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NEUTRALIZING ANTIBODY TO POLIOVIRUSES IN NORMAL HUMAN URINE *

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Among the viruses that have been isolated from urine during the course of human infections are those of lymphocytic choriomeningitis (1), mumps (2), measles (3), rabies (4), and the Coxsackie- (5), salivary gland- (6), and adenoviruses (7). These viruses have usually been recovered during the systemic phase of each disease, but it is not known whether they actually produce infection of the kidney or other parts of the genitourinary tract. Many others, including polioviruses, have not been isolated from human urine; a few attempts in this laboratory to cultivate these viruses from urine in acute cases of poliomyelitis have failed, and we are unaware of any reports of successes. It is known, however, that the presence of antibody, even in amounts which are not detectable by conventional methods, may prevent disease and the detection of virus (8). It has also been shown that γ-globulins are among the biocolloids of normal human urine (9). The possibility that antibodies to polioviruses might be present in human urine, therefore, seemed worth exploring. The demonstration of poliovirus-neutralizing antibodies in the urine of some normal individuals was reported recently in a brief preliminary communication (10). