ACUTE DISSEMINATED ENCEPHALOMYELITIS FOLLOWING IMMUNIZATION WITH HOMOLOGOUS BRAIN EXTRACTS

I. Studies on the Role of a Circulating Antibody in the Production of the Condition in Dogs*

By LEWIS THOMAS, M.D., PHILIP Y. PATERSON, M.D., and BETTY SMITHWICK

(From the Division of Infectious Disease, Department of Medicine, Tulane University School of Medicine, New Orleans)

(Received for publication, May 8, 1950)

It is known that the brain tissue of various mammalian species contains an antigenic component which confers immunological organ-specificity, but not species-specificity, on extracts of this organ. The injection of suspensions of heterologous or homologous brain into rabbits (1–4) and monkeys (5) results in the appearance of a complement-fixing antibody which reacts with extracts of brain from various unrelated animals. Except for cross-reactions with testis and corpus luteum, reported by Lewis (6, 7), the antibody appears to be specifically directed against brain. The peculiar capacity of this tissue to call forth in the same species an immunological response ordinarily reserved for foreign substances has never been satisfactorily explained. It has been suggested that the component involved is of the nature of a haptene, since fresh homologous brain extracts require foreign serum or other adjuvants in order to act as antigens. The relation of this immunologic phenomenon to disseminated, so called "allergic" encephalomyelitis has not yet been established.

The experimental condition was first described in 1935 by Rivers and Schwentker (8), who observed that repeated injections of brain tissue extract in monkeys, continued over a long period of time, resulted in a demyelinating encephalopathy bearing certain pathological resemblances to multiple sclerosis. The same authors (4) reported the occurrence of paralysis in rabbits following immunization with autolyzed or vaccinia-infected rabbit brain, or fresh brain combined with foreign serum. An antibrain antibody was demonstrated in the sera of immunized rabbits, but its significance could not be evaluated because of the absence of definite histological lesions in paralyzed animals.

Recently several groups of workers (9–16) have reported the production of a greatly accelerated form of demyelinating encephalopathy in monkeys, guinea pigs, rabbits,

* This investigation was aided by grants from the National Multiple Sclerosis Society and the Life Insurance Medical Research Fund.

and mice, by the incorporation of homologous brain extracts in the adjuvant mixture of oil and dead tubercle bacilli which was devised by Freund (17). The augmenting effect of adjuvants, and the incubation period of 3 or more weeks between the time of injection and the onset of the experimental condition, have been cited as evidence in support of the view that the encephalopathy is "allergic," involving a reaction between autologous antibrain antibody and a constituent of living white matter. However, there is as yet no *direct* evidence that the condition is caused by an antibody. In guinea pigs, a species highly susceptible to the experimental condition, several investigators (11, 12, 15) have been unable to demonstrate a circulating antibody following immunization with guinea pig brain; animals given heterologous brain produced an antibody which did not appear related to the condition. Hill (18) has described a complement-fixing antibody for saline extracts of homologous brain in guinea pigs, but correlation of this antibody with encephalomyelitis was not demonstrated. Working with monkeys, Kabat and his associates (19) were unable to transfer the condition by the injection of massive quantities of serum from immunized to normal animals. Kopeloff and Kopeloff (5) showed that monkeys are capable of forming a complement-fixing antibody for homologous brain, but the relationship of the antibody to the demyelinating condition was not clarified.

The present paper is concerned with a study of experimental encephalomyelitis in dogs, and with the role of a circulating antibrain antibody in the condition.

Materials and Methods

Dogs.—Mongrel dogs of various ages and sizes were obtained in the city of New Orleans and its environs and were quarantined in the animal house for at least 1 month before use. A few of the test animals were born and raised in the animal quarters. The dogs were kept in individual cages and maintained on a diet consisting of a cooked mixture of horsemeat and commercial dog food (kibble-meal). Each dog was examined for neurological abnormalities at least once each day during the period of immunization. Spontaneous encephalomyelitis or paralysis did not occur in any of the stock animals during this investigation.

Antigens and Adjuvants.—Brain tissue was obtained from normal healthy dogs which had been under observation in quarantine for a period of at least 1 month. Stock supplies of normal brain were frozen for storage at −20°C. between experiments. Weighed samples of sterile tissue were emulsified in physiological saline as 50 per cent suspensions, in a Waring blendor, and the gross particles allowed to settle out by standing or by light centrifugation. The suspensions were combined with adjuvants as described below. Antigens made from twenty-five individual normal dog brains were used in the experiments to be reported. In all dogs which received two or more injections, different brain tissues were used for preparing successive antigens.

The method described by Freund (17) was employed for the preparation of antigen-adjuvant mixtures. One part of arlacel-A[1] was mixed with nine parts of bayol-F[2] containing 0.5 mg. per ml. of dry, heat-killed tubercle bacilli, and the combination was added to an equal volume of brain tissue suspension. The whole mixture was emulsified by drawing in and out

[1] Arlacel-A is the trade name for mannide monooleate, supplied through the courtesy of the Atlas Powder Co., Wilmington.

[2] Supplied through the courtesy of Esso Standard Oil Co., New York.

of a 20 ml. syringe, yielding a white creamy suspension. The antigens were prepared under aseptic conditions and used on the day of preparation. The dogs were given a subcutaneous injection of 1 ml. of the antigen-adjuvant mixture in each of two areas along the back. The injections were repeated at intervals of 2 to 5 weeks, until neurological symptoms occurred. Three dogs were given comparable injections of the adjuvant mixture without brain extract for serological controls.

Sera.—All dogs were bled from the jugular vein or directly from the heart at intervals of 7 to 10 days throughout the course of immunization. The serum was obtained as soon as possible after clotting and stored frozen at −20°C.

