A COMPLEMENT FIXATION TEST FOR POLIOMYELITIS*

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A simple, rapid and practical method for the diagnosis of poliomyelitis has long been sought. The neutralization test in monkeys has been too costly for routine application and has been employed primarily as a research tool in studies on the immunology, pathogenesis, and epidemiology of this disease. The important finding of Enders and his associates (1, 2) that the poliomyelitis viruses can be cultivated in tissue cultures, has permitted the use of tissue culture methods for *in vitro* assay of poliomyelitis antibody. A refinement of this technique lies in the recently described metabolic-inhibition test (3). Both procedures, however, are relatively intricate, require special facilities and trained personnel, and do not lend themselves readily to the small laboratory. Consequently, some simpler *in vitro* technique such as the complement fixation method would appear desirable.

Complement fixation techniques, using infected mouse brain (4–6), cotton rat brain (7–9), and tissue culture fluids (10–13) as a source of antigen, have been described. Purification (lipoid extraction, ultracentrifugation, methanol precipitation) of the rodent brain antigen is required and even so there is considerable difficulty with heterotypic fixation with human sera. Also, brain antigens can be prepared only from Lansing-like (Type II) viruses, which are propagable in rodents. The same difficulty with heterotypic reactions, although not to the same extent, has been encountered with tissue culture antigens. Other obstacles lie in the fact that in some procedures the antigens require concentration through ultrafiltration (10) and the very small amount of concentrated material obtained necessitates a micro method of conducting the complement fixation test; *e.g.*, the method of Fulton and Dumbell (14). Other workers (12, 13) have employed the unconcentrated tissue culture fluid as a source of antigen and also used the drop method of fixation. In the latter case, the avidity for complement of the various test and control antigens, and of the serum, has to be ascertained and the interpretations based on differences in avidity as well as the actual titer of the serum (12).

The complement fixation methods described in the literature are thus beset with difficulties associated with heterotypic reactions, and while these can be

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avoided by certain manipulations, considerable experience is required to avoid pitfalls; in addition, the drop method, as is true of any micro method, requires more care and skill than does a macro method in which a greater margin of error is permissible without unduly affecting the final results.

We considered, therefore, that a macro method would be desirable not only because it is more easily and more quickly performed on a large scale, but also because the technique of such a method is familiar to most workers in clinical and public health laboratories. The procedure finally evolved is essentially the same as that employed in this laboratory for the routine diagnosis of viral and rickettsial diseases.[1] The present communication describes the test and presents evidence for its sensitivity and specificity as derived from examination of patients with poliomyelitis, as well as patients with other neurotropic viral infections.

Materials and Methods

Specific Antigens.—The antigens utilized in these preliminary studies consisted of unconcentrated fluids harvested from monkey kidney tissue cultures infected with poliomyelitis virus. The fluids were obtained from the Connaught Laboratories, and their preparation has been described by Farrell *et al.* (15). The Type I virus was represented by the Mahoney strain, the Type II virus by the MEF-1 strain, and the Type III virus by the Saukett strain.

The potency of each antigen was determined in "checkerboard" or "block" titrations with hyperimmune monkey serum, and it was found that, on the whole, the 0.2 ml. of undiluted tissue culture fluid used in the test represented slightly more than 1 unit, but less than 2 units of antigen, if 1 unit is described as that dilution of antigen which gives three plus or four plus fixation with the highest dilution of immune serum. This will be described further below under Experimental.

Control Antigens.—All sera were examined for possible non-specific complement-fixing activity by testing them against a control antigen prepared from the fluid and disintegrated cells of uninfected monkey kidney cultures. These antigens were used undiluted in 0.2 ml. amounts.

Complement.—Lyophilized complement was used throughout this work; it was reconstituted in sterile distilled water as needed. The complement was titrated in the presence of the specific antigen, and 2 *exact* units in a volume of 0.2 ml. were used in the tests. The incubation period for the titration was 1 hour at 37°C.

Hemolysin.—2 units of hemolysin in 0.25 ml., as determined by titration at 37°C. for 30 minutes with 1:30 complement (0.2 ml.) and a 2.0 per cent sheep cell suspension (0.25 ml.), were used.

Sensitized Cells.—Sensitized cells were prepared by mixing equal volumes of a 2 per cent suspension of sheep erythrocytes and a dilution of hemolysin containing 2 hemolytic units in a volume of 0.25 ml. The mixture was allowed to stand at room temperature for 10 minutes before use.

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[1] The details of the complement fixation method used in this laboratory are available in mimeographed form and will be supplied on request. Our method is also described in the chapter on influenza in the forthcoming 2nd edition of Diagnostic Procedures for Viral and Rickettsial Diseases published by the American Public Health Association.

Sera.—Monkey hyperimmune sera (16) for each of the three immunologic types of virus were obtained from The National Foundation for Infantile Paralysis through the courtesy of Dr. Henry W. Kumm and Dr. Herbert Wenner. These sera, which were in lyophilized form, were reconstituted with physiological saline with due precaution to maintain sterility; the reconstituted serum was kept in the refrigerator at 4°C. These immune sera were used as controls in each test and, in addition, high titered human sera for each of the three virus types were used as controls whenever possible.

The test sera examined in this work came from 37 patients with a clinical diagnosis of poliomyelitis and from 33 patients shown by laboratory methods to be suffering from central nervous system disturbances produced by the viruses of Western equine encephalitis, St. Louis encephalitis, or mumps. There were 10 individuals with St. Louis encephalitis, 11 with Western equine encephalitis, and 12 with mumps encephalitis.

