We are also indebted to Miss Rosemary O'Bryan for very able technical assistance.

1. Hirst, G. K., *J. Exp. Med.*, 1942, v76, 195.
2. ———, *ibid.*, 1942, v75, 49.
3. Friedwald, W. F., Miller, E. S., and Whatley, L. R., *ibid.*, 1947, v86, 65.
4. Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 312.
5. Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *ibid.*, 1949, v72, 227.
6. McCrea, J. F., *Austr. J. Exp. Biol. Med. Sci.*, 1948, v26, 355.
7. Gottschalk, A., and Lind, P., *Brit. J. Exp. Path.*, 1949, v30, 85.
8. Dinter, Z., *Tierärztliche Umschau*, 1949, p. 229.

Received July 11, 1955. P.S.E.B.M., 1955, v90.

Importance of Optimum Amounts of Virus to Demonstrate Neutralizing Antibody Rise in Convalescent Poliomyelitis Sera.* (21969)

ROBERT WARD, CHARLES C. CHANG, AND DORA L. RADER.
(Introduced by L. Emmett Holt, Jr.)
From the Department of Pediatrics, New York University College of Medicine, and Bellevue Hospital, New York City.

A study of 38 patients infected with poliomyelitis viruses showed a 5-fold or greater increase in the homologous antibody titer in 35 (92%). This finding was at variance with that of other workers(1,2), who postulated that the rise in neutralizing antibodies occurred so rapidly that often no increases in titer were demonstrable. Thus Miller and Wenner, using a virus concentration of 32 $TCID_{50}$† in their neutralization tests, observed a 10-fold rise in homotypic antibody in only 3 of 14 patients(2). Tests for homologous antibody were carried out in only 7 patients, none of whom showed a similar rise. Melnick and his associates, with a virus inoculum of 100 $TCID_{50}$, reported rises of homotypic antibody in 15 (37%) of 41 patients from whom poliomyelitis viruses had been isolated(1). Homologous antibody formation was not studied by these workers.

This report presents evidence that the demonstration of increases of homologous antibody in patients infected with poliomyelitis viruses is related to the dose of virus used in the neutralization test.

Materials and methods. Data are derived from 38 patients studied in New York City in 1953, a non-epidemic year.‡ Their stools were collected within 2 weeks of onset of illness and after concentration in the Spinco high speed centrifuge, yielded poliomyelitis viruses as follows: Type 1, 25 patients; Type 2, 2 patients; Type 3, 11 patients. All but 4 showed some degree of weakness or paralysis. Serum specimens were collected at frequent intervals and tested for neutralizing antibodies against the patient's own virus and the 3 prototypes. To be reported here are the results of tests carried out on mixtures of homologous virus and serum obtained usually in the 1st or 2nd week of illness, in the 3rd or 4th week, and lastly at about the 12th week. Trypsinized monkey kidney cells grown in tissue culture in glass tubes§ and examined microscopically for cytopathogenic changes constituted the test object for isolation of virus and for neutralization tests. Neutralization tests were carried out usually with serial 4-fold dilutions of serum, inactivated at 56°C for 30 min. Serum dilutions were mixed with an equal vol-

* Aided by a grant from the Sister Kenny Foundation.

† $TCID_{50}$—Tissue culture infective doses, 50% end point.

‡ We are grateful to the staffs of Willard Parker, Mt. Sinai, Babies', and Flower-5th Avenue Hospitals for supplying material for this investigation.

§ For the past year tubes containing cultures of monkey kidney cells supplied by Microbiological Associates, Inc., Bethesda, Md., have been used.

TABLE I. Relation of Increase in Neutralizing Antibody and Dose of Virus Used in Test. 38 patients.

Day of illness 1st serum obt.	Virus type in stool	Dose of virus in neut. test*	Antibody fold-increase		
7	1	3.2	500		
6	3	2.7	400		
5	3	4.0	200		
4	2	2.3	200		
2	1	2.7	200		
11	1	2.5	60		
8	3	3.7	50		
7	1	4.2	40		
11	1	3.7	40		
3	1	3.0	30		
7	1	3.5	25		
8	1	2.7	25		
18	3	3.9	25		
7	3	1.2	25		
8	1	3.7	16+?	76%	
5	3	3.2	16		
10	1	3.5	16		
7	1	4.5	12+?		92%†
7	1	1.5	12+?		
8	1	4.5	12		
8	3	2.5	12		
3	1	3.2	12		
8	1	2.0	10+?		
9	3	4.2	10		
5	3	4.0	10		
4	1	4.0	10		
6	3	3.0	10		
4	1	2.5	10		
14	2	1.0	10		
8	1	2.7	7		
9	1	4.7	7		
18	1	2.7	6	16%	
18	1	3.2	5		
9	1	2.5	5		
16	1	3.5	5		
7	1	4.0	3		
14	3	2.7	2		
10	1	4.9,3.5,2.4,1.2	0		

