A SPECIFIC COMPLEMENT-FIXATION TEST FOR INFECTION WITH POLIOMYELITIS VIRUS*

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In the preceding paper (1) the process through which the MEF1 strain of Lansing-type poliomyelitis virus was adapted to newborn mice has been described. In a previous publication (2) a complement-fixing antigen obtained with the newborn mouse-adapted strain was reported. It is the purpose of the present paper to describe in more detail the complement-fixation (CF) test for infection with poliomyelitis virus and to report the results of tests with sera from several animal species, including man.

Materials and Methods

Virus.—The MEF1 strain (3) of Lansing-type poliomyelitis virus adapted to newborn mice, designated hereafter the adapted strain, was used throughout for the preparation of poliomyelitis antigen and mouse hyperimmune serum. The MEF1 standard strain, that is to say, the one which is being propagated currently in the laboratory and passaged only in adult mice and the stock Lansing strain (Armstrong (4)) were employed for the preparation of immune sera in other species, and for neutralization tests.

The Nakayama strain of Japanese B encephalitis virus (J.B.E.) and the McMillan strain (5) of Western equine encephalitis virus (W.E.E.) were employed for the preparation of control antigens and immune sera. The J.B.E. virus had an unknown but large number of mouse passages; the W.E.E., from 6 to 10.

Antigen.—The material from which the MEF1 antigen was prepared consisted of brain and spinal cord tissue deriving from newborn mice. 3- and 4-day-old mice were inoculated intracerebrally with 0.02 ml. of a 10^{-1} dilution of the adapted virus. If 10^{-2} dilution was injected the result was a delayed incubation period. When the mice first became paralyzed, on the 2nd, 3rd, or 4th day following inoculation, they were exsanguinated[1] and the relatively blood-free central nervous system (CNS) tissue was harvested by means of an incision of the cranium

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[1] All such operations were carried out with the aid of deep ether anesthesia.

and spinal column thus exposing the tissues. It was later found that the more potent antigens were prepared from material collected on the 2nd day after inoculation; perhaps a still earlier period might yield better results—a matter now being investigated. The tissues were immediately frozen by placing them in a dish in a solid CO_2 alcohol bath and the pooled tissues from a number of mice were kept frozen at −20°C. until ready for use. This procedure was followed since harvested tissues held at room temperature for periods of about 1 hour softened rapidly and did not yield effective antigens.

Active antigens were first prepared with CNS of the 21st mouse passage of the adapted virus; no previous passage had been tested. From the 21st passage to the present 70th, usable antigens have been secured consistently.

20 to 25 gm. of tissue obtained from 80 to 100 mice sufficed for a single preparation of antigen. It was produced by a method previously described, (6), carried out as follows:—

The tissues were thawed, then homogenized, and rapidly extracted with 20 volumes of chilled acetone in an ice-packed Waring blendor. Following centrifugation the sediment was reextracted with acetone, once with a mixture of equal parts of acetone and anhydrous ethyl ether, and twice more with ether. Each of these successive extractions was allowed to proceed for 20 minutes during which time the bottle containing the material was held in an ice bath. After the last ether extraction, the residual ether was evaporated under an oil pump vacuum. The dry residue was then resuspended in physiological saline solution, the proportion used being 1 ml. of saline solution for each gram of wet tissue weight. The material was shaken by hand a few times, kept at 4°C. overnight, and centrifuged at 10,600 R.P.M. for 1 hour in an electrically driven angle head centrifuge. The supernatant fluid which constituted the antigen was pipetted off, merthiolate in final dilution of 1:10,000 added, and the antigen kept frozen at −20°C. The average yield of antigen was 0.6 ml. per gm. of tissue and its appearance slightly opalescent.

An identical procedure was followed for the preparation of control antigens from normal looking mice or from mice injected with J.B.E. or W.E.E. viruses. For those obtained from normal, uninfected mice, the CNS of animals 5 to 8 days old were used. When antigens were prepared with J.B.E. and W.E.E. viruses, the mice were 3 or 4 days old when injected with the former and 4 to 6 days old with the latter. The two control viruses were not propagated serially in newborn mice, but were derived from suspensions of the standard virus propagated in 3- to 4-week-old animals. Cord and brain tissue were harvested on the 4th day after injection of J.B.E. virus and on the 2nd day following injection of W.E.E. virus, at which times the inoculated mice showed signs of active infection.

Sera.—Sera from the following animal species have been tested: mouse, cotton rat, *rhesus* monkey, chimpanzee, and man.