All sera, after dilution to 1:4 or 1:8 in Kolmer saline solution[2] were inactivated at 60°C. for 30 minutes.

Procedure for Complement Fixation Test.—Serial twofold dilutions of each serum were prepared in Kolmer saline solution.[2] Each dilution was dispensed into three series of tubes so that tests against each of the three virus types could be run concurrently. In addition, the first two dilutions of each serum were tested for anticomplementary activity in the absence of antigen and for non-specific complement-fixing activity with the control antigen. Antigen was then added in a volume of 0.2 ml. followed by 0.2 ml. of complement dilution containing 2 exact units. Fixation was allowed to proceed at 4°C. overnight (approximately 18 hours). The tubes were then warmed in a 37°C. water bath for 10 minutes, 0.5 ml. of sensitized cells was added to each tube, and secondary incubation at 37°C. was then conducted for 15 to 30 minutes, depending upon the time required for the complement control tubes to show clearing. The complement controls consisted of a series of four tubes containing antigen, sensitized red cells, and 2 units, 1½ units, 1 unit, or ½ unit of complement, respectively. The tubes containing 1, 1½, and 2 units should show clearing after 15 to 30 minutes of incubation, and as soon as this occurred the tests were read. The titer of a serum was the highest dilution (initial, *not* final) showing three plus or four plus fixation with the specific antigen.

EXPERIMENTAL

Potency of Antigens.—The sensitivity of a complement fixation method depends, in part, upon the potency of the antigens employed. The antigenic capacity of the infected tissue culture fluids was determined in "checkerboard" titrations, using type-specific hyperimmune monkey sera. The results of one such titration, employing Type I tissue culture antigen, are shown in Table I. It will be observed that when the fluid was used undiluted, fixation occurred through a serum dilution of 1:128, and that when the fluid was diluted 1:2, fixation occurred through a serum dilution of 1:32, i.e., this minimal dilution resulted in a fourfold decrease in the serum titer (sensitivity). If 1 unit of antigen is defined as that dilution of the fluid which gives 4+ or 3+ fixation with the highest dilution of immune serum, then the undiluted tissue culture fluid represented slightly more than 1 unit, but less than 2 units. The fact that the unitage was greater than one is indicated by the appreciable amount of fixation which occurred when the fluid was used diluted 1:2.

[2] Kolmer saline solution was used as the diluent for all reagents in the complement fixation test, and was prepared by adding 1.0 ml. of 10 per cent magnesium sulfate solution to 1.0 liter of 0.85 per cent sodium chloride solution.

Since, for purposes of standardization and for subsequent comparisons, it is desirable to know the exact concentration of reagents employed in a test, several attempts were made to concentrate the antigen by centrifugation for prolonged intervals at high speed *in vacuo*. While some increase in potency could be achieved, even to the extent of obtaining a preparation that would give 2 units in 0.2 ml., and aside from the fact that the concentrated antigens tended to be slightly anticomplementary, the small extent to which the sensitivity of the test was increased did not warrant the labor involved.

TABLE I

Example of Block Titration of Antigen, Using Mahoney Strain, Type I, Tissue Culture Antigen and Homologous Monkey Immune Serum

Antigen (dilutions of tissue culture fluid)	Dilutions of monkey immune serum, Type I					
	1:16	1:32	1:64	1:128	1:256	1:512
Undiluted	4*	4	4	4	1	0
1:2	4	3	±	0	0	0
1:4	0	0	0	0	0	0
None	0	0	0	0	0	0

* 4 = four-plus fixation, etc.

TABLE II

Effect of Increasing Concentrations of Antigen on Extent of Fixation. Mahoney Strain, Type I, Virus Tissue Culture Fluid and Homologous Monkey Immune Serum

Undiluted tissue culture fluid antigen	Dilution of monkey immune serum, Type I					
	1:16	1:32	1:64	1:128	1:256	1:512
ml.						
0.1	4	2	0	0	0	0
0.2	4	4	4	3	0	0
0.3	4	4	4	4	2	0
0.4	4	4	4	4	2	0
None	0	0	0	0	0	0

The possibility was therefore considered of increasing the sensitivity of the test by using larger volumes of undiluted culture fluid; *i.e.*, 0.3 ml. or 0.4 ml. instead of the usual 0.2 ml. The results of one of several "checkerboard" titrations, using undiluted tissue culture antigen and type-specific monkey hyperimmune serum, are shown in Table II. It will be noted that 0.1 ml. of antigen gave relatively little fixation as compared with that obtained with 0.2 ml. of antigen; with the latter amount, there was an eightfold increase in the serum endpoint titer as compared with the former amount. The use of 0.3 or 0.4 ml. amounts of antigen did not increase the serum titers over those obtained with 0.2 ml.; as will be seen in Table II, the larger amounts of antigen gave the same degree of fixation as did 0.2 ml. with serum diluted 1:128, and gave only 2+ fixation with a serum dilution of 1:256.

While increasing the amount of antigen through the use of larger volumes of undiluted culture fluid in the system did not serve to increase the extent of fixation in the several experiments in which monkey sera were employed, the use of larger volumes of antigen served, in some instances, to increase the sensitivity of the method when human sera were examined. This is shown in Table III, which presents the results obtained with serum specimens from 10 patients with poliomyelitis when the sera were tested against varying amounts of undiluted tissue culture fluid. The amount of fixation obtained with the sera of the first two patients in Table III was unaffected by increasing the amount of antigen in the test. In the case of the next four individuals in the table, the use of larger volumes of antigen resulted in a two-fold increase in the serum titers, and in the remaining four patients, the serum titers were increased fourfold or more.

While the sensitivity of the test could thus be sharpened, at least in some instances, through the use of 0.3 or 0.4 ml. of the tissue culture antigen, it was found that 0.4 ml. amounts proved to be undesirably anticomplementary, and 0.3 ml. amounts were slightly anticomplementary. To avoid difficulties associated with anticomplementary activity, it seemed desirable, at least in this preliminary work, to use only 0.2 ml. of undiluted fluid as the antigen, and the experiments described below are all based on the use of this amount. Nevertheless, the data obtained through the use of the larger volumes of culture fluid antigen suggest that the sensitivity of the complement fixation method used here could be appreciably increased, if the potency of the antigen could be increased. Experiments on this aspect are currently under way.

Examination of Sera from Patients with a Clinical Diagnosis of Poliomyelitis.—The preliminary assays described above showed that infected tissue culture fluids possessed complement-fixing reactivity when used under conditions that would prevail in the complement fixation technique it was desired to employ. To gain some information on the usefulness and reliability of this technique for the diagnosis of poliomyelitis, antigens representing the three known immunologic types of poliomyelitis virus were used to examine paired or multiple serum specimens from 27 patients in whom a clinical diagnosis of poliomyelitis had been made. The findings are presented in Table IV, which also gives the results of complement fixation tests done with the same sera against the viruses of mumps, Western equine encephalitis, and St. Louis encephalitis. Tests with these latter viruses were performed because the mumps virus is by no means an infrequent incitant of central nervous system disturbances in California (17) and because, in certain parts of the state, poliomyelitis, and Western equine and St. Louis encephalitis are endemic, and their seasons of prevalence essentially coincide (18). It will be noted that in none of the 26 patients from whom sufficient serum was available to perform these tests, was there any serologic evidence of infection with the viruses of mumps, Western equine encephalitis, or St. Louis encephalitis. In every instance but one, the

TABLE III

Effect of Increasing Concentrations of Poliomyelitis Tissue Culture Antigen on the Extent of Fixation with Human Sera

Patient	Serum taken, days after onset	Amount undiluted antigen	Complement fixation titers		
			Type I	Type II	Type III
		ml.			
1. Do.Kn.	7	0.2	<4	<4	<4
	7	0.3	<4	<4	<4
	32	0.2	8	<4	<4
	32	0.3	8	<4	<4
2. Mi.Cu.	4	0.2	<4	<4	<4
	4	0.3	<4	<4	<4
	21	0.2	8	<4	<4
	21	0.3	8	<4	<4
3. Cu.Ho.	4	0.2	<4	<4	<4
	4	0.3	<4	<4	<4
	25	0.2	<4	<4	4
	25	0.3	<4	<4	8
4. An.Re.	7	0.2	<4	<4	<4
	7	0.4	<4	<4	<4
	21	0.2	8	<4	<4
	21	0.4	16	<4	<4
5. Su.Me.	7	0.2	<4	<4	<4
	7	0.3	<4	<4	<4
	21	0.2	8	<4	<4
	21	0.3	16	<4	<4
6. Hu.Po.	30	0.2	32	<4	<4
	30	0.3	64	<4	<4
7. Ka.Ke.	6	0.2	<4	<4	<4
	6	0.3	16	<4	<4
	24	0.2	8	<4	<4
	24	0.3	32	<4	<4
8. T.Go.	7	0.2	<4	<4	<4
	7	0.3	<4	<4	<4
	44	0.2	8	<4	<4
	44	0.3	32	<4	<4
9. M.Su.	4	0.2	<4	<4	<4
	4	0.4	<4	<4	<4
	21	0.2	8	<4	<4
	21	0.4	32	<4	<4
10. E.Bo.	15	0.2	<4	<4	<4
	15	0.4	<4	8	<4
	34	0.2	<4	16	<4
	34	0.4	<4	64	<4

complement-fixing titers against these agents were less than 1:8, *i.e.*, negative[3]; the exception was patient 24, whose sera gave slight fixation with the Western equine encephalitis complement-fixing antigen, but the antibody level encountered is not significant for Western equine encephalitis and, moreover, there was no rise in titer.

A single stool specimen was available from each of the 27 patients listed in Table IV. From 12 of these individuals, a cytopathic agent was recovered in tissue culture (monkey kidney, human embryo, or HeLa cells), identified as a poliomyelitis virus, and typed. In the remaining 15 individuals, attempts to isolate an agent from the stool were negative.