* Expressed as negative log. † 92% of patients.

ume of a selected dilution, usually 1:500, of the patient's virus. The latter was titrated simultaneously in each test against normal monkey serum. The mixtures were incubated at room temperature for one hour. 0.1 cc of each dilution was pipetted into tissue culture tubes containing 0.9 cc of standard medium.‖ As a rule 3 tubes per dilution were used. In some of the "box" titrations 2-fold dilutions of serum were mixed with decimal dilutions of virus and inoculated into 6 tubes per dilution. All tubes were examined daily under the microscope for cytopathogenic effects. The seventh day reading was taken as final. Antibody titers were calculated by the method of Reed and Muench and expressed as the 50% serum dilution endpoint (log of reciprocal).

Results. Table I shows the relation of degree of antibody rise and dose of virus used in the test. A 5-fold or greater increase in homologous antibody obtained in 35 (92%) of 38 patients. Twenty-nine patients (76%) had antibody rises from 10 to 500-fold. In 6 patients (16%) increases from 5 to 7-fold were obtained. It is evident that antibody increments as great as 200-fold were obtained with as much as 4 logs of virus. The average amount of virus used in all the tests was about 3 logs. Substantial increases in antibody were demonstrated even when the first serum specimen was obtained as late as the second or third week of illness. In the group whose antibody rises were under 10-fold the first serum was obtained on an average of the 12th day of illness, whereas in the group of 29 patients whose antibody rises were 10-fold or greater, the first serum was drawn on an average of 7.2 days after onset. If the reverse calculation is made, it will be seen that the average antibody rise is 95-fold in patients whose first serum was obtained in the first 7 days of illness as compared with an average of 16-fold for those whose first serum was obtained between the 8th and 18th days.

"Box" Neutralization Tests with Homologous Virus. During the beginning of this investigation in most of the individual tests a single relatively large amount of virus was used against serial dilutions of serum. It seemed possible that the greater number of definite antibody rises observed might be related to the larger doses of virus employed as compared with those used by previous observers. This possibility was investigated by simultaneous titrations of virus and serum (so-called "box" titration) from 3 patients—2 infected with Type 1 (A and B) and one with Type 3 (C). The results ap-

‖ The "standard medium" consisted of Earle's balanced salt solution, lactalbumin hydrolysate 0.5% and calf serum, 2%. 50 units of penicillin and 50 mg of streptomycin per ml of medium were added. Recently 20 units of mycostatin (Squibb) per ml were added which has helped considerably to suppress the growth of yeasts.

TABLE II. "Box" Neutralization Tests.

Patient	Day of illness serum drawn	A: Logs of *homologous* virus in test: .5	.6	.7	1.5	1.6	1.7	2.5	2.6	2.7	3.5	3.6	3.7	4.5	4.6	4.7	5.6	B: Logs of *homotypic* virus in test: .4	.6	1.4	1.6	2.4	2.6	3.4	3.6	4.4	4.6	5.4
		Antibody titer (log of dilution)																										
A	3	3.4+?			2.3			.6, 1.2			.6			<.6 <1.2														
	80	3.5			2.8			2.6, 2.4			1.7																	
B	9		3.2+?*			2.2			1.6			1.1			<.6		<.6	3.0+		2.6		2.0		1.1–?		.5–?		<.6
	82		3.6			3.4			2.8			2.8			1.1		1.0	4.0		3.2		2.5		2.1		.9		<.6
C	15			2.6+?			2.2			1.7			1.1			<.6			2.8†		2.0		1.7		1.2		.5	
	93			3.2			2.8			2.2			2.2			1.1			3.2		2.7		2.4		2.2		1.5	
	Fold increase	0	2–?	?	3	16	4	100, 16	16	3	12	50	12	?	4	4	3	0	2½	4	5	3	5	10+?	10	2+?	10	0

* 6 tissue culture tubes per dilution of serum.
† *Idem*
; serial 2-fold dilutions of serum.

pear in Table II (Section A). It is evident that little or no antibody rise was demonstrable with the smallest doses of virus (.5, .6, .7 log). With 1 log higher dose only 1 patient (B) showed a significant rise; with 2 logs, 2 patients, and with 3 logs, all 3 patients showed substantial rises. When the sera were "overwhelmed" with 4.5-5.6 logs of virus, no significant antibody increase resulted. In the test with patient B's virus and serum the accuracy was enhanced by using 6 tubes per dilution. In the case of patient C, a 12-fold antibody increase was the maximum achieved. This occurred with 3.7 logs of virus. It may be that this relatively small antibody rise is accounted for by the lateness of obtaining the acute phase serum, *e.g.*, 15th day of illness. In any event, the result of this test shows a trend in keeping with the pattern of the other two.