Mouse.—Hyperimmune sera were prepared with the MEF1-adapted strain, J.B.E., and W.E.E. viruses. The procedure of immunization was as follows:—

A 10^{-1} suspension of brain and cord tissues from newborn mice infected with the respective virus was prepared in saline solution. Groups of 25 mice, 45 to 60 days old, were immunized by intraperitoneal injection of 0.5 ml. of this suspension three times at 7 day intervals. 10 to 12 days following the last treatment, the mice were bled from the heart under ether anesthesia. CF antibody was usually present at that time; if not, an additional injection or two of virus was given and the mice bled again 10 to 12 days after the last dose. They could be reinjected and bled six or seven times at reasonable intervals for an ample supply of immune sera. With J.B.E. and W.E.E. viruses, however, the mice were given 2 intraperitoneal injections of formalin-inactivated virus on the 8th and 10th day prior to the first inoculation of active virus, each dose being 0.25 ml. of a 10^{-1} suspension. This was done in order to prevent deaths which would result from an initial introduction of active virus.

Cotton Rat.—Cotton rat hyperimmune sera after being kept frozen for over 1 year have been found to contain specific CF antibody. The available sera were derived from a group of 13 cotton rats which had been immunized as follows:—

Mouse passage Armstrong strain was transmitted four times serially in cotton rats; brain and cord from passages 3 and 4, in a 10^{-1} suspension, were given intramuscularly, 0.5 ml. into each thigh, and 23 days later the rats were bled, the bloods pooled, and the serum kept frozen. 52, 74, and 81 days after the first injection, they were reinjected intraperitoneally with 2 ml. of a similar suspension; 10 days after the last injection, or 91 days from the beginning of immunization, the animals were bled again and the sera likewise stored at −20°C. The two pools of immune sera including several samples of sera derived from the same animals prior to immunization, were available for the CF tests. The particular interest in the former is that they were prepared with a strain of Lansing virus different from the MEF1.

Monkey.—Twenty-six different samples of *rhesus* monkey sera were available. Of these, 10 were paired sera from 5 animals; 14 were single samples from individual monkeys and 2 were pooled specimens from normal animals. The previous course of events in the monkeys is set down in Table IV. It will be noted that the animals were injected, or immunized, or convalescent from infection with the following strains of poliomyelitis virus: MEF1 and Armstrong, both Lansing type, as already stated; MV, also a Lansing type; Brunhilde, a non-Lansing virus; and Leon, also a non-Lansing virus, but serologically unrelated to the Brunhilde strain (7).

Chimpanzee.—Serial bleedings of 5 chimpanzees which received poliomyelitis virus orally were available. One of them, "Rosebud," was fed a non-Lansing-type virus (Pittsfield strain) first and a Lansing type (YSK) later. The other 4 were first fed YSK virus and later the Brunhilde. Detailed accounts of the sequence of events are shown in Table V.

Man.—Sera from 76 individuals were tested both by neutralization and CF methods. Of these, 41 were apparently normal individuals with no history of poliomyelitis; they were designated controls and 35 were persons recently attacked by poliomyelitis. Among the latter, a number supplied serial sera; another group contributed paired sera, and the remainder had been bled only once, during convalescence. The results of the neutralization tests were given by the laboratories which supplied the sera, or were determined in this laboratory. Furthermore, when paired or serial sera were tested, only the result of the CF and neutralization tests on one sample will be discussed here; the question of the development of CF antibody with time and its possible practical use as a diagnostic test is to be taken up in another publication.

All sera, human and animal, were inactivated at 60°C. for 20 minutes for the CF test (8) and at 56°C. for 30 minutes for neutralization, following the routine procedure of this laboratory.

Hemolytic System.—Commercially available sheep erythrocytes and antisheep hemolysin were used. The former, a stabilized suspension of washed and pooled cells, was prepared as a 3 per cent suspension and the hemolysin was diluted adequately so as to have 3 minimal hemolytic doses in the required volume. Cells and hemolysin were mixed and held at room temperature for 15 minutes before they were added to the other materials of a test.

Complement.—Fresh guinea pig serum was used occasionally as complement; ordinarily, however, a commercially available lyophylized preparation was employed. The latter had the advantage of a fairly constant strength, thereby insuring greater uniformity in the tests. The titers of the antigens were generally low; moreover, the antigens showed occasionally an enhancement of hemolysis by complement. Under these conditions an excess complement was likely to result in incomplete fixation or yield low readings. For these reasons it was found necessary to titrate the complement accurately and to add in the test no less than 1.6 units and no more than 2 units of complement, preferably, 1.8 units. Physiological saline solution,

0.85 per cent sodium chloride, was the diluent not only of complement but of all the materials of the test. Because of its importance, the titration of complement will be described here in detail.

