FRANCIS L. BLACK*
JOSEPH L. MELNICK

Section of Preventive Medicine, Yale University School of Medicine

THE SPECIFICITY OF THE COMPLEMENT FIXATION TEST IN POLIOMYELITIS**

Infection with poliomyelitis virus gives rise to complement-fixing (C-F) antibodies which may be detected with antigens prepared either from the brains of infected mice or tissue culture fluids, as shown with Type II by Casals *et al.*[1,2] and with all three types by Svedmyr *et al.*[9] These workers used sufficient antigen in each test to obtain maximum sensitivity. With this antigen concentration the presence of antibody could generally be detected within the first week after onset of the disease, but the sera usually gave similar C-F titers against antigens of the three types of poliomyelitis virus regardless of the type involved in the infection. It has now been found possible to obtain a more specific reaction in patients with the disease by a method in which not more than one conventional unit of antigen is used per test. In order to detect fixation with this low level of antigen, dilutions of serum were titrated at several complement concentrations by the plate method of Fulton and Dumbell.[8]

METHODS AND MATERIALS

The general technique of the plate C-F method as used in this laboratory has been described previously.[4] This method has already been used in poliomyelitis work by Svedmyr *et al.*,[9] and by Le Bouvier.[5] Instead of glass capillaries, the dropping pipettes used in this study consisted of No. 19 needles, with the bevelled tip removed,[9] fitted to "oiling tubes."† The unit in which *complement* was used was the greatest amount of freshly reconstituted, lyophilized guinea pig serum in a 20-cm. drop that caused no visible hemolysis in the test system. This "minimal unit" is approximately one-third of a conventional 100% lytic unit. Serum and antigen titrations were carried out against two, three, and four of these units and the controls were tested against one, two, and three units.

---

* Fellow of the Rockefeller Foundation.

** Aided by a grant from the National Foundation for Infantile Paralysis.

† Macalaster Bicknell Co. Inc., New Haven, Conn.

Received for publication April 6, 1954.

Antigens were prepared from virus grown to high titer (10^6 to 10^8 TC_{50} doses per ml.) in bottle-cultures of kidney tissue[6] and of HeLa cells.[8]* The Brunhilde and W-S (Type I), Y-SK (Type II), and Leon (Type III) strains were found to be satisfactory for the preparation of antigens. Fluids

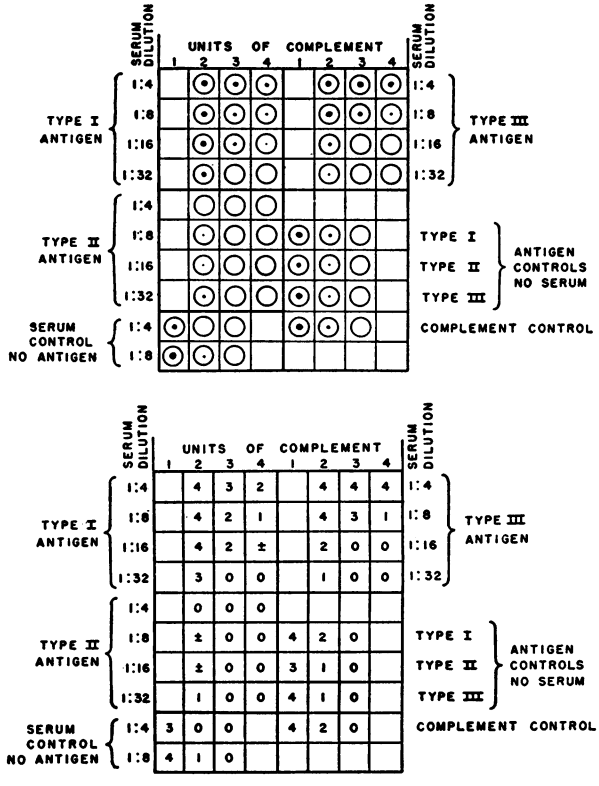

FIG. 1. Appearance of a typical plate at time of reading, and corresponding fixation scores.

from the tissue cultures were harvested when most of the cells had lysed (after 16 to 64 hours exposure to virus depending on the inoculum). These fluids were lightly centrifuged to remove cellular debris and were generally used without further treatment. The amount of antigen required to fix one minimal unit of complement in excess of the greatest amount fixed by any of the controls was termed one minimal unit. This is between one-half and

* Some of the HeLa bottle-cultures were obtained ready for use from Microbiological Associates, Bethesda, Maryland.

one-fourth of a conventional unit. Fluids containing less than two units per 20 cm. drop could be concentrated by centrifugation at 40,000 R.P.M. (about 106,000 x gravity) for two hours, and resuspension of the pellet in a small volume of diluent, but this was usually not necessary. Occasional lots were found anticomplementary in which case they could often be made serviceable by heating at 56° C. for 30 minutes.[9] Heating the antigens had the added advantage of rendering them non-infectious.