Complement Fixation Tests.—Antigen, serum, and complement, in volumes of 0.2 ml. each, were mixed in appropriate dilutions and held at 37°C. for 1 hour; sensitized sheep cells were then added to each tube in a volume of 0.6 ml., consisting of 0.4 ml. of 2 per cent thrice washed cells and 0.2 ml. of rabbit amboceptor diluted to contain 4 units. After 30 minutes of additional incubation at 37°C., the results were read. The extent of hemolysis was recorded numerically, with 4+ indicating complete hemolysis and 0 indicating no hemolysis. Arbitrarily, 2+ hemolysis was selected as the standard end-point of complement fixation. All sera were inactivated by heating at 56°C. for 30 minutes before use. Guinea pig complement was used in an amount sufficient to contain 2 units per tube. The customary anticomplementary controls for sera and antigen, as well as hemolytic controls for unknown antigens, were included in all tests. It was found of importance to include normal dog sera as controls in all tests of tissue antigens. In most instances a sample of serum obtained before immunization was included in each test with the serum of an immunized dog; in some tests a pool of normal dog serum was employed as the normal control. Because of frequent anticomplementary reactions with undiluted serum, fixation in a serum dilution of 1–4 was taken to be the minimum titer of significance.

Tissue Studies.—Dogs were sacrificed by exsanguination, at varying times after the appearance of neurological signs. The brain, spinal cord, and samples of other tissues were fixed in 10 per cent formalin. Sections of brain and cord were stained with hematoxylin and eosin, and Smith's myelin stain. Tissues which were to be used for subsequent serological and biochemical studies or for inoculation into other animals were stored in a −20°C. refrigerator.

EXPERIMENTAL

The Production of Disseminated Encephalomyelitis in Dogs

In a group of fifty-five dogs, injected with homologous brain tissue antigen for varying periods of time, thirty-five animals developed symptoms indicating neurological disturbance. The distinguishing features of the illness were similar in all animals, although varying degrees of severity were encountered.

The onset was characterized by the sudden appearance of ataxia, which was followed within a few hours by paralysis of varying extent. The abruptness with which symptoms appeared, and the rapidity of their development were notable features of the condition. Several animals developed paralysis of all extremities within as short a time as 1 hour after having been in apparent good health. Two animals became completely blind during a phase of the condition, but regained vision within several days. In some dogs the neurological symptoms were transient; ataxia and weakness, sufficient to produce an awkward gait with occasional falling, occurred for a period of 1 or 2 days, after which the animals again behaved in a normal fashion. Death which appeared to be a direct result of the encephalomyelitis occurred in eight dogs. Several animals remained alive during a period of total paralysis lasting 3 or 4 days and then recovered completely; in some dogs the process of recovery seemed to occur almost as quickly

and suddenly as the condition itself. Nine recurrences of the condition were observed in animals which received reinjections of antigen after spontaneous recovery from paralysis.

Detailed histopathological studies were made in fifteen animals, at various intervals after the appearance of neurological symptoms.

The lesions in the central nervous system were similar to those which have been described by others in monkeys (8, 21). Focal areas of myelin sheath destruction were seen throughout the brain and spinal cord, with the cervical and thoracic spinal cord being most extensively involved. In almost every instance the lesions were closely related to blood vessels, and the walls of the vessels themselves showed conspicuous abnormalities. The veins, venules, and capillaries were commonly involved, with damage to veins being the most pronounced. The walls were infiltrated by polymorphonuclear neutrophils and lymphocytes, and surrounding the vessels were dense accumulations of lymphocytes and large epithelioid cells apparently of adventitial origin. Necrosis of all elements of the vessels was occasionally seen in small venules and capillaries, sometimes attended by perivascular hemorrhages. In all areas where myelin sheath destruction was evident, there was also extensive inflammatory cell infiltration. Pure demyelination unaccompanied by inflammation was not seen. Involvement of nerve cells in the inflammatory reaction was apparent in some areas, but in general the lesions were confined to the white matter. In some animals the destruction was severe enough to cause the loss of all recognizable tissue elements with cavitation. In all instances a moderate degree of leptomeningitis was present, characterized chiefly by lymphocytes. No involvement of nerve roots after their emergence from the spinal cord was evident. Organs and tissues other than the central nervous system showed no histological abnormalities.

The total incidence of the experimental condition in dogs was 63.6 per cent, a figure which is somewhat lower than that reported by workers with other species (9–13). These results represent the accumulated instances of paralysis in fifty-five dogs during a period of 12 months, with repeated immunizations at 2 to 5 week intervals throughout this time. The time of occurrence of paralysis is illustrated in Table I, in which it will be seen that during the first 8 weeks of immunization only sixteen of the fifty-five dogs, or 29 per cent, developed the condition. In twelve animals symptoms did not appear until immunization had been carried on for 6 months or longer. In only eight animals did paralysis occur following a single injection of antigen.

In view of the irregularity with which the condition occurred and the comparatively long periods of immunization required, it was of interest that the "incubation" period, computed as the number of days between the last injection and the onset of neurological symptoms, was extremely uniform. As is shown in Table I, the onset of paralysis in all thirty-five dogs occurred between 6 and 15 days following the most recent injection of antigen. This is especially noteworthy in the eight dogs which developed symptoms after a single injection of antigen; five became ill between the 6th and 9th days following injection. Similarly, in the animals receiving multiple injections, more than 60 per cent became paralyzed within 9 days following their most recent

inoculation. The total number of injections given and the length of time between the last two injections had no evident effect on the incubation period.

Three control dogs were given repeated inoculations of the adjuvant mixture without brain tissue for a period of 8 months. No neurological symptoms nor evidences of encephalomyelitis occurred in these animals. Several animals were given repeated injections of 20 ml. of 10 per cent suspensions of homologous brain in saline and observed for a period of 2 months; no symptoms appeared in these dogs.

TABLE I

Time of Occurrence of Paralysis in 35 Dogs after Immunization with Brain-Adjuvant Antigen

No. of injections before paralysis	No. of paralyzed dogs	Time of onset of paralysis													
		No. of wks. after first injection				No. of days after last injection									
		0–2	2–8	8–24	24–48	6	7	8	9	10	11	12	13	14	15
1	8	6*	2			1*	1	2	1			1			2
2	3		3			1	1		1						
3	4		4				1		1	1	1				
4	5		1	4		2	2					1			
5	2			2		1			1						
6	2			1	1			1		1					
7	4				4		1	1		1			1		
8	2				2		1				1				
9	2				2			1		1					
10	1				1							1			
11	1				1		1								
12	1				1		1								
Total.....	35	6	10	7	12	5	9	5	4	4	2	3	1		2

* Figures refer to number of dogs becoming paralyzed at indicated time.

Late in the course of these experiments, it was found that arlacel-A was not essential for the production of the condition. Thirteen dogs were given repeated intradermal injections of homologous brain extract combined with paraffin oil and tubercle bacilli without arlacel-A, and five became paralyzed within 3 months. The incubation periods were similar to those encountered following injection of the complete adjuvant mixture. It is of particular interest that two dogs became paralyzed 6 and 7 days, respectively, after a single injection of the oil suspension without arlacel-A.

Five animals were injected with the adjuvant mixture containing brain tissue from a dog which had died as the result of the experimental condition. One of these animals became paralyzed 7 days after the injection, and the others remained well during a period of 8 weeks thereafter. Saline suspensions of brain and cord from several dogs with paralysis were inoculated into other animals, in attempts to demonstrate the presence of an infectious agent. Guinea pigs, mice, hamsters, and young puppies were given injections by intracerebral and intraperitoneal routes. Cultures of blood and tissue from the affected dogs were also made. In no instance was a transmissible agent encountered by these methods.

Because of the reported occurrence of a spontaneous demyelinating condition in dogs (22) continual observation was made of the other dogs in the animal house during the course of these investigations. Spontaneous "postdistemper" paralysis was not seen in any of the stock animals. Two dogs with hind-leg paralysis following respiratory infection were brought to the laboratory from outside sources. Histological sections of the brain and cord of these animals revealed lesions in the white matter which bore a striking resemblance to the experimental condition under study. Intracerebral and intraperitoneal inoculation of brain tissue from these animals into adult dogs, puppies, guinea pigs, and mice yielded negative results.

Antibrain Antibody in the Serum of Immunized Dogs

Reactions with Aqueous Tissue Extracts.—Preliminary complement fixation tests with antigens prepared from aqueous or saline extracts of normal brain tissue yielded variable and evidently non-specific results, in which significant differences were not consistently demonstrable between normal and immunized dog sera. These reactions were similar to those described by Kidd and Friedewald (23) between normal serum and the sedimentable component of various tissue extracts.

Elimination of the non-specific component was accomplished by heating and high speed centrifugation, in the following manner. Ten per cent aqueous suspensions of normal dog brain tissue were prepared in a Waring blendor, heated at 70°C. for 1 hour, and then centrifuged in an angle centrifuge at 14,000 R.P.M. for 30 minutes. The resultant almost water-clear supernatant fluid was then used as antigen in the complement fixation tests with sera from normal and immunized dogs.

Fourteen samples of normal serum yielded negative results. Tests of sera obtained at 10 day intervals in a group of thirty immunized dogs revealed the appearance of an antibody which reacted with the antigen in serum dilutions of between 1–8 and 1–512. In one animal, antibody was demonstrable on the 20th day following immunization; in the majority of instances it appeared between the 3rd and 4th week. Illustrative results of the complement fixation test with the aqueous antigen are presented in Table II, in which the reactions with sera obtained at various intervals from four immunized dogs are shown.

Aqueous extracts of brain tissue prepared in the above manner frequently became anticomplementary or developed the property of reacting non-specifically with normal dog serum after storage for several days in the refrigerator. It was therefore necessary to make fresh batches of antigen for each day of testing.

Reactions with Alcohol Extracts of Brain.—

Dog brain tissue was emulsified in distilled water in a Waring blendor and lyophilized. The dried material was added to absolute ethyl alcohol to make a 10 per cent suspension by weight, mixed in a Waring blendor, and allowed to stand for 24 hours at 37°C. The suspensions were then centrifuged, the clear supernatant fluid diluted 1–100 in physiological saline, and serial twofold dilutions were prepared from this for titration of antigenicity in complement fixation tests.

Results which were typical of those obtained in numerous tests with normal and immune dog serum are illustrated in Table II. Antigen dilutions of 1–100 or higher were not anticomplementary and gave no non-specific reactions with normal dog serum. In the presence of sera from immunized dogs, complement fixation occurred in antigen dilutions as high as 1–100,000. The extracts retained their specific activity during storage for several weeks

TABLE II

Complement Fixation Tests with Dog Sera before and after Immunization with Brain-Adjuvants, Using Homologous Aqueous and Alcohol-Soluble Antigens

Dog. No.	Days of immunization	Serum dilution													
		Aqueous antigen*							Alcohol antigen‡						
		1–4	1–8	1–16	1–32	1–64	1–128	1–256	1–4	1–8	1–16	1–32	1–64	1–128	1–256
[illegible]	0	2§	0	0	0	0	0	0	0	0	0	0	0	0	0
[illegible]	28	4	4	4	4	4	4	2	4	4	4	4	4	4	0
[illegible]	112	4	4	4	4	4	4	4	4	4	4	4	4	4	0
[illegible]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
[illegible]	28	4	4	4	4	4	4	2	4	4	4	4	4	0	0
[illegible]	84	4	4	4	4	0	0	0	4	4	4	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
[illegible]	72	0	0	0	0	0	0	0	0	0	0	0	0	0	0
[illegible]	166	4	4	4	4	4	0	0	4	4	4	4	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31–15	30	4	4	4	2	0	0	0	4	4	4	0	0	0	0
	90	4	4	4	4	4	4	4	4	4	4	4	4	4	2

* 10 per cent water extract of whole dog brain, heated at 70°, centrifuged at 14,000 R.P.M.