Rehydrated lyophilized complement was diluted 1:29 in saline solution designated as original dilution of complement; adequate amounts of this dilution and of saline solution were placed in a set of 8 master test tubes, to produce a graduated series containing 0.16, 0.14, 0.12, 0.10, 0.08, 0.07, 0.06, and 0.04 ml. of the original dilution in each 0.3 ml. From these master tubes 0.3 ml. of each dilution was transferred to selected test tubes, 13 × 100 mm. To each tube of the latter series 0.2 ml. of saline solution was added, then 0.2 ml. of the cells plus hemolysin. After incubation at 37°C. for 30 minutes, the reaction was read; the smallest amount of complement giving complete hemolysis constituted one unit. The dilutions had been so calculated that ordinarily the unit of complement was 0.10 ml; therefore, in the test 0.18 ml. had to be added which was equivalent to 1.8 units.

The titration just described was the preliminary titration of complement; it indicated the amount to be used in the test. However, the true potency of the complement, as well as the anticomplementary power of the antigens, was determined in a second or final titration which was run along with the test proper in the following manner:—

One set of tubes for each antigen to be tested and an additional set for control, *i.e.* without antigen, were prepared. 0.3 ml. of the mixture in the master tubes was delivered in succession to each tube in each set, thus affording several series containing from 0.16 ml. to 0.04 ml. of complement, as just described. To each of the tubes of the first set, the saline solution controls, 0.2 ml. of saline solution was added; to the tubes in the next set, 0.2 ml. of an antigen to be tested, continuing in this way until all the antigens of the test were added to their own row of tubes. These sets, which, in effect, constituted a titration of complement in the presence of each antigen along with a saline control of complement, and the tubes of the test itself, were placed in the refrigerator at 4°C. for 18 hours. After this incubation and 30 minutes at room temperature the cells plus hemolysin were added and following incubation in the water bath for 30 minutes at 37°C., the test was read. By this procedure, the amount of free complement was determined in the presence of each antigen at the time when the cells plus hemolysin were added to the test proper. This procedure would show up any possible anticomplementary behavior of the antigens.

No loss of complement activity under the conditions described has been observed; on the contrary, the titer of complement in the presence of the antigens was often higher than it had been in the preliminary titration. As a result, the amount of free complement at the end of incubation in the first or fixation phase was, as a rule, between 1.8 and 2.0 units.

Table I illustrates the result of a typical titration of complement. Since experience showed that hemolytic or anticomplementary effect of the antigens could not be revealed in preliminary titrations they were consequently omitted. In the example tabulated, the unit of complement was contained in 0.10 ml.; 0.18 ml. was therefore used in the test. The final titration indicated that 1.8 units of complement were free in the presence of MEF1 and J.B.E. antigens as well as in the control having no antigen, and that 1.5 units were free with W.E.E. antigen.

Test.—All sera were tested with two control antigens along with the MEF1 antigen, and in addition, for anticomplementary power, *i.e.*, in the absence of an antigen. Serial dilutions of serum were prepared, beginning with dilution 1:2 and carrying them up to 1:32 or 1:64. 0.1 ml. of each dilution was delivered into the test tubes, of which there were 4 sets, one having no antigen; another, MEF1; a third, J.B.E.; and the last, W.E.E. antigen. Next, 0.2 ml. of the corresponding antigen was added, or in the control, 0.2 ml. of saline solution. The adequate amount of complement as determined by the preliminary titration was then introduced and the volume in the tubes was adjusted to 0.5 ml. with saline solution. The tubes containing

serum, antigen, and complement in a volume of 0.5 ml. were held at 4°C. for 18 hours, after which at room temperature for 30 minutes. Then were added 0.1 ml. of a 3 per cent suspension of sheep cells and 0.1 ml. of hemolysin diluted so as to have 3 minimal hemolytic doses. The tubes now contained a final volume of 0.7 ml. and were incubated in a water bath at 37°C. for 30 minutes. The reaction was read visually, 4+ representing no hemolysis and 0, complete hemolysis. A positive titer was defined as the highest dilution of serum giving a 2+ or better fixation.

TABLE I

Titration of Complement

Rehydrated lyophylized complement diluted 1:29

Antigen*	Preliminary								Final							
	Amount of diluted complement, *ml.*															
	0.16	0.14	0.12	0.10	0.08	0.07	0.06	0.04	0.18	0.16	0.14	0.12	0.10	0.08	0.07	0.06
None, saline	0	0	0	0	±	1	2	3	0	0	0	0	0	±	2	3
MEF1									0	0	0	0	0	1	2	3
J.B.E.									0	0	0	0	0	2	3	3
W.E.E.									0	0	0	0	±	1	3	4

* J.B.E., Japanese B encephalitis; W.E.E., Western equine encephalomyelitis.

TABLE II

Titrations of MEF1 Antigens with Hyperimmune MEF1 Mouse Serum

Serum dilution	Antigen in dilutions														
	Brain and cord, A				Brain, B				Cord, B					J.B.E.	W.E.E.
	1*	2	4	8	1	2	4	8	1	2	4	8	16	1	1
4*	4	4	1	0	4	4	2	0	4	4	4	±	0	0	0
8	4	4	0	0	4	4	0	0	4	4	3	0	0	0	0
16	4	3	0	0	4	4	0	0	4	4	3	0	0	0	0
32	4	3	0	0	4	4	0	0	4	4	2	0	0	0	0

4*, 1* = dilution factors of serum or antigens.

Table II shows the result of "box" titrations of 3 MEF1 antigens and mouse hyperimmune serum and the way such a test is carried out. Antigen A was the regular antigen derived from pooled cord and brain tissue; its titer was 1:2. Antigens B were derived from the same animals by harvesting, on one hand, the cord and brain stem and, on the other, the rest of the brain tissue. The titer of the antigen made from cord and brain stem was 1:4, that of the one prepared from brain was 1:2 to 1:4. The titer of the serum used in this test was at least 1:32, for no further dilutions were tested. No reaction whatever took place with the J.B.E. and W.E.E. antigens prepared from brain and cord infected with the corresponding virus.

Neutralization Test.—The neutralizing antibody of the Lansing type was disclosed by the method already described (8–10), the test being performed either with the Armstrong, or the standard MEF1 strain, and with dilutions of virus or dilutions of serum.

EXPERIMENTAL

Specificity of the Test.—An antigen prepared with MEF1 virus adapted to newborn mice and a technique for its use in a complement-fixation test to detect infection with a poliomyelitis virus were devised by application of the methods just described. Now the prime question was whether such an antigen and technique were specific for poliomyelitis virus, at least for the homologous Lansing-type strains. This question was answered by the results of repeated tests with Lansing-type hyperimmune mouse and cotton rat sera and with sera obtained from convalescent and immunized monkeys, as will be noted in Tables II to IV.

TABLE III

Complement-Fixation Tests with Immune Sera from Various Species and the MEF1 Virus

Serum	Antigens			
	None	MEF1	Japanese B	Western equine
Mouse, MEF1	0	64*	0	0
" , Japanese B	0	0	128+	0
" , Western equine	0	0	0	128+
Cotton rat, Armstrong	0	64	0	0
Monkey 1108, MEF1	0	8	0	0
" 19, Armstrong	0	16	0	0
" 16, Brunhilde	0	2		0

* 64 = reciprocal of highest dilution of serum giving a 2+ or better fixation; 0 = no fixation in 1:2 dilution of serum.

In Table II will be seen that complement fixation occurred only with an antigen prepared from MEF1 infected tissues, even though the Japanese B and Western equine encephalitis virus antigens added as controls were made in precisely similar ways as was the MEF1 preparation. The mouse serum, moreover, was shown to have antibody by means of a neutralization test for it neutralized 100, or more, LD_{50} of Lansing-type viruses, *i.e.*, to the possible limit of the test. It is also interesting to note that the MEF1 antigen made from spinal cord tissue alone exhibited a greater power of fixation than did the ones prepared from brain or from brain plus cord.

In Table III will be observed again that cotton rat and mouse Lansing-type hyperimmune sera in dilutions of 1:64 fixed complement specifically by the MEF1 antigen and not by those highly potent heterologous ones of Japanese B and Western equine encephalitis viruses. Additional evidence in Tables III and IV is brought forward to demonstrate that not only was hyperimmune serum obtained from the mouse and cotton rat positive but also from monkeys artificially immunized against poliomyelitis virus and convalescent from experimental poliomyelitis infection. Finally, one can see from these tab-

ulated results a certain even though slight degree of cross-reaction between the MEF1 or Lansing type and the Brunhilde, a matter which will be discussed later.