Sera were titrated with two minimal units of antigen per test drop against three concentrations of complement. The titer of a serum was taken as the highest dilution to fix more than one minimal unit of complement in excess of the amount fixed by either serum or antigen control. (For example in the Type I titration illustrated in Figure 1, the greater amount of fixation is in the antigen control. The highest serum dilution to give fixation of more than one unit in excess of this control is 1 : 16 and the serum titer is 16.) As well as the titer, it was possible to determine from such a two-dimensional titration, the total amount of complement fixed. This character of the serum, its *avidity* for complement, is partially independent of the titer. An expression of the avidity dependent on both the titer of the serum and the amount of complement fixed was calculated as follows: (a) the score for the degree of fixation was summed for all serum dilutions up to the titration endpoint, and from this was subtracted (b) the number of dilutions tested up to the endpoint, multiplied by the sum of the fixation score for the control showing the greater degree of fixation. Serum scores, (a) above, were calculated for the titrations with two, three, and four units of complement and the control scores (b) for one, two, and three units and thus the avidity was positive only when more than one unit of complement was fixed. (In the Type I example again, the value for (a) is 22, and for (b) the subtrahend, based on the antigen control, is 6 x 3 or 18. The avidity score is therefore four. The avidity for the Type III titration in Figure 1 is ten, although the titer is only eight.) With the human sera the avidity was found more reproducible and showed fewer erratic variations between serial serum samples than the simple serum titer.

RESULTS

Standard monkey sera. Monkey sera prepared and kindly supplied by Wenner *et al.*[10] were titrated in two-fold dilutions against various antigens at several concentrations. The results are given in Table 1. The titers obtained with the higher antigen concentrations were comparable to those found in the laboratories of Wenner and of Enders, and the slight crossing between the Type II serum and Type I antigen was confirmed.[10] The titers

found when approximately two minimal units of antigens were used were somewhat lower. Approximately two units were used in the studies on human sera reported below.

TABLE 1. TITER OF STANDARD POLIOMYELITIS SERA AGAINST DIFFERENT CONCENTRATIONS OF ANTIGENS

Antigens			*Titer of standard monkey sera*		
Virus strain	*Cell culture used*	*No. of minimal units*	*I*	*II*	*III*
Brun.	Kidney	3.5	>64	<4	<4
WS	HeLa	3.0	32	<2	<2
Brun.	Kidney	2.7	32	<2	<2
WS	HeLa	2.1	32	<4	<4
Brun.	Kidney	2.0	16	<2	<2
WS	HeLa	1.5	32	<4	<4
Brun.	Kidney	1.3	16	<2	<2
YSK	Kidney	3.5	4	>64	<4
YSK	HeLa	3.0	2	32	<2
YSK	Kidney	2.7	tr*	16	<2
YSK	HeLa	2.1	tr	16	<4
YSK	Kidney	2.0	tr	8	<2
YSK	HeLa	1.5	<8	<8	<4
YSK	Kidney	1.3	tr	4	<2
Leon	Kidney	4.0	<4	<4	>64
Leon	Kidney	3.3	2	<2	64
Leon	Kidney	2.5	<4	<4	32
Leon	HeLa	2.0	....	<2	16
Leon	Kidney	1.7	<8	<4	16
Leon	HeLa	1.4	....	<2	16
Leon	HeLa	1.0	....	<2	tr

*tr indicates trace (less than 2).