‡ 10 per cent alcohol extract of lyophilized dog brain, diluted 1–100 in physiological saline.

§ Numbers refer to degree of fixation of complement. 4 = complete fixation; 0 = no fixation.

at 37°C. When stored in the refrigerator or at room temperature, some preparations underwent a sharp loss of antigenicity which was associated with the appearance of a visible precipitate; heating of the extract at 56°C. put the material back in solution and restored the antigenic titer.

It was of interest to determine whether the antibody reacting with the aqueous and alcoholic antigens represented a single antibody. For this purpose, cross-absorption tests were set up in the following manner:

A 1 ml. aliquot of serum from an immunized dog was mixed with the sediment obtained by centrifuging 10 ml. of a 10 per cent aqueous suspension of normal dog brain at 14,000 R.P.M. At the same time another aliquot of the same serum was mixed with the dried residue

from a 10 ml. sample of alcohol extract. Both mixtures were placed in the refrigerator for 18 hours, then centrifuged at 14,000 R.P.M., and the supernatant serum tested. A control sample of unabsorbed serum stored and centrifuged in the same manner, was included in the test.

Results which illustrate those obtained in several experiments are shown in Table V, which includes other absorption data to be discussed later. It will be seen that the absorption of immune serum with either the aqueous or alcohol-soluble material removed all of the demonstrable antibody for both antigens.

The antibody which reacted with the aqueous and alcohol-soluble antigens was not affected by heating at 60° and 62°C. for 30 minutes; it was partly inactivated at 65° and eliminated at 70°C. Fractionation of a sample of immune serum by ammonium sulfate precipitation revealed that the antibody was contained in the globulin fraction.

Sources of Alcohol-Soluble Antigen.—

It has been reported by others that the tissue component responsible for allergic encephalomyelitis is present in adult brain tissue from various mammalian species, in peripheral nerve (20), and is not inactivated by treatment with 10 per cent formalin or by prolonged autolysis (19). It has also been shown that the tissue component is not present in other organs, and is absent from brain tissue from newborn animals (19). It seemed of importance to learn whether the alcohol-soluble antigen which reacted in complement fixation tests was similarly distributed. Accordingly, alcohol extracts were prepared from various samples of brain, nerve, and other tissues and tested as antigens in the presence of immune and normal dog serum. The results are included in Table III. It will be seen that complement fixation reactions which were comparable to those obtained with dog brain antigen were elicited with extracts of brain from other mammalian species, as well as with peripheral nerve from dog and guinea pig sources. An autolyzed guinea pig brain, which had been allowed to stand at 37°C. for 2 weeks, and a sample of dog brain which had been stored in 10 per cent formalin for 1 month, each yielded the alcohol-soluble antigen in amounts comparable to fresh brain. No antigen was demonstrable in brain extracts from newborn dogs, nor in other organs of adult animals including heart, lung, liver, kidney, spleen, testis, and adrenal. The cross-reactions reported by others between brain and testis (6) could not be demonstrated in alcohol extracts of dog, rabbit, guinea pig, and human testis.

Properties of Antigen.—

The solubility of the antigenic component in normal dog brain in various lipid solvents was studied.

Ten per cent extracts of lyophilized brain were prepared with ethyl ether, petroleum ether, acetone, benzene, and chloroform. Since some of the solvents were unsatisfactory for the

preparation of saline dilutions, each extract was dried and the residue redissolved in absolute ethyl alcohol.

It was found that the titers of antigen obtained with each of the solvents were similar, and were approximately the same as those with the original alcohol extracts.

TABLE III

Sources of Alcohol-Soluble Antigen for Complement Fixation with Immunized Dog Serum

Antigen*		Immune serum		Normal serum	
Tissue	Species	Antigen titer‡	Serum titer	Antigen titer	Serum titer
Brain	Dog	12,800	128	0	0
"	Guinea pig	6,400	128	0	0
"	Rat	3,200	128	0	0
"	Mouse	3,200	128	0	0
"	Rabbit	6,400	128	0	0
"	Man	12,800	128	0	0
Peripheral nerve	Dog	6,400	128	0	0
" "	Guinea pig	6,400	128	0	0
Autolyzed brain	" "	6,400	128	0	0
Formalinized brain	Dog	6,400	128	0	0
Newborn "	"	0	0	0	0
Lung	"	0	0	0	0
Liver	"	0	0	0	0
Spleen	"	0	0	0	0
Kidney	"	0	0	0	0
Heart	"	0	0	0	0
Erythrocytes	"	0	0	0	0
Skin	"	0	0	0	0
Adrenal	"	0	0	0	0
"	Guinea pig	0	0	0	0
Testis	Dog	0	0	0	0
"	Rabbit	0	0	0	0

* Each antigen consists of 10 per cent extract of whole tissue in absolute alcohol, diluted 1–100 in physiological NaCl.

‡ Titer indicated by reciprocal of highest dilution of antigen or serum causing fixation of complement.

Antigen titers determined in presence of 1–20 dilution of serum. Serum titers determined in presence of 1–100 dilution of antigen.