Taking first the 12 individuals from whom a poliomyelitis virus was recovered, and hence could serve to confirm the serologic findings, it will be noted that in every instance the type of virus recovered from the stool corresponded with the serologic findings. Thus, 8 individuals (patients 11 to 18) showed a fourfold or greater rise in complement-fixing antibody titer to the Type I virus, and a Type I virus was recovered from the stools. Patient 20, whose stool yielded a Type I virus, showed a marked rise in complement-fixing antibody titer to the same type; he also showed a minimal serologic response to Type II virus, but while the titer rose slightly at this minimal level, it subsequently decreased. Patient 19, from whose stool a Type I virus was recovered, showed a complement-fixing titer of 1:16 against all three types of the poliomyelitis virus. However, examination of the recovery phase serum specimens showed that while the titer to Type I virus had remained stationary, the titer to Types II and III had decreased. Patients 21 and 22 showed a fourfold or greater rise in antibody titer to Types II and III, respectively, which corresponds with the immunologic virus types recovered from the stools of these patients. In essence, therefore, an antibody rise of sufficient magnitude to permit diagnosis occurred in 11 of the 12 individuals, and in the twelfth individual, the homotypic antibody response remained at a stationary level, the heterotypic antibody level decreasing. While the number of individuals examined is small, in each case the serologic results agreed with the virus isolation results.

As concerns the 15 individuals in whom a clinical diagnosis of poliomyelitis had been made and from whose stools no virus was recovered, 7 (patients 23, 24, 29, 30, 34, 36 and 37) showed a fourfold or greater rise in complement-fixing antibody against the Type I virus. Patient 28, paralytic, had an antibody titer of less than 1:8 to Type II and Type III viruses, and a stationary titer of 1:32 to the Type I virus. Patient 25, also paralytic, had a titer of less than 1:8 to the Type II virus, and an essentially stationary titer to Types I and III, the titer to the Type I being considerably higher than to the Type III virus. Patient 32 had a titer of less than 1:8 to the Type III virus, a fourfold or higher rise to the Type II virus with a subsequent decline in the titer, and a relatively high but stationary titer to the Type I virus. If any interpretation is permissible on the basis of relatively high stationary titers, the findings may be interpreted in the case of these three patients as pointing to infection with the Type I virus.

[3] In the complement fixation test used in this laboratory, the lowest serum dilution examined as routine is 1:8.

TABLE IV

Results of Complement Fixation Tests on Individuals with a Clinical Diagnosis of Poliomyelitis

Patient	Age	Sex	Days after onset	Clinical type of poliomyelitis	Virus isolated from stool	Poliomyelitis complement fixation titers			Neurotropic viruses complement fixation titers against		
						Type I	Type II	Type III	Mumps	WEE	SLE
11. Ti.Go.	10 mos.	M	7	Paralytic	Type I	<4	<4	<4	Not tested*		
			44			8	<4	<4	Not tested*		
12. An.Da.	11 mos.	M	4	Paralytic	Type I	<8	<8	<8			
			30			64	<8	<8	<8	<8	<8
13. La.Ga.	3 yrs.	M	5	Paralytic	Type I	<8	<8	<8			
			10			<8	<8	<8			
			15			16	<8	<8	<8	<8	<8
14. Ji.Mo.	5 yrs.	F	3	Non-paralytic	Type I	<8	<8	<8	<8		
			11			16	<8	<8	<8	<8	<8
			19			8	<8	<8	<8	<8	<8
15. An.Re.	5 yrs.	F	7	Paralytic	Type I	<8	<8	<8			
			21			16	<8	<8	<8	<8	<8
16. Fr.Ca.	8½ yrs.	M	7	Non-paralytic	Type I	<8	<8	<8			
			20			128	<8	<8	<8	<8	<8
17. Dw.De.	10 yrs.	M	9	Paralytic	Type I	<8	<8	<8			
			30			64	<8	<8	<8	<8	<8
18. Ge.En.	10 yrs.	F	8	Paralytic	Type I	<8	<8	<8	<8		
			21			128	8	8	<8	<8	<8
19. WI.La.	13 yrs.	M	2	Abortive	Type I	16	16	16	<8		
			15			16	4	8	<8	<8	<8
20. Jo.Ho.	25 yrs.	F	4	Paralytic	Type I	<8	<8	16			
			34			128	16	16	<8	<8	<8
			43			128	8	16	<8	<8	<8
21. Ed.Bo.	6 yrs.	M	15	Paralytic	Type II	<8	<8	<8			
			29			<8	8	<8	<8	<8	<8
			34			<8	32	<8	<8	<8	<8
22. Pa.Ha.	14 mos.	M	15	Paralytic	Type III	<8	<8	<8			
			27			<8	<8	32	<8	<8	<8

TABLE IV—*Continued*

Patient	Age	Sex	Days after onset	Clinical type of poliomyelitis	Virus isolated from stool	Poliomyelitis complement fixation titers			Neurotropic viruses complement fixation titers against		
						Type I	Type II	Type III	Mumps	WEE	SLE
23. Ma.Su.	3½ yrs.	F	7	Not stated‡	None	<4	<4	<4			
						16	<4	<4	<8	<8	<8
24. Ca.Re.	3½ yrs.	F	5	Paralytic	None	<8	<8	<8		16	
			10			64	<8	<8		8	
			15			64	<8	<8	<8	8	<8
25. Mi.Ga.	7 yrs.	M	2	Paralytic	None	32	<8	8			
			7			32	<8	16			
			19			64	<8	16	<8	<8	<8
26. Ba.Ca.	7 yrs.	F	7	Non-paralytic	None	<4	<4	<4		<8	
			10			32	16	32	<8	<8	<8
			21			64	16	64		<8	<8
27. Mi.Pa.	8 yrs.	M	1	Not stated	None	<4	<4	<4			
			12			<4	<4	<4	<8	<8	<8
28. No.Je.	11 yrs.	M	9	Paralytic	None	32	<8	<8			
			28			32	<8	<8	<8	<8	<8
29. Br.Ca.	16 yrs.	M	3	Paralytic	None	<8	<8	<8			
			7			<8	<8	<8			
			14			<8	<8	<8	<8	<8	<8
			21			16	<8	<8	<8	<8	<8
30. Su.Me.	22 yrs.	F	4	Abortive	None	<4	<4	<4			
			21			16	<4	<4	<8	<8	<8
31. Do.Wi.	23 yrs.	F	3	Not stated	None	<4	<4	<4	<8		
			21			<4	<4	<4	<8	<8	<8
32. Ma.Pi.	23 yrs.	F	3	Abortive	None	32	<8	<8			
			17			64	16	<8	<8	<8	<8
			27			32	8	<8			
33. Ea.Ed.	25 yrs.	M	4	Paralytic	None	<8	<8	<8			
			14			8	<8	<8	<8	<8	<8
			27			<8	<8	<8	<8	<8	<8

TABLE IV—*Continued*

Patient	Age	Sex	Days after onset	Clinical type of poliomyelitis	Virus isolated from stool	Poliomyelitis complement fixation titers			Neurotropic viruses complement fixation titers against		
						Type I	Type II	Type III	Mumps	WEE	SLE
34. Mi.Na.	28 yrs.	M	3	Non-paralytic	None	8	4	4	<8		
			14			16	<4	4			
			21			16	4	4	<8	<8	<8
			44			16	4	4			
			60			32	4	4			
35. Ro.Un.	29 yrs.	M	3	Non-paralytic	None	<4	<4	<4			
			9			4	<4	<4	<8	<8	<8
36. Fr.Tr.	32 yrs.	M	3	Paralytic	None	<4	<4	<4			
			13			8	<4	<4	<8	<8	<8
37. La.Sc.	34 yrs.	M	5	Paralytic	None	<8	<8	<8			
			7			<8	<8	<8			
			11			8	<8	<8	<8	<8	<8
			21			128	<8	<8			

* Insufficient serum.

‡ Not stated, information not provided by attending physician.

Patient 26 had a fourfold or greater rise in titer to all three virus types. The significance of the results is difficult to assess since, although fixation with the Type II virus might be described as due to a heterotypic response, the rise in antibody titer to the Type I and Type III viruses was of equal magnitude. In the case of patients 27 and 35, no antibody was demonstrable to any of the three virus types. While it is possible that these patients did not have poliomyelitis, it is also possible that the second blood specimen was taken too early after the onset of the illness for antibodies to have appeared (the second serum specimens were obtained 12 and 9 days respectively after the onset). In patient 33, also, the serologic findings were essentially negative although the individual was paralyzed, and the third serum specimen examined was taken 27 days after the onset of the illness. The findings in the fourth individual of the group in whom the serologic results were negative perhaps have no bearing on the other findings discussed here since no information could be obtained as to the clinical form of the disease diagnosed in this patient as poliomyelitis, and it is not impossible that he was suffering from some illness other than poliomyelitis.

In summary, in this group of 27 individuals, it was possible in 18 instances to make a diagnosis of poliomyelitis on the basis of a rise in complement-fixing antibody titer and, in four additional individuals, on the basis of a

high stationary antibody titer. In three individuals, no complement-fixing antibodies could be detected in the serum, and in two of these, at least, it is possible that the second blood specimen was taken too early after the onset of the illness for antibodies to have appeared. In one individual, there was a high and equal antibody response to two types of the poliomyelitis virus, and in one individual, in whom no antibody was demonstrable, it is not impossible that the illness may have been other than poliomyelitis.

Specificity of the Poliomyelitis Complement Fixation Reaction.—The application of any diagnostic technique to a new and unknown area requires that something be known of the specificity of the reactions encountered. Consequently, the sera from patients with central nervous system disturbances resulting from infections with the viruses of mumps, Western equine encephalitis, or St. Louis encephalitis, were tested against the poliomyelitis antigens to ascertain whether non-specific or cross-reactions occur. The findings are presented in Tables V, VI, and VII.

Table V lists 12 individuals in whom a serologic diagnosis of mumps infection was made on the basis of a rise in hemagglutination-inhibiting antibody and in complement-fixing antibody to the soluble antigen of the mumps virus. It will be observed that 9 of the 12 patients had complement-fixing antibody titers of less than 1:4 to all three types of the poliomyelitis virus. The remaining three patients had stationary titers of 1:4, 1:8, and 1:16 respectively, against Type I poliomyelitis virus. Such *low* stationary complement-fixing antibody titers are encountered in diseases other than poliomyelitis, *e.g.*, influenza, mumps, Western equine encephalitis, and Q fever, and in our experience, have no diagnostic significance (19) in these diseases. Whether the low poliomyelitis antibody titers, however, represent residual antibody from prior experience with the virus, or are due to an anamnestic response, is uncertain at this stage of our knowledge.