TABLE IV

Complement-Fixation Tests with Monkey Sera

Lab. No.	Designation of sera	Animal convalescent (C), immunized (I), or normal (N)	Length of time from onset in convalescents	Strain	CF antigen				
					MEF1	J.B.E.	W.E.E.	Normal	None
			days						
1	E483	I		Leon	0	0	0		0
2	D95	I		Brunhilde	0	0	0		0
3	MV D586	I		MV	8	0	0		0
8	K1816	I		Lansing	8	0			0
1108	1108	C	162	MEF1	16	0	2	0	0
1109	1109	C	92	"	16			0	
19	A1519	C	41	Lansing	16		0		0
20	A1503	C	41	"	2		0		0
178	2275a	C	2	"	0	0	0		0
179	" b	C	26	"	0	0	0		0
7	K3178	C	?	Minnesota	16		4		0
16	A1329	C	64	Brunhilde	2		0		0
17	A1322	C	68	"	0		0		0
18	A1325	C	67	"	0	0			0
170	A215a	C	0	"	0	0	0		0
171	" b	C	21	"	2	2	2		0
172	B216a	C	0	"	0	0	0		0
173	" b	C	20	"	0	0	0		0
174	C225a	C	1	"	0	0	0		0
175	" b	C	20	"	0	0	0		0
176	E207a	C	2	"	0	0	0		0
177	" b	C	19	"	0	0	0		0
3711	3711	C	241	"	8	0	0		0
4	NMS B8	N			0	0	0		0
15	Pool 2	N			0		0		0
4889	4889	N			0	0			0

It would thus appear from these repeated results that the complement-fixation test depending on the use of MEF1 virus antigen and immune serum deriving from mice, cotton rats, and monkeys is specific. Now that specific means for detection of an immune antibody were at hand, the next step was to determine how complement fixation could be used to detect antibody in a number of monkey, chimpanzee, and human sera which were then available.

Monkey Sera.—The results of the tests with 26 samples of monkey serum are shown in Table IV. While the specificity of the reaction was clearly ap-

parent, now and again a non-specific reaction occurred (Nos. 7 and 171) as one might expect in a series of tests. Such odd results could be easily detected and interpreted through the use of control antigens, as was done here. It will be observed that with immune sera, only the sera from animals immunized with the Lansing type, including the serologically related MV strain, reacted with the MEF1 antigen. Convalescent sera from monkeys injected with Lansing type gave positive reactions when the animals were bled 41 days or longer after onset of paralysis, while no reaction occurred with the serum of an animal bled on the 26th day after onset.

With respect to the possibility of a cross-reaction occurring between Lansing-type and non-Lansing-type viruses, sera from convalescent monkeys infected with the Brunhilde strain were negative in all cases bled within 68 days after onset. The reaction shown by serum 7 was non-specific and that given by serum 16 had too low a titer to be significant. On the other hand, serum 3711 showed repeatedly a positive reaction. This serum was obtained from a monkey which had no Lansing-neutralizing antibody and was injected with MEF6 virus, known not to be a Lansing type (3), and the animal was bled 241 days after onset. A suggestion appeared here of a cross-reaction between the MEF1 antigen and a non-Lansing type-convalescent serum. Moreover, this occurred in an animal bled 241 days after onset, while monkey 179 injected with Lansing-type virus was negative when bled only 26 days after onset. The problem which is left for further study is the time after onset of illness when a positive complement fixation can be secured.

Chimpanzee Sera.—Serial bleedings from 5 chimpanzees were generously supplied by Dr. Melnick, who also provided the data reported in Table V on the type of virus given orally, the time of bleeding, and the results of the neutralization tests. As shown in the table the animals developed complement-fixing antibody against the MEF1 virus when first given YSK strain (a Lansing type) and later, Brunhilde virus. Again the specificity of the test is here demonstrated, although the problem of cross-reaction between Lansing and non-Lansing types could not be resolved because of the lack of an orderly sequence in feeding both types of virus. Chimpanzee Rosebud, however, which received a non-Lansing virus first, failed to react with MEF1 antigen. The positive reaction here with Japanese B encephalitis virus antigen was specific since the animal had been previously exposed to this virus.

Human Sera.—Human sera were also studied to disclose (*a*) whether man develops specific complement-fixing antibody against MEF1 virus and (*b*) whether any evidence can be deduced to detect cross-fixation between Lansing and non-Lansing types of poliomyelitis virus. Other studies, *e.g.* development of the antibody with time and in different clinical types of the disease, as well as the possibility of using this test for diagnosis of an early stage of poliomyelitis are still being carried on.