The antigen was resistant to mild acid hydrolysis. A solution of antigen in alcohol was refluxed for 1 hour, using 1 part of 2 N HCl for 3 parts of antigen, and the antigenic activity was compared with that of an untreated sample. No impairment in activity resulted from this procedure, as is illustrated in Table IV, and in several instances the titer was slightly increased following hydrolysis.

Saponification of the alcohol-soluble antigen was performed in the following manner:—

A 25 ml. sample of alcohol extract from lyophilized dog brain was mixed with 10 ml. of saturated sodium hydroxide, and the mixture was refluxed for 1 hour. The unsaponifiable fraction was then extracted in ether, washed several times with water, recovered from the ether by evaporation, and redissolved in the original volume of alcohol.

The results are illustrated in Table IV. All the antigen appeared to be contained in the unsaponifiable fraction of the alcohol extract. A similar

TABLE IV

Antigen and Serum Titers in Complement Fixation Tests with Various Brain Extracts

Antigen	Immune serum		Normal serum	
	Antigen titer*	Serum titer‡	Antigen titer*	Serum titer‡
Alcohol brain extract, untreated§	12,800	64	0	0
" " " acid-hydrolyzed	12,800	64	0	0
" " " unsaponifiable fraction	12,800	128	400	8
" " " after digitonin precipitation	12,800	64	0	0
Unsaponifiable fraction, " " "	12,800	64	400	8
Calf white matter lipid, acetone-soluble‖	6,400	128	0	0
" " " " , ‖ unsaponifiable fraction	12,800	128	400	8
Proteolipid A, acetone extract‖	200	32	0	0
" B, " "‖	0	0	0	0
Liponucleoprotein, acetone extract‖	200	32	0	0
Cholesterol¶	200	32	200	32

* Antigens titrated in presence of 1–10 dilution of serum.

‡ Serum titrated in presence of 1–200 dilution of antigen.

§ 10 per cent extract of lyophilized dog brain in alcohol.

‖ 1 per cent extract of dry lipid made in acetone, evaporated, and redissolved in alcohol.

¶ 1 per cent solution of cholesterol (C.P.) in alcohol.

procedure was carried out with the acetone-soluble fraction of a sample of white matter lipid from calf brain, and the results were the same. In some instances, it was noted that saponification caused the appearance of non-specific reactions between the unsaponifiable fraction and normal dog serum, but these were not sufficiently marked to interfere with the detection of specific antigen in the preparations.

The identity of the antigen in the unsaponifiable fraction with that present in the whole aqueous and alcohol extracts was demonstrated by absorption tests, employing the procedure described above. Aliquots of immune serum were absorbed with dried residue from the unsaponifiable fractions, residue from untreated alcohol extract, and the sedimented tissue from an aqueous

extract of whole brain. The results are illustrated in Table V. Absorption with the unsaponifiable fraction removed all demonstrable antibody for the aqueous antigen as well as for the alcohol-soluble antigen. Conversely each of the latter two antigens removed all the antibody reacting with the unsaponifiable fraction. These results indicated that the same antibody was involved in the reaction of immune serum with the three preparations.

Considerable amounts of cholesterol were present in the alcohol extracts of brain and in the unsaponifiable fraction, as indicated by strongly positive Liebermann-Burchard reactions. Evidence that cholesterol was not the antigen under study was obtained in the following observations. No differences were observed in the reactions of immune and normal dog sera in complement fixation tests with alcoholic solutions of cholesterol (C.P. grade), although both

TABLE V

Absorption of Immune Dog Serum with Various Brain Tissue Components

Serum absorbed with	Serum tested against			
	Aqueous extract	Alcohol extract	Unsaponifiable fraction	Cholesterol
Aqueous extract	0*	0	0	32
Alcohol "	0	0	0	32
Unsaponifiable fraction	0	0	0	32
Cholesterol‡	128	64	64	32
Unabsorbed control	128	64	64	32

* Numbers refer to reciprocals of highest serum dilution producing complement fixation.
‡ 10 mg. C.P. cholesterol used for absorption with 1 ml. serum.

types of sera produced non-specific complement fixation (Table IV). Absorption of immune serum with cholesterol did not affect the titer of antibody for alcohol extracts of brain as is shown in Table V. Removal of cholesterol by precipitation with digitonin, employing equal volumes of 10 per cent brain extract in absolute alcohol and 1 per cent digitonin in 90 per cent alcohol, resulted in no reduction in the antigenic titer of the extract. Similarly, digitonin precipitation of the unsaponifiable fraction did not affect the antigen. These results are illustrated in Table IV.

Several samples of purified lipid fractions derived from calf brain tissue were obtained through the courtesy of Dr. Jordi Folch-Pi of Harvard Medical School. These materials included total white matter lipid from which proteolipids had been removed, proteolipid A, proteolipid B, and liponucleoprotein (24). Alcohol and acetone extracts of dried materials from each of these fractions were tested in the complement fixation test with normal and immune dog serum. The results are shown in Table IV. It will be seen that a high degree of antigenic activity was demonstrable in extracts of the white matter lipid

preparation, yielding results which were comparable to those obtained with the alcohol-soluble antigen from dog brain. Small amounts of antigen were also demonstrable in proteolipid A and liponucleoprotein, but not in proteolipid B. Repeated extraction of the white matter lipid with acetone resulted in the complete removal of antigen; the acetone-insoluble residue was inactive.

Flocculating Antibody in Immune Dog Serum.—

A flocculation reaction was demonstrated between the serum of immunized dogs and the alcohol-soluble antigen, by a modification of the standard Kahn procedure (25). The following method was employed.