The results of poliomyelitis complement fixation tests on the sera of 11 individuals in whom a serologic diagnosis of Western equine encephalitis had been made are shown in Table VI. In only 2 of these 11 individuals were antibodies against poliomyelitis virus encountered, and in both instances, they were against the Type I virus. One patient, De.Mo., had a stationary complement-fixing antibody titer of 1:8, a titer whose significance, as mentioned above, is uncertain from the data thus far available. The other patient, K.Ul., however, had a rise in titer from less than 1:4 to 1:32; *i.e.*, an eightfold or greater rise, and one that is considered significant. While this may quite conceivably represent an anamnestic reaction, it would appear more probable that the patient had suffered a dual infection with the viruses of Western equine encephalitis and of poliomyelitis. The infection in this child was encephalitic rather than poliomyelitic in nature and suggests that the Western equine virus was responsible for the symptoms and physical signs, and that the

TABLE V

Results of Poliomyelitis Complement Fixation Tests on Patients with CNS Disease Caused by Mumps Virus

Patient	Age	Clinical diagnosis	Days after onset	Mumps complement fixation titer*	Poliomyelitis complement fixation titers		
					Type I	Type II	Type III
	yrs.						
F.Pe.	2	Mumps encephalomyelitis	8	<8	<4	<4	<4
			18	32	<4	<4	<4
L.Cr.	3	Mumps encephalomyelitis	5	<8	4	<4	<4
			15	32	4	<4	<4
T.Mu.	4	Poliomyelitis	2	<8	<4	<4	<4
			18	32	<4	<4	<4
M.Da.	5	Mumps	2	16	<4	<4	<4
			21	64	<4	<4	<4
R.Ma.	6	Mumps meningitis	1	<8	<4	<4	<4
			12	32	<4	<4	<4
Da.Ti.	7	Mumps encephalomyelitis	3	<8	16	<4	<4
			28	32	16	<4	<4
Mi.Th.	7	Mumps encephalomyelitis	7	<8	<4	<4	<4
			34	64	<4	<4	<4
Ro.Se.	7	Poliomyelitis	4	<8	<4	<4	<4
			15	256	<4	<4	<4
Da.Fr.	8	Mumps encephalomyelitis; poliomyelitis	4	<8	<4	<4	<4
			16	32	<4	<4	<4
J.Be.	9	Mumps encephalomyelitis	1	<8	<4	<4	<4
			11	32	<4	<4	<4
J.Ro.	14	Mumps encephalomyelitis	3	<8	8	<4	<4
			13	128	8	<4	<4
Ru.Ma.	52	Poliomyelitis; encephalomyelitis	1	<8	<4	<4	<4
			14	64	<4	<4	<4

* Sera were tested against the S (soluble) antigen.

poliomyelitis virus played a lesser role and perhaps induced only a subclinical infection (this illustrates the difficulties encountered, as emphasized by Sabin

et al. (20) and by Lennette (21)), in arriving at a laboratory diagnosis in patients from geographic areas where two different viruses, each capable of

TABLE VI

Results of Poliomyelitis Complement Fixation Tests on Patients with WesternEquine Encephalitis

Patient	Age	Clinical diagnosis	Days after onset	WEE complement fixation titer	Poliomyelitis complement fixation titers		
					Type I	Type II	Type III
K.Ta.	3 wks.	Encephalitis	4	<8	<4	<4	<4
			11	128	<4	<4	<4
Th.Ma.	1 mo.	Encephalitis	8	<8	<4	<4	<4
			32	256	<4	<4	<4
De.St.	6 wks.	Encephalitis	4	<8	<4	<4	<4
			21	128	<4	<4	<4
K.Ul.	2 mos.	Encephalitis	4	<8	<4	<4	<4
			40	256	32	<4	<4
Ca.La.	3 mo.	Encephalitis	20	<8	<4	<4	<4
			35	128	<4	<4	<4
T.Fl.	3 mo.	Encephalitis	3	<8	<4	<4	<4
			41	256	<4	<4	<4
I.Ca.	5 mo.	Encephalitis	5	<8	<4	<4	<4
			16	64	<4	<4	<4
Fr.Ab.	3 yrs.	Encephalitis	3	<8	<4	<4	<4
			14	256	<4	<4	<4
Da.Es.	8 yrs.	Encephalitis	5	<8	<4	<4	<4
			15	128	<4	<4	<4
De.Mo.	14 yrs.	Poliomyelitis	3	<8	8	<4	<4
			31	256	8	<4	<4
Ha.Ma.	67 yrs.	Encephalitis	1	<8	<4	<4	<4
			14	256	<4	<4	<4

producing inapparent as well as overt infections, are being disseminated concurrently.

Of the 10 patients with St. Louis encephalitis, 7 had no demonstrable antibody against any of the three types of poliomyelitis virus. Patient Ra.Aq. had a stationary titer of 1:8 against the Type I virus. Patient Ro.Mc. had

the equivalent of a fourfold rise in titer to Type II and Type III viruses. However, in the light of our experience with the complement fixation test in other neurotropic virus diseases, interpretation of a fourfold rise in titer

TABLE VII

Results of Poliomyelitis Complement Fixation Tests on Patients with St. Louis Encephalitis