TABLE V

Complement-Fixation Tests with Chimpanzee Sera

Poliomyelitis virus given orally (Dr. Melnick's series)

Lab. No.	Animal	Date of bleeding (B), or viral exposure (V)	Strain and length of time from viral exposure	Neutralization *vs.* Lansing virus	CF antigen				
					MEF1	J.B.E.	W.E.E.	Normal	None
			days						
89	Rosebud	3-10-47 B	−2	0	0	0			0
		3-12-47 V	Pittsfield						
90		7-31-47 B	140		0	0			0
84		1-13-48 B	300	0	0	8			0
		1-16-48 V	YSK						
110		6-15-48 B	450 Pittsfield	1:550	0	8			0
			150 YSK						
165	21	8-15-50 B	−1	0	0	0		0	0
		8-16-50 V	YSK						
164		10-4-50 B	49		0	0		0	0
		11-4-50 B	80	1:10					
		11-14-50 V	Brunhilde						
83		12-13-50 B	119 YSK		2	0		0	0
			29 Brunhilde						
169	22	8-15-50 B	−1	0	0	0		0	0
		8-16-50 V	YSK						
105		10-4-50 B	49		2–4	0		0	0
		11-4-50 B	80	>1:100					
		11-14-50 V	Brunhilde						
167		12-13-50 B	119 YSK		32	0		0	0
			29 Brunhilde						
108	23	8-15-50 B	−1	0	0	0		0	0
		8-16-50 V	YSK						
102		10-4-50 B	49		4–8	0		0	0
		11-4-50 B	80	>1:100					
		11-14-50 V	Brunhilde						
87		12-13-50 B	119 YSK		4–8	0		0	0
			29 Brunhilde						
86	24	8-15-50 B	−1	0	0	0	0		0
		8-16-50 V	YSK						
101		10-4-50 B	49		2	0	0		0
		11-4-50 B	80	>1:100					
		11-14-50 V	Brunhilde						
88		12-13-50 B	119 YSK		8	0	0		0
			29 Brunhilde						

That human sera reacted specifically with the MEF1 antigen is demonstrated in Tables VI and VII. In both one notes that positive reactions occurred only with MEF1 antigen; no fixation whatever or of any significant degree was observed with Japanese B or Western equine encephalitis antigens.

TABLE VI

Complement-Fixation Tests with Human Sera

Serum		Neutralization index for Lansing-type virus	Antigens			
Poliomyelitis convalescent			None	MEF1	Japanese B	Western equine
Child	22	> 80	0	16*	0	0
	23	5	0	2	0	0
	24	< 4	0	32+	0	0
	25	< 4	0	32+	0	0
	26	16	0	32+	0	0
	27	< 4	0	2	0	0
	28	< 4	0	16	0	0
	29	10	0	0	0	0
	30	> 80	0	2	0	0
	31	< 4	0	16	0	0
	182	12	0	16	0	0
	184	10	0	64	0	0
"Normal"						
Adult	46	>100	0	8	0	0
	47	100	0	0	0	0
	48	>300	0	0	0	0
	49	>100	0	0	0	0
	50	>300	0	16	0	0
	51	>100	0	8	0	0
	52	>100	0	0	0	0
	53	>100	0	0	0	0
	55	>100	0	0	0	0
	56	>300	0	4	0	0
	58	>300	0	4	0	0
	60	>100	0	4	0	0

* 16 = reciprocal of highest dilution of serum giving a 2+ or better fixation; 0 = no fixation in 1:2 dilution of serum.

In Table VI will be seen the results of tests with sera from 12 children, aged 1 to 9 years, obtained some time during convalescence from paralytic poliomyelitis. The isolation of virus was not attempted and its type therefore not known. Since 10 of the cases exhibited negative or questionable neutralizing antibody against the Lansing virus, it was reasonable, and conformable with epidemiological practice, to assume that the epidemic occurring in New York

in 1950, from which the present patients were selected was not caused by a Lansing-type virus. As the table reveals, complement-fixing antibody was detected in high titer in a number of instances. By the way of contrast, of 19 children of similar ages, having no Lansing-neutralizing antibody and no history of an attack by poliomyelitis, the serum of only one showed positive complement fixation with the MEF1 antigen.