Varying dilutions of a 10 per cent alcohol extract of lyophilized dog brain were made in physiological saline solution. The tubes were shaken for 3 minutes, and the highest concentration of antigen showing no spontaneous flocculation was employed as antigen. Sera were inactivated by heating at 56°C. for 30 minutes. Varying quantities of the diluted antigen, ranging from 0.05 ml. to 0.0125 ml., were mixed with 0.15 ml. of serum and shaken for 3 minutes, after which readings of flocculation were made.

TABLE VI

Flocculation Reaction with Normal and Immune Dog Sera

Dog No.	Day of immunization	Volume of antigen			Complement fixation titer*
		0.05 ml.	0.03 ml.	0.01 ml.	
31–11	0	0‡	0	0	0
	120	4	4	4	128
31–15	0	0	0	0	0
	140	3	4	4	64
50–40	0	0	0	0	0
	80	2	4	4	32

* Reciprocal of highest dilution producing fixation of complement.

‡ Degree of flocculation. 4 = complete aggregation with clear supernate; 0 = no flocculation.

The results obtained with three pairs of sera from immunized dogs are summarized in Table VI. In each instance the baseline serum was negative in the flocculation test, while the sera obtained following immunization, which produced complement fixation with the alcohol-soluble antigen, yielded strongly positive flocculation reactions.

Identity of the flocculating and complement-fixing antibody was demonstrated by absorption tests with several samples of immune dog serum in the following manner. A sample of immune serum was absorbed for 18 hours with a dry aliquot of the unsaponifiable fraction of the antigen, prepared as described

above. After absorption the serum was centrifuged at high speed, decanted, and tested for flocculation and complement fixation in the usual manner. Absorption with the unsaponifiable fraction removed the antibody responsible for the flocculation reaction as well as that which reacted in the complement fixation test. This observation indicated a close relationship between the antibodies involved in the two reactions.

Further evidence for this relationship was obtained by the observation that the antigen which reacted in complement fixation was contained in the insoluble floccules formed in the flocculation test. This was demonstrated in the following manner:—

A mixture of immune serum with the alcohol-soluble antigen was prepared in a proportion to yield optimal flocculation, and then centrifuged at low speed. The sedimented floccules were washed four times in physiological saline by high centrifugation and then extracted with absolute ethyl alcohol, at 37°C. for 1 hour. The alcohol-soluble material was tested as antigen in the complement fixation test with serum from normal and immunized dogs.

Immune sera reacted with the flocculated antigen in titers identical to those produced with the original alcohol extracts of whole brain. No reactions with normal sera were observed.

The foregoing observations were interpreted as indicating that the antibody participating in the complement fixation reaction was similar to that causing flocculation, and that the antigens involved were similar materials.

Attempts to Produce Encephalomyelitis with the Unsaponifiable Fraction of Brain Lipid.—

Other workers have described conflicting results in the production of allergic encephalomyelitis in animals by the use of various fractions of brain extracted with lipid solvents (12, 16, 19). It seemed important to learn whether the unsaponifiable fraction of brain lipid, previously shown to contain all the antigenicity found in whole alcohol extracts, had the property of producing the disease. Accordingly four dogs were inoculated with 25 mg. of dried residue from the unsaponifiable alcohol-soluble extract, in combination with tubercle bacilli and bayol. Three injections were given, at 2 week intervals. One dog died suddenly 6 days following the third inoculation, without any premonitory neurological symptoms. No histological lesions were found in sections of the brain and spinal cord of this animal. The other three animals remained well during the following 2 months. It is noteworthy that each of these dogs developed a complement-fixing antibody for alcohol extracts of homologous brain, similar to that encountered in animals immunized with whole dog brain in adjuvants.

In view of the difficulty with which the condition was produced in dogs even when whole brain suspensions were used, the significance of these negative re-

sults was in doubt. It was therefore decided to employ guinea pigs as the subject animal, since their susceptibility to the condition was reported to be considerably greater.

Twenty-four guinea pigs were inoculated with a suspension of the unsaponifiable fraction of dog brain lipid combined with adjuvants, prepared in the same fashion as described previously. Each guinea pig received approximately 10 mg. of the brain material in a volume of 1 ml. with tubercle bacilli in a final concentration of 0.25 mg. per ml. Between 8 to 30 days following injection, eight of the twenty-four pigs developed sudden weakness of the hind-legs, followed by coma and epileptiform convulsions leading to death in 24 hours. Brain and spinal cord tissues from each of the animals showed neuropathological changes similar to those described by others in allergic encephalomyelitis of guinea pigs, consisting of cuffing of small blood vessels, infiltration by round cells in and about the walls of veins and venules, and a mild degree of lymphocytic meningitis.

The rapidity with which animals died following the onset of symptoms was unlike the course of the condition in guinea pigs injected with whole brain. Moreover, the more chronic, granulomatous blood vessel lesions described by Lumsden (20) in guinea pigs with encephalomyelitis were not seen in the animals given the unsaponifiable brain fraction. It cannot be concluded from these experiments that the unsaponifiable fraction has the capacity to produce the same encephalopathy as whole brain.

The Relation of the Complement-Fixing Antibody to the Occurrence of Allergic Encephalomyelitis

No definite relationship between the appearance or titer of antibody and the occurrence of the encephalomyelitis was demonstrable. Some dogs developed antibody shortly before the onset of the state, while others exhibited equal or higher levels of antibody for many weeks before becoming paralyzed. Twelve animals became paralyzed without having had demonstrable antibody at any time during immunization. In several animals, the antibody appeared, disappeared, and reappeared during immunization without any accompanying symptoms suggesting brain disturbance. Of greater importance was the fact that antibody was not present in any serum taken within the first 3 weeks after immunization, yet eight dogs became paralyzed in this time.

In Table VII a summary is given of the maximum observed titers, the titers within 1 to 2 weeks before paralysis, and the titers on the day of paralysis. The absence of any consistent correlation with the condition is self-evident.