Patient	Age	Clinical diagnosis	Days after onset	SLE complement fixation titer	Poliomyelitis Complement fixation titers		
					Type I	Type II	Type III
	yrs.						
Ra.Li.	2	Encephalitis; poliomyelitis	4	<8	<4	<4	<4
			25	64	8	16	16
De.Br.	3	Encephalitis	3	<8	<4	<4	<4
			14	64	<4	<4	<4
G.Wa.	7	Poliomyelitis	25	<8	<4	<4	<4
			44	64	<4	<4	<4
Ja.Be.	9	Poliomyelitis	3	<8	<4	<4	<4
			18	64	<4	<4	<4
Ra.Aq.	10	Encephalitis; poliomyelitis	4	<8	8	<4	<4
			19	64	8	<4	<4
Ro.Mc.	21	Encephalitis	8	<8	<4	<4	<4
			25	128	4	8	8
Wi.Fl.	42	Encephalitis; poliomyelitis	6	<8	<4	<4	<4
			14	128	<4	<4	<4
Jo.Ma.	63	Encephalitis	4	<8	<4	<4	<4
			21	128	<4	<4	<4
Al.De.	69	Encephalitis	4	<8	<4	<4	<4
			12	64	<4	<4	<4
S.Pe.	69	Encephalitis	20	32	<4	<4	<4
			28	128	<4	<4	<4

as a positive, at these borderline levels, requires caution (19), especially in the absence of a clinical and epidemiologic history on the patient, or absence of other, confirmatory laboratory evidence. Patient Ra.Li. had at least a fourfold rise in titer to the Type I virus, and at least an eightfold rise to both Types II and III. The rise in titer to all three virus types, and especially a rise

at the low levels indicated, suggests either an anamnestic response or even a dual infection with the viruses of St. Louis encephalitis and of poliomyelitis, the sera giving fixation not only against the infecting type of poliomyelitis virus but also against the heterologous types.

It would appear from these limited experiments that the complement fixation test using poliomyelitis antigens is essentially highly specific for poliomyelitis, and that while some specific fixation may occur with antigens for other neurotropic diseases, the fixation consists essentially of stationary titers at a low, and diagnostically non-significant, level. Where rises in complement-fixing antibody titer to two different viruses, *e.g.*, poliomyelitis and Western equine encephalitis, are encountered, it seems more reasonable, in the light of present limited information, to attribute such a situation to a dual infection rather than to an anamnestic rise in antibody to one agent produced by clinical infection with another (20, 21).

DISCUSSION

From the time of the first isolation of the virus by Landsteiner and Popper in 1908, and until relatively recently, investigations on poliomyelitis have been impeded by the fact that the monkey constituted the only reliable means for isolation of the virus and for antibody assay. The cumbersomeness and costliness of such a diagnostic and epidemiologic tool is well illustrated by the train of events which followed the discovery that certain Type II viruses, *viz.*, the Lansing and MEF-1 strains, are propagable in the Swiss mouse; epidemiologic studies especially were amplified and intensified, using the Type II virus as a model. The advent of the newer tissue culture techniques served further to open whole new approaches to the study of poliomyelitis, since it became possible for the first time to cultivate all three known immunologic types of the virus outside the animal host and to undertake virus isolation attempts and antibody assays on a scale hitherto impossible.

Despite the widened scope and the amplitude of the studies made possible by these tissue culture methods, the need for a simple *in vitro* method of antibody assay, especially for diagnosis, is still compelling.

The complement fixation test described by Casals *et al.* (5), and by Lahelle (9), utilized antigens prepared from rodent brains infected with Type II viruses. Despite purification, however, the use of brain antigens was attended with the occurrence of considerable heterotypic fixation. Subsequent investigators turned their attention to tissue culture materials, not only because the culture fluid, relatively free of extraneous proteins, might prove to be a source of more potent antigen but also because such fluids might provide a source of specific antigen to all three known immunologic types of the virus rather than to only one.

Svedmyr et al. (10) found that tissue culture fluids were comparatively weak as complement-fixing antigens and resorted to an ultrafiltration technique to concentrate the active material. Concentration, however, brought about an undesirable

amount of anti-complementary activity and heterotypic fixation could not be completely avoided. Also, the comparatively small amount of concentrated antigen obtained from rather large volumes of starting material made it necessary to utilize the Fulton-Dumbell technique of fixation, with all the difficulties inherent in microtechniques. Melnick and his associates (12, 13), using unconcentrated tissue culture fluid as an antigen and the fixation method of Fulton and Dumbell, expressed their results by a method which took into account, and removed from consideration, the effects of non-specific or cross-fixation. More than passing care and skill are required in the execution of this test.

The complement fixation test reported on here is essentially the one used in this laboratory for large-scale diagnosis of a diversity of viral and rickettsial disease. Since it is, except for minor modifications, essentially the technique used in clinical and public health laboratories, it thus possesses the not inconsiderable advantage of eventually being incorporated (after considerably more is known, of course, as to its specificity, accuracy, and reliability with respect to poliomyelitis) into the diagnostic routine of the clinical, hospital, or public health laboratory. This was considered a basic desideratum when the work was undertaken and we believe, in the light of the findings reported here, that this objective is attainable, although, as just mentioned, additional investigation and experience derived through practical application of the method are required. Certainly a more potent antigen is desirable, and it is to be hoped that this can be achieved without recourse to concentration methods; experimental work on this facet of the problem is currently under way.

One of the major disadvantages of the neutralization test for the diagnosis of poliomyelitis lies in the fact that in this disease as in certain others, *e.g.*, Western equine encephalitis (22, 23), neutralizing antibody appears very early, so that by the time the patient is first seen, the titer is at, or near, the maximal level and thus renders difficult or impossible the demonstration of a diagnostically significant rise in titer (23). This does not appear to be true of the complement-fixing antibody since, as will be noted from the several tables in this report, antibody in the acute phase blood specimen was not demonstrable in a preponderance of the patients, and when it was demonstrable, it was present at a very low level. This slow appearance of complement-fixing antibody, also noted by others (6, 11–13), makes it possible to detect diagnostic increases in antibody titer, and is thus an advantage in favor of the complement fixation method as a diagnostic procedure. Unfortunately, as is evident from the several tables, and as has been reported by others (6, 11–13), complement-fixing antibody may be rather late in appearing so that this advantageous factor is offset by another which militates against arriving at a serodiagnosis relatively early in the disease.

Little difficulty was encountered with heterotypic reactions, but it must be emphasized that the number of individuals examined was comparatively

small, and the actual extent to which heterotypic reactions might be expected must await the results of a considerably wider experience. With respect to specificity, in so far as other neurotropic diseases are concerned, examination of serum specimens from a small group of patients with Western equine or St. Louis encephalitis, or with mumps encephalitis, indicates that little trouble need be expected from this quarter. In the three instances in which there was a rise in complement-fixing antibody titer not only to the poliomyelitis virus but also to the Western equine or St. Louis encephalitis viruses, the possibility of dual infections cannot be ruled out, since the individuals came from an area where all three diseases are endemic and the causal viruses are disseminated at the same time of the year. In such situations, it is necessary not only to set up a battery of serologic tests for a variety of agents, especially those known to be operating in the particular area, but also to attempt isolation of the virus; on the basis of data derived from such laboratory studies, together with information obtained from the clinical and epidemiologic histories, it will be possible to appraise the role of each agent in the causation of overt or inapparent infections and to obtain more precise knowledge of the epidemiology of the diseases caused by these agents.

SUMMARY

A macroscopic (tube) complement fixation test for poliomyelitis, using infected tissue culture fluids, is described.

The test was applied to 27 individuals with a clinical diagnosis of poliomyelitis. In 18 patients it was possible to make a laboratory diagnosis of poliomyelitis on the basis of a rise in complement-fixing antibody titer and in 4 others on the basis of a high stationary antibody titer. One individual gave a high and equal antibody response to two virus types, 3 others had no detectable antibody, and 1 appeared not to have poliomyelitis.

Heterotypic reactions were encountered, but gave little difficulty in interpreting homologous responses. In those patients from whom a virus had been recovered, the serologic findings corresponded to the virus type recovered.

The possible occurrence of dual infections with the viruses of poliomyelitis and Western equine and St. Louis encephalitis is discussed.

BIBLIOGRAPHY

1. Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, **109**, 85.
2. Robbins, F. C., Enders, J. F., Weller, T. H., and Florentino, G. L., *Am. J. Hyg.*, 1951, **54**, 286.
3. Salk, J. E., Youngner, J. S., and Ward, E. N., *Am. J. Hyg.*, 1954, **60**, 214.
4. Loring, H. S., Raffel, S., and Anderson, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 385.
5. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.*, 1951, **94**, 123.
6. Casals, J., Olitsky, P. K., and Sabin, A. B., *J. Exp. Med.*, 1952, **96**, 35.

7. Pollard, M., *Proc. Soc. Exp. Biol. and Med.*, 1951, **78**, 388.
8. Pollard, M., *J. Immunol.*, 1951, **67**, 463.
9. Lahelle, O., *Am. J. Hyg.*, 1951, **54**, 391.
10. Svedmyr, A., Enders, J. F., and Holloway, A., *Proc. Soc. Exp. Biol. and Med.*, 1952, **79**, 296.
11. Svedmyr, A., Enders, J. F., and Holloway, A., *Am. J. Hyg.*, 1953, **57**, 60.
12. Black, F. L., and Melnick, J. L., *Yale J. Biol. and Med.*, 1954, **26**, 385.
13. Melnick, J. L., Ramos-Alvarez, M., Black, F. L., Girardi, A. J., and Nagaki, D., *Yale J. Biol. and Med.*, 1954, **26**, 465.
14. Fulton, F., and Dumbell, K. R., *J. Gen. Microbiol.*, 1950, **92**, 483.
15. Farrell, L. N., Wood, W., Franklin, A. E., Shimada, F. T., and MacMorine, H. G., *Canad. J. Pub. Health*, 1953, **44**, 273.
16. Wenner, H. A., Miller, C. A., Kamitsuka, P., and Wilson, J. C., *Am. J. Hyg.*, 1954, **59**, 221.
17. Lennette, E. H., unpublished data.
18. Lennette, E. H., and Longshore, W. A., Jr., *California Med.*, 1951, **75**, 189.
19. Lennette, E. H., *in* The Dynamics of Virus and Rickettsial Infections, (F. W. Hartman, F. L. Horsfall, Jr., and J. G. Kidd, editors), New York, The Blakiston Company, Inc., 1954, 364.
20. Sabin, A. B., Horsfall, F. L., Jr., Meyer, K. F., Scott, T. F. McN., and Snyder, J. C., *in* Viral and Rickettsial Infections of Man, T. F. Rivers, editor, Philadelphia, J. B. Lippincott Company, 1952, 2nd edition, 172.
21. Lennette, E. H., *in* Diagnostic Procedures for Viral and Rickettsial Diseases, (T. Francis, editor), New York, American Public Health Association, 2nd edition, in press.
22. Meiklejohn, G., Rust, E., and Brunetti, O., *J. Immunol.*, 1949, **62**, 293.
23. Lennette, E. H., Nyberg, M. C., Barghausen, D. M., Chin, R., Fujimoto, F. Y., and Itatani, M. K., *California Med.*, 1953, **79**, 78.