Table VI also summarizes the results of a test with 12 sera from adult individuals, aged 20 years or older, who were apparently normal and had no

TABLE VII

Complement-Fixation Tests on Sera from Poliomyelitis Cases

Lansing-virus neutralization tests negative

Lab. No.	Name	Age	Diagnosis	Length of time from onset	Type as reported	CF antigen				
						MEF1	J.B.E.	W.E.E.	Normal	None
		yrs.		*days*						
9	Wa.	?	?	?	Brunhilde ?	4	0			0
37	Gr.	?	Paralytic	24	"	4	0	0		2
120	Be.	?	?	?	Not reported	2			0	0
76	Ho.	4	Non-paralytic	250	Brunhilde-like	2	0	0		0
81	Ri.	1.5	Paralytic	14	"	8	0	0		0
79	Ob.	13	"	14	"	8	0	2		0
72	Fi.	8	"	27	"	8	0	0		0
23	M.P.	4	"	36	Not reported	2	0	0		0
27	D.E.	6	"	80	" "	2	0	0		0
28	J.J.	6	"	49	" "	32	0	0		0
31	K.L.	5	"	85	" "	16	0	0		0
24	M.M.	4.5	"	88	" "	32	0	0		0
25	E.R.	1.8	"	135	" "	32	0	0		0
184	Ha.	2	"	3	" "	64	0	0		0

history of a prior attack of paralytic poliomyelitis. They were chosen because it was predicted that neutralizing antibody of Lansing type would be present, since this antibody is distributed among the population in a manner that increases with age, until adolescence or adulthood, when almost all individuals have it (11–13). As expected, all had a high level of Lansing-type neutralizing antibody; there were fewer CF positives, and at a lower level, than were found among those obtained from poliomyelitis convalescents.

In another series of experiments (Table VII) the possibility of cross-fixation between non-Lansing-type antibody and MEF1 antigen is strengthened. Of 14 sera, some of which are recorded in Table VI, none had Lansing-type neutralizing antibody. Furthermore, sera 76, 81, 79, and 72, for which we are in-

debted to Dr. A. B. Sabin, were obtained from patients having had poliomyelitis caused by the Brunhilde strain for this virus, so identified, was not only recovered from the patients but their sera exhibited with the passage of time a rising titer of neutralizing antibody against their own homologous strain of virus (14). The results again indicate that cross-fixation may exist between non-Lansing type antibody and MEF1 antigen.

Finally, a table was constructed to summarize the combined results of tests on single sera (Table VIII). In this table the results are shown of complement-fixation and neutralization tests on the serum obtained from 35 poliomyelitis convalescents and from 41 individuals having no history of poliomyelitis. Although the different groups of sera are small, the data in Table VIII point to

TABLE VIII

Summary of Complement-Fixation and Neutralization Tests

Diagnosis and No. of cases	Neutralization test, Lansing virus			Complement-fixation test, MEF1 antigen		
	+	±	0	+	±	0
Poliomyelitis	7			5	1	1
35		11		8	1	2
			17	8	7	2
Normal	22			6	6	10
41		0			0	
			19	1	2	16

Neutralization index of 50 or more = +; of 10–49 = ±; complement-fixation test, positive serum dilution of 1:8 or higher = +; of 1:2 or 1:4 = ±.

the fact that poliomyelitis patients with positive and questionable neutralizing antibody against Lansing virus had a positive complement fixation in a high proportion of instances (13 of 18); and of the remaining 17 with no Lansing-neutralizing antibody 8 had definite fixation, 7 slight fixation, and 2 none. The greater number of positive complement fixations with the sera of apparently normal persons, occurred in those in which Lansing-neutralizing antibody was present (6 of 22). In the absence of Lansing-neutralizing antibody, only occasionally (1 of 19) did a positive fixation appear. To summarize, 21 of 35 poliomyelitis cases, or 60 per cent, exhibited fixation in dilution of 1:8 or higher, while 7 of 41 normal individuals, or 17 per cent, had a similar titer of complement-fixing antibody; the latter group comprised chiefly adults who harbored Lansing-type neutralizing antibody.

DISCUSSION

Attempts to develop a complement-fixation antigen with Lansing-type virus have been reported previously by Loring, Raffel, and Anderson (15). Through

the use of a concentrated, purified preparation positive results with rat, monkey, and human sera were obtained but a certain degree of non-specificity was apparent in some of their results. In our laboratory previous attempts throughout several years consistently failed. The reason for lack of success was believed to be that antigenic substance was not present in the preparations in sufficient concentration and hence it was assumed that to detect a reaction, antigens of a higher potency had to be prepared. Two possibilities were considered: (1) to increase the amount of antigen in the tissues and (2) to resuspend the tissues in a smaller volume of diluent than heretofore had been used. It was thought that these two conditions might be produced simultaneously in newborn mice.

It was known (16) that by passage of Japanese B and Western equine encephalitis viruses in newborn mice an increase of pathogenicity and a higher level of complement fixation could be obtained from their CNS tissues. It had also been found that such tissues could be emulsified in a smaller volume of diluent than those of adults, yielding an extract which was not anticomplementary. Thus, adult mouse CNS tissue (6) could be readily resuspended in 3 volumes of diluent but not in less, while newborn mouse CNS tissue could be emulsified in only 1 volume of diluent, without introducing an anticomplementary effect. This higher concentration can be explained perhaps on the basis of the chemical composition of the CNS tissues of adult and newborn mice. Folch (17) found that the brain tissue of 4-day-old mice contained: water, 87.8 per cent; proteins, 8.7 per cent; and total lipids, 3.5 per cent. Most of the lipids were phosphatides; no cerebrosides were present. In contrast, adult mouse brain contained: water, 78.1 per cent; proteins, 10.6 per cent; and lipids, 11.3 per cent. Since less solids, particularly less lipids, were present in the newborn mouse brain tissue, it was to be expected that with respect to the wet weight of the original tissue more concentrated extracts having no anticomplementary effect could be prepared. As the tests reported here show, this proved to be the case.

It is questionable whether the MEF1 strain, once adapted to the newborn mouse (1), has resulted in an increase in its infectivity nor is the cause known which underlies the correlation between the adaptation of the MEF1 strain and the development of a potent antigen. Newborn mouse tissue, however, is apparently essential since the adapted virus when passaged in adult mouse cerebral tissue failed to yield an effective antigen. Investigations are now in progress to determine whether the use of adapted virus as a complement-fixing antigen is based on qualitative or quantitative changes in the virus.

As reported in the present paper titers of antigens have been low, the latter can be diluted only 1:2 or 1:4 if they are still to be effective. Attempts are being made to increase the titers by passage of the adapted virus in other strains of mice. Already, by using wild mice, a strain being propagated by Dr. H. A. Schneider of this Institute, complement-fixing antigens have been obtained with titers of 1:8 and 1:16 respectively. If this apparently significant finding proves

generally true, antigens can be prepared in wild mice which will have a binding power such as characterizes the encephalitis-virus antigens, now established as wholly reliable and useful for specific diagnosis of infections (6, 8). By the use of such potent MEF1 antigens, the 1:2 and 1:4 reactions recorded in the tables might conceivably have been extended to a higher level with result in findings thus definitely positive.

In the present work a specific complement-fixation reaction has been demonstrated between antibody in the serum of man and of some lower animals and the MEF1 (a Lansing-type virus) antigen. Now the problem is whether such an antigen will detect only type-specific antibody or will be inclusive, that is to say, react with antibody against other types of the poliomyelitis virus. The fact that the sera were positive which were obtained from patients who had no Lansing-neutralizing antibody but from whom a Brunhilde strain was isolated, proving to be related to their illness, seems to indicate that a cross-reaction may have existed between the Lansing and the Brunhilde types. This aspect of the problem, however, requires further study before a definite conclusion can be reached, and further study is also needed to discover whether the complement-fixation test can be applied practically to the diagnosis of poliomyelitis. The development of the antibody with passage of time, its persistence, its occurrence in different clinical types of the disease, and the conditions under which a rise in titer can be demonstrated in paired sera, acute and convalescent, are also problems still under investigation.

It is of interest that sera from normal individuals had complement-fixing antibody in 7 of 41 instances. The significance of these positives has not yet been determined; it is thought, however, that in 6 positive cases of 22 normal persons in whom neutralizing antibody of the Lansing-type was present, the fixation antibody may indicate a recent subclinical infection, or, possibly, a repeated infection with virus of this type. Of the 19 individuals that had no neutralizing antibody against the Lansing virus, only one was positive and 2 exhibited low or questionable reactions. It is assumed that these individuals had not been infected with the Lansing virus and that these positive reactions point to subclinical infection with virus of another type.

CONCLUSIONS

A complement-fixing antigen has been developed, using as source of material CNS tissue from newborn mice infected with the newborn mouse–adapted strain of the Lansing type, MEF1 virus. With this antigen, specific reactions have been obtained with sera from mice, cotton rats, and monkeys immunized with the Lansing-type virus, and from monkeys and chimpanzees convalescent from infection with this virus.

Twenty-one of 35 human sera obtained from individuals convalescent from poliomyelitis were positive and 6 of 22 from apparently normal persons having

Lansing-neutralizing antibody, while this held true for only 1 of 19 from those having no Lansing-neutralizing antibody.

The fact that positive results were found in sera from patients having an infection with poliomyelitis virus of the Brunhilde type and at the same time no Lansing-neutralizing antibody brings up the possibility of the existence of a cross-reaction in complement fixation between the two types.

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