A comparable series of antibody titrations was undertaken in nineteen dogs which did not become paralyzed following immunization for periods varying from 30 to 300 days. It is obvious that this cannot be considered a true control group, since any or all of the dogs might have become paralyzed eventually if immunization had been continued for a long enough time. With this reservation, the results of antibody determinations in these nineteen dogs were com-

pared with those in thirty-five paralyzed dogs. The results are shown in Table VIII. It will be seen that the incidence of demonstrable antibody was actually

TABLE VII

Occurrence of Anti-Brain Antibody before and at Time of Paralysis in Immunized Dogs

Dog No.	Maximum antibody titer	Antibody titer 1-2 wks. before paralysis	Antibody titer on day of paralysis
1	64	8	8
2	32	4	4
3	0	0	0
4	64	0	4
5	0	0	0
6	0	0	0
7	256	16	0
8	0	0	0
9	32	0	16
10	64	16	64
11	16	16	4
12	256	64	64
13	32	8	8
14	16	0	16
15	64	0	0
16	8	0	4
17	16	16	4
18	64	16	16
19	4	0	0
20	32	0	32
21	8	0	4
22	0	0	0
23	128	32	128
24	16	16	0
25	16	0	16
26	0	0	0
27	32	0	32
28	0	0	0
29	0	0	0
30	0	0	0
31	8	0	0
32	0	0	0
33	16	0	0
34	0	0	0
35	0	0	0

higher in the non-paralyzed animals, whether one considers the level of antibody at any time during the course of immunization, or at the time of paralysis. The lower incidence of antibody is more striking in the sera drawn at the time of onset of paralysis. The percentage of dogs showing relatively high titer

antibody, *i.e.* 1–32 or higher, was considerably higher in the non-paralyzed group than in the dogs with the encephalomyelitis, and was lowest in the sera taken at the time of paralysis. These differences may be partly accounted for by the fact that some of the paralyzed animals developed the condition before the expected time of maximum antibody formation, including the eight dogs in which paralysis occurred within 3 weeks after the first injection.

TABLE VIII

Occurrence of Antibody after Immunization with Homologous Brain-Adjuvants, in Paralyzed and Non-Paralyzed Dogs

Group	No. of dogs	Antibody	No.	Per cent
Paralyzed	35	Demonstrable antibody at some time during immunization	23	66
		Demonstrable antibody at time of paralysis	17	47
		Antibody 1–32 or higher at some time during immunization	13	37
		Antibody 1–32 or higher at time of paralysis	5	14
Non-paralyzed	19	Demonstrable antibody	14	74
		Antibody 1–32 or higher	10	53

DISCUSSION

The assumption that the demyelinating condition under study is an allergic phenomenon has become generally accepted to the extent that the term "allergic encephalomyelitis" has come into common usage. On certain grounds it seems a reasonable assumption. It is known that brain tissue contains an organ-specific antigen, and the formation of antibody against homologous organ extracts has been demonstrated in some species. The method by which the condition is produced bears in outline a resemblance to conventional immunization procedures. Saline extracts of brain tissue alone cause demyelination only after many months and many injections, as was shown by Rivers and Schwentker (8). The addition of adjuvants, which markedly enhances antibody formation in other immunological systems, accelerates the condition. Good, Campbell, and Good (13) have presented substantial evidence for the inhibition of the condition in guinea pigs by large doses of salicylate and *p*-aminobenzoic acid; these authors suggest that the formation of autoantibody may be impeded by the drugs. The presence of plasma cells in the damaged areas of brain has been cited by Campbell and Good (26) as indirect evidence for the allergic mechanism.

Proof of the allergic pathogenesis would require not only the demonstration of antibody involved but also a direct correlation between the presence of antibody and the occurrence of the encephalomyelitis. Serological studies such as those employed in the present investigation might fail to provide the needed evidence, since the issue may be determined by tissue antibody rather than by circulating antibody, and determinations of the latter may not give information about the existence of the former. With this important reservation, the data presented here lend no support to the allergic theory. An antibrain antibody has been demonstrated in the serum of dogs following immunization with homologous brain antigens, but a definite relationship between this antibody and the condition could not be established. In some animals the antibody appeared in high titer several months before the onset of paralysis, while in others the encephalomyelitis occurred at a time when antibody was not demonstrable. It is conceivable that the same antibody may have been present in brain tissue at the time of the pathological disturbance, in concentrations not dependent on the level of circulating antibody, but no information concerning this point is available.

The possibility must be considered that the antibody in dog serum may represent an immunological event which is irrelevant to the condition. If, as has been suggested (12), several different kinds of antibody appear following immunization with homologous brain, the problem of relevance would become more complex. The results in this study suggest that only one antibody is implicated in the complement fixation and flocculation reactions, and that this antibody is directed against a component in the unsaponifiable fraction of brain lipids. The observation that a condition resembling allergic encephalomyelitis could be produced in guinea pigs by the injection of this fraction constitutes inadequate circumstantial evidence, since the actual identity of the condition with that caused by whole brain antigens is in question. However, other points of similarity between the complement-fixing antigen and the component causing the condition may represent more than coincidence. Both have been shown, either in the present study or by others (4, 19, 20) to be present in adult brain and peripheral nerve, absent from fetal or newborn brain, and resistant to high temperatures, autolysis, and formaldehyde fixation.

