the case, however, it might be expected that other highly vascular tissues should also react. Reactions with kidney, however, were infrequent and relatively weak. This is not unexpected, since the antigens employed in the tests were prepared from whole lyophilized organs and the proportions of vascular tissue might vary from one to the other. Further studies using better sources of vascular endothelium such as the isolated glomeruli of Solomon *et al.* (32) might lead to a more definite identification of the antigen in brain and placenta responsible for this reaction.

Antibodies to other constituents of the brain tissue may also be formed but these are less common. Individual sera reacting strongly only with fetal rabbit brain (Table VII, Nos. 7 and 1-89) or only with monkey brain and peripheral nerve (Table IV, Nos. 2-02, and 5-88), or predominantly with brain and posterior pituitary (Table I, Nos. 1-65, and 5-55) or with brain but slightly or not at all with posterior pituitary (Table I, Nos. 5-07, 5-84, and 5-88)

serve as examples. Another type is represented by those sera which react with corpus luteum (Table III). The nature and significance of these antibodies are as yet unknown.

The complex nature of the antibody response to the injection of brain tissue should emphasize the difficulty of attributing any complement fixation reaction in serum to nervous tissue. The three sera from human beings given rabies vaccine (25) apparently produced antibodies to an antigen other than those of the neural elements of brain tissue. The extent to which antibody formation to antigens other than those specific for nervous tissue was a complicating factor in the studies on the relationship of complement-fixing antibodies to encephalomyelitis in dogs (27) and in mice (28) cannot be evaluated except by extensive serological studies. Since these latter animals were subjected essentially to the same immunizing procedure, it is probable that they too developed a multiplicity of antibodies.

The relation of the various complement-fixing antibodies to the encephalomyelitis is very difficult to determine. The antibody fixing complement with placenta can readily be excluded since it has been produced with non-encephalitogenic emulsions such as fetal rabbit brain and lung (Tables IV to VII). With respect to the complement-fixing antibodies to brain, the 89 sera from monkeys injected with encephalitogenic emulsions fell into 4 groups distributed as follows:—

Disseminated encephalomyelitis	Complement fixation		
	+	−	Totals
+	48	21	69
−	5	15	20
Total	53	36	89

From these data, it would appear that statistically there is a high degree of correlation between the appearance of complement-fixing antibodies to brain and the occurrence of disseminated encephalomyelitis. This, however, may merely be a consequence of the animals having responded generally in parallel to more than one of the antigens in the brain emulsion and does not necessarily in any way imply any etiological relationship between the complement-fixing antibodies and the encephalomyelitis. Indeed the existence of two groups, one showing complement-fixing antibodies but no clinical or histological evidence of encephalomyelitis (5 animals) and another larger group (21 animals) showing no complement-fixing antibodies but definite clinical and histological evidence of encephalomyelitis, weighs in the direction of no etiological relationship between the two phenomena. The first of these two groups, those with no encephalomyelitis but with complement-fixing anti-

bodies, does not contribute as convincingly to the evidence against an etiological relationship as it might, since of the 5 animals involved only 1 showed stronger complement fixation to brain than to placenta, 1 showed reactions of equal intensity to both, and the remaining 3 reacted more strongly to placenta than to brain. The possibility that 3 or 4 of these 5 might therefore have only antibody to the placental antigen and not to brain, cannot be excluded; were this the case 4 of these animals would fall into the group negative for both encephalomyelitis and complement fixation to monkey brain.

With respect to the animals showing encephalomyelitis an attempt to correlate the complement-fixing antibodies with the severity of the clinical disease gave the following results:

Severity of disease	Complement fixation reaction			
	−	+	++	+++
−	1*	3	3	0
+	0	3	2	0
++	8	9	7	5
+++	13	9	6	4

* No symptoms observed in this animal; few lesions histologically.

The data clearly show the absence of correlation between the degree of complement fixation and the severity of the encephalomyelitis; of especial significance is the finding that all but one of the 22 animals that showed no complement-fixing antibodies showed moderate or severe disease.

The data were scrutinized with respect to the comparability of the times at which the serum samples for complement fixation were taken in relation to the appearance of clinical symptoms. The median day for the appearance of symptoms in 197 monkeys with encephalomyelitis studied in this laboratory during the past 5 years was 30 with the first and third quartiles falling on days 25 and 44 with symptoms occasionally occurring up to 164 days. For the 68 animals with encephalomyelitis in this study the median day was 29 and the first and third quartiles on the 24th and 40th day respectively, symptoms occurring as late as 116 days; the incubation period in the experimental group of animals developing the disease was, therefore, representative. For the complement fixation studies the median day on which the serum samples were taken was 42 with the first and third quartiles occurring 27 and 82 days after the first injection: The bulk of the samples were taken in the 2 weeks immediately following the day of onset of symptoms. Of the 68 animals showing symptoms of encephalomyelitis all but 15 were examined in complement fixation tests within 1 week of the onset of symptoms. The 16 animals showing neither encephalomyelitis nor complement-fixing antibodies were bled from 28 to 291 days after the first injection.

It is also of interest that 4 animals which had shown signs of encephalomyelitis and had gone into a remission showed complement fixation reactions 180, 233, 239, and 259 days after the first injection of brain emulsion although they appeared well; at necropsy these animals showed histological evidence of encephalomyelitis.

The findings are probably best summarized by stating that the data show no correlation between the various complement-fixing antibodies and the encephalomyelitis and consequently provide no support for an hypothesis that the complement-fixing antibodies to brain which are demonstrable in the serum of monkeys injected with brain emulsions plus adjuvants are etiologically responsible for the encephalomyelitis.

SUMMARY

1. Animals injected with emulsions of monkey brain with adjuvants show a complex pattern of antibody response as determined by complement fixation tests.

2. Organ-specific complement-fixing antibodies to constituents of brain tissue may be formed which fix complement with brain tissues of various animal species but fail to react with other organs or with rabbit placenta.

3. Antibodies may be formed to some constituent of brain other than nervous tissue. It would seem that these can be detected by the strong complement fixation given with rabbit placenta.

4. Sera from individual animals may contain antibodies to the brain or placenta constituents, to both, or to neither. Occasional individual sera show unique patterns of antibody response as determined with various additional antigens such as fetal brain, posterior pituitary, or peripheral nerves.

5. No evidence of any etiological relationship between the development of encephalomyelitis and the complement-fixing antibodies to brain demonstrable in the sera could be found. The complement-fixing antibody to the placental constituent was unrelated to the encephalomyelitis.

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