*Sera from poliomyelitis patients.** Acute and convalescent sera from 49 patients diagnosed as poliomyelitic were tested for C-F antibodies against the three types of antigen. These sera had previously been tested for neu-

*Material obtained from patients in the Lock Haven, Pennsylvania, epidemic of 1952. Detailed virological study in which Drs. Manuel Ramos Alverez and Anthony J. Girardi actively participated will be presented in a separate paper.

tralizing antibody, and stools from most of the patients had been tested for virus.[11] Both Type I and II viruses, but no Type III virus had been isolated from the stools. Some examples of the results are given in Table 2, and the

TABLE 2. EXAMPLES OF DATA OBTAINED ON LOCK HAVEN CASES

Patient no.	Type of virus isolated	Clinical evidence of paralysis	Date of serum*	Complement fixation: Titers I	Titers II	Titers III	Avidity I	Avidity II	Avidity III	Neutralization titers I	Neutralization titers II	Neutralization titers III
83	I	+	7	0	0	16	0	0	2	160+	20	0
			49	32	0	4	15	0	1	160+	40	0
			70	32	0	0	19	0	0	160+	160+	0
93	II	+	3	0	0	0	0	0	0	0	10	0
			33	0	32	0	0	15	0	0	100+	0
			44	0	32	0	0	31	0	0	100+	0
8	Neg.	—	3	0	0	0	0	0	0	100+	0	0
			21	4	0	0	1	0	0	100+	0	0
			50	16	0	0	7	0	0	100+	0	0

* Days after onset.
0 indicates less than 4.
32 indicates 32 or greater.

TABLE 3. SUMMARY OF RESPONSES IN C-F ANTIBODIES IN POLIOMYELITIS PATIENTS

Type of virus isolated	I	I	II	II	0	0
Paralysis	+	—	+	—	+	—
Antibody responses	No. of patients in each category					
Against one type	15	12	4	1	0	4
Against two types	3	0	1	0	0	0
Against three types	0	0	0	0	0	0
Against no type	0	1	1	2	2	3

types of response found in all the sera are classified in Table 3 by type of disease. All patients from whom virus was isolated had homotypic neutralizing antibodies in their serum and frequently this was already at high titer at the time of the first serum collection (0-14 days after onset), e.g. No. 83. In contrast homotypic C-F antibodies were rarely detectable in the first

serum sample by the method which has been described. Of the 40 patients studied from whom virus was isolated only three (2 Type I, 1 Type II) had demonstrable homotypic C-F antibodies at the time the first specimen was collected, 0-14 days after onset. In six cases the antibodies first became demonstrable in the third specimen more than 30 days after onset. More heterotypic responses were found in the neutralizing than in the C-F antibodies. There was no significant difference in the response of nonparalytic and paralytic patients regardless of whether infected with Type I or II viruses.

On the basis of the findings of Casals, Svedmyr, and their co-workers,[2,9] it was expected that the highest titers of C-F antibody would be found in the sera collected in the first few weeks after onset. However, in the present study homotypic C-F antibody was rarely found before seven days after the onset of the disease and often increased after the third week. Because of this it was possible to demonstrate an increase in titer more frequently than might otherwise have been the case. On the other hand it was not possible with this method to demonstrate such a rise until some weeks after the individual had become sick.

The above investigators had found that many persons infected with Type I poliomyelitis developed C-F antibodies against Type II antigen. Yet in our study only one individual infected with Type I virus showed a Type II C-F response, nor did the reverse heterologous response, Type I C-F antibodies in Type II infection, occur more frequently. These differences between the results of our own studies and those from the two other laboratories appear to be due to the smaller amounts of antigen used in the present work. Casals used 8 to 16 units of antigen in each test and Svedmyr an approximately equivalent amount, whereas the amount of antigen used in our study was not more than one conventional unit. Serum samples from poliomyelitis patients have been exchanged with Dr. John F. Enders and the results obtained by the method described in the present report proved more specific than those obtained with concentrated antigens.[9]

Sera from children inoculated with formalinized Type I virus. The details of this experiment[11] will be reported elsewhere; here we merely wish to call attention to the application of the complement fixation test to this type of investigation. Thirty apparently healthy children aged 12 to 17 were tested for C-F antibodies at the time of inoculation and 3 and 20 weeks after inoculation with a formalin-inactivated Type I virus preparation, similar to that used by Salk.[7] At the time of inoculation Type I C-F antibodies were not present at a detectable level in any individual, and Types II and III antibodies were each present in only one individual. The changes found are

given in Tables 4 and 5. Where antibodies appeared, they reached a peak titer of eight or greater in all but three instances. The responses in this group occurred, with but three exceptions, only against those types with which there had been previous experience as indicated by the neutralization tests that had been made before inoculation. Two of the three exceptions

TABLE 4. CHANGES IN C-F ANTIBODIES IN CHILDREN AFTER INOCULATION WITH TYPE I FORMALIN-INACTIVATED VIRUS

Type of antibodies	*Neutralizing antibodies prior to inoculation*	*Number without C-F antibodies pre-inoc.*	*C-F antibody response*	
			positive	*negative*
I	—	10	1	9
	+	20	8	12
II	—	15	1	14
	+	15	9	6
III	—	13	1	12
	+	13	7	6

TABLE 5. CHANGES IN TYPES I AND II C-F ANTIBODIES IN CHILDREN AFTER INOCULATION WITH TYPE I FORMALIZED VACCINE

Result of neutralization tests made prior to inoc.		*Total number of cases*	*I Increased II Negative**	*II Increased I Negative*	*I and II Increased*	*Both negative*
Type I	*Type II*					
—	—	7	0	0	1	6
+	—	8	4	0	0	4
—	+	3	0	2	0	1
+	+	12	1	4	3	4
Total		30	5	6	4	15

* Negative indicates serum titer of less than 2.

occurred in one individual where Types I and II antibodies appeared in the 20-week sample. The findings in healthy children inoculated with formalin inactivated virus were in marked contrast to those in poliomyelitic patients reported above. In the latter the response was usually only homotypic with the type of virus causing the infection. In the "vaccine" study where all the responses were caused by Type I virus material the

antibodies to the three types of virus responded similarly. The Type III response was somewhat weaker than the Types I and II reaching lower maximal titers (median for Type III was 8; for Types I and II, 32) and falling off more rapidly. Table 5 shows that the presence of Type I neutralizing antibody had no significant effect on the probability of a Type II C-F response and vice versa. The Type III C-F response was also independent of Types I and II neutralizing antibodies, and Types I and II C-F responses of Type III neutralizing antibodies, but to have included these combinations would have overburdened the table. The reason for the contrasting nonspecificity of the response in the group of children in whom antibodies were stimulated artificially as compared to the response in the natural infections, has not been found as yet.

SUMMARY

A complement fixation test for poliomyelitis which uses minimal quantities of complement and antigen has been described and its application noted in two phases of poliomyelitis investigations: serodiagnosis of infection and antibody response to "vaccination": (i) Results of tests for C-F antibodies for the three types of virus are reported on specimens obtained from 49 poliomyelitis patients. The results show a homotypic C-F titer rise in 90% of the patients from whom virus was isolated, and a heterotypic rise in 10%. (ii) Sera from 30 children before and after inoculation with formalin-inactivated Type I virus were tested for C-F antibodies. Of these, 60% showed a post-inoculation increase in Type I, II, or III antibodies. Some showed C-F responses to antibodies of more than one type. Nearly all of the increases occurred in individuals who possessed pre-inoculation neutralizing antibodies.

REFERENCES

1 Casals, J., Olitsky, P. K., and Anslow, R. O.: A specific complement fixation test for infection with poliomyelitis virus. J. Exp. M., 1951, *94,* 123.

2 Casals, J., Olitsky, P. K., and Sabin, A. B.: Development, persistence and significance of Type 2 poliomyelitis complement fixing antibody in man. J. Exp. M., 1952, *96,* 35.

3 Fulton, F. and Dumbell, K. R.: The serological comparison of strains of influenza virus. J. Gen. Microb., Lond., 1949, *3,* 97.

4 Kraft, L. M. and Melnick, J. L.: Immunological reactions of the Coxsackie viruses. J. Exp. M., 1950, *92,* 483.

5 Le Bouvier, G. L.: Observations on the poliomyelitis complement fixation test. Brit. J. Exp. Path., 1953, *36,* 300.

6 Morann, G. L. and Melnick, J. L.: Poliomyelitis virus in tissue culture VI. Use of kidney epithelium grown on glass. Proc. Soc. Exp. Biol., N. Y., 1953, *84,* 558.

7 Salk, J. E.: Studies in human subjects on active immunization against poliomyelitis. J. Am. M. Ass., 1953, *151,* 1081.

8 Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (Strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exp. M., 1953, *97,* 695.

9 Svedmyr, O., Enders, J. F., and Holloway, A.: Complement fixation with the three types of poliomyelitis viruses propagated in tissue culture. Am. J. Hyg., 1953, *57,* 60.

10 Wenner, H. A., Miller, C. A., Kamitsuka, P., and Wilson, J. C.: Preparation and standardization of antiserums prepared with the three known types of poliomyelitis viruses. Am. J. Hyg., 1954, *59,* 221.

11 Yale Poliomyelitis Study Unit, unpublished work.