The time of occurrence of paralysis in immunized dogs is difficult to explain on the basis of a circulating antibrain antibody. Of the total group of thirty-five dogs with paralysis, six developed the encephalomyelitis in the 2nd week following immunization, at a time when no antibody was demonstrable. Five of these animals became paralyzed between the 6th and 9th day after the injection. A similar incubation period was observed in animals in which two or more injections of brain-adjuvant mixture were required. It is more difficult to explain the occurrence of the condition following injection of brain tissue in paraffin oil without the emulsifying agent customarily employed in adjuvant

mixtures. In experiments currently in progress, no circulating antibody has been demonstrable in seven dogs injected with this type of material during a 3 month period of immunization.

It is known that local antibody production against various antigens may begin within a day or so of immunization, and it is conceivable that an antibody may appear within the central nervous system before it is demonstrable in the circulating blood. In other circumstances, local concentrations of tissue antibody are caused by the presence of antigen within the tissue. In the condition under study, this would imply either that antigen from the injected brain-adjuvant mixture was deposited in brain tissue, or that a component of living intact brain tissue was capable of providing a local antigenic stimulus. Both possibilities are of speculative interest but inaccessible, at present, to experimental proof.

The possibility that the condition may be due to the action of a toxin has been mentioned by others (12, 20), and cannot be excluded by the available data. It is conceivable that a component of brain which is both toxic and antigenic could be released under conditions favoring autolysis, such as might exist locally in the injected brain-adjuvant mass. In this case, the antibody might represent a protective reaction. This would be compatible with the relative resistance of dogs as compared with other species, the lower incidence of antibody at the time of paralysis, and the short incubation period for the condition in dogs. It might also account for the observation by Schwentker and Rivers (4) that autolyzed brain extracts produced paralysis and antibody formation in rabbits, while fresh brain had no effect. Although no direct evidence for a toxin has been obtained in the present study, the matter warrants further exploration.

The nature of the antigen in the complement fixation reaction, and its relation to the component of brain which produces the encephalomyelitis, constitute the core of the problem. When these materials are obtainable in chemically isolated form, it should be possible to determine whether the serological phenomenon is an aspect of disseminated encephalomyelitis, and whether the condition is actually caused by antibody.

SUMMARY

1. A severe demyelinating condition characterized by ataxia and paralysis, in some instances leading to death, was produced in thirty-five of a total of fifty-five dogs following immunization with homologous brain tissue combined with Freund's adjuvants. In more than 30 per cent of instances paralysis did not occur until immunization was continued for 6 or more months. Only eight dogs became paralyzed after a single injection of antigen. The condition appeared between 6 and 15 days after the last injection in all animals, irrespective of the total number of injections or the duration of immunization.

2. An antibody which reacted in complement fixation tests with aqueous and alcoholic extracts of homologous brain tissue was demonstrable in the majority of immunized dogs, whether or not the animals became paralyzed. It appeared during or after the 3rd week of immunization, and its occurrence or titer could not be correlated with the incidence of the encephalomyelitis. In general, there were fewer dogs with demonstrable antibody in the paralyzed group than in the non-paralyzed group.

3. A flocculation reaction with alcohol extracts of homologous brain was demonstrated in the serum of immunized dogs. The antigen and antibody involved were apparently identical with those responsible for the complement fixation reactions.

4. The brain tissue component which reacted as antigen in the complement fixation test was present in adult brain from several mammalian species, and peripheral nerve. It was not present in the brain of newborn dogs nor in other unrelated organs. It was demonstrable in brain tissue which had been allowed to autolyze, or treated with 10 per cent formalin. It was not impaired by boiling, or by acid hydrolysis, and was contained in the unsaponifiable fraction of brain lipids. It was separable from cholesterol by digitonin precipitation of the latter.

5. Immunization of dogs with the unsaponifiable fraction of homologous brain, in adjuvants, caused the appearance of antibrain antibody similar to that in animals injected with whole brain. Encephalomyelitis was not observed during a 2 month period of immunization with this fraction.

6. In guinea pigs, an injection of the unsaponifiable fraction of brain, in adjuvants, was followed by fatal meningoencephalitis, but the identity of the state with that caused by whole brain antigens was not established.

BIBLIOGRAPHY

1. Brandt, R., Guth, H., and Mueller, R., *Klin. Woch.*, 1926, **5**, 655.
2. Witebsky, E., and Steinfeld, J., *Z. Immunitätsforsch.*, 1928, **58**, 271.
3. Lewis, J. H., *J. Immunol.*, 1933, **24**, 193.
4. Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.
5. Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944. **48**, 297.
6. Lewis, J. H., *J. Immunol.*, 1934, **27**, 473.
7. Lewis, J. H., *Am. J. Path.*, 1941, **17**, 725.
8. Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, **61**, 689.
9. Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.
10. Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.
11. Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.
12. Alvord, E. C., *J. Immunol.*, 1949, **61**, 355.
13. Good, R. A., Campbell, B., and Good, T. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 341.
14. Morrison, L. R., *Arch. Neurol. and Psychiat.*, 1947, **58**, 391.

15. Koprowski, H., and Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69,** 472.
16. Olitsky, P. K., and Yager, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70,** 600.
17. Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 548.
18. Hill, K. R., *Bull. Johns Hopkins Hosp.*, 1949, **84,** 285.
19. Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1948, **88,** 417.
20. Lumsden, C. E., *Brain*, 1949, **72,** 198.
21. Wolf, A., Kabat, E. A., and Bezer, A. E., *J. Neuropath. and Exp. Neurol.*, 1947, **6,** 333.
22. Innes, F. R. M., *Proc. Roy. Soc. Med.*, 1940, **33,** 171.
23. Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **76,** 543.
24. Folch, J., and Uzman, L. L., *Fed. Proc.*, 1948, **7,** 155 (abstract).
25. Eagle. H., Laboratory Diagnosis of Syphilis, St. Louis, C. V. Mosby Co., 1937.
26. Campbell, B., and Good, R. A., *Arch. Neurol. and Psychiat.*, 1950, **63,** 298.