"Box" Neutralization Tests with Homotypic Virus. It was important to learn if this apparent relation between antibody rise and dose of virus would apply to *homotypic* as well as to homologous virus. Similar "box" titrations were performed with the sera of 2 patients, B and C, mixed with Type 1 (Mahoney) and Type 3 (Leon) viruses, respectively. To increase the accuracy of the tests, these box titrations were carried out with serial 2-fold dilutions of serum against decimal dilutions of virus and the mixtures were inoculated into 6 tissue culture tubes per dilution. The results are presented in Table II (Section B). As with homologous viruses, little or no antibody rise resulted with the smallest doses of homotypic virus. With 3.4 and 3.6 logs, 10-fold or higher increments were obtained. Unlike the other tests in which the largest doses of virus either obliterated or diminished the difference in antibody level between the two sera, in the case of patient C's sera and the heaviest concentration of Leon virus (4.6 logs), a 10-fold increase was still demonstrable. Then actual titers are not very different, however, from those obtained with C serum and homologous virus (Section A). In that test when a comparable dosage (4.7 logs) of C virus was used, even though it seemed to result in a 4-fold antibody rise, the titers of each serum (0.6 and 1.1) did not differ significantly from those obtained with Leon virus (0.5 and 1.5).

From the results of these 2 tests with homotypic viruses, it would appear that the relation of antibody increase to virus dose was not limited to the patient's own virus, but a similar pattern was obtained with homotypic viruses, Mahoney and Leon, respectively.

Discussion. The results obtained in individual tests with various virus strains and sera indicated a relationship between increase in neutralizing antibody and concentration of virus used in the test. This was confirmed by "box" neutralization tests in which virus and 2 serum specimens from the same patient were titrated simultaneously. In the zones of antibody excess and virus excess, respectively, there was little or no apparent increase in antibody from acute to convalescent phase of illness. In the middle dosage range of about 3 logs of virus regular antibody increases were demonstrable. The explanation of this phenomenon is not clear. The question arises as to whether antibodies found in the acute stage differ qualitatively or quantitatively from those found in convalescence. Work is being done currently on the dynamics of virus-antibody union which may shed light on the problem. For example, is the union between virus and "late" antibody more stable than that which occurs with "early" antibody? Are there detectable differences in the velocity of the reaction? Does "late" antibody have a greater avidity for virus than "early" antibody? The results of the present report have been obtained entirely with the use of cultures of monkey kidney cells as the test object of virus activity. It is important that Steigman and Sabin(3) and Winsser and Sabin(4), who carried out quantitative neutralization tests in monkeys, observed substantial homologous antibody increases between 16 and 32-fold with amounts of virus ranging from 160 to 1000 PD_{50}. The results of the present study indicate that the relation between antibody rise and virus dose is not limited to homologous systems. The acute and convalescent sera of 2 patients (one with a Type 1 infection, the other Type 3) showed similar antibody trends against the homotypic virus, Mahoney and Leon, respectively.

Even though the mechanism by which it works is not understood at present, the phenomenon appears to have important practical as well as fundamental implications. The neutralization test may still prove to be an important diagnostic tool provided the dose of virus is properly adjusted. It would appear that approximately 1000 $TCID_{50}$ is the optimum dose of virus. Another factor of importance in demonstrating antibody increase is the time of drawing the "acute" serum sample—the earlier the better. The study of basic problems concerned with the kinetics of the poliomyelitis virus-antibody reaction will be advanced by the use of Dulbecco's new plaque method(5).

Summary. The formation of neutralizing antibodies against their own virus was studied in 38 poliomyelitis patients. In the sera of 35 (92%), 5-fold or greater antibody increases were demonstrated. Twenty-nine (76%) exhibited rises between 10 and 500-fold. The average dose of virus used in separate tests was about 3 log dilutions. "Box" neutralization tests revealed that little or no antibody increase was demonstrable in the zones of antibody or virus excess. Substantial antibody rises were obtained in the middle zones, when between 2.5 and 4.6 log dilutions of virus were used. A similar trend of antibody increase was found with homotypic virus studied in two individuals. These results seem to indicate a relation between increase in neutralizing antibody and concentration of virus in the test. The mechanism by which this phenomenon operates is not understood but it appears to have both fundamental and practical implications.

1. Melnick, J. L., Ramos-Alvarez, M., Black, F. L., Girardi, A. J., and Nagaki, D., *Yale J. Biol. and Med.*, 1954, v26, 465.

2. Miller, C. A., and Wenner, H. A., *Pediatrics,* 1954, v14, 573.

3. Steigman, A. J., and Sabin, A. B., *J. Exp. Med.,* 1949, v90, 349.

4. Winsser, J., and Sabin, A. B., *ibid.,* 1952, v96, 477.

5. Dulbecco, R., and Vogt, M., *ibid.,* 1954, v99, 167.

Received July 18, 1955. P.S.E.B.M., 1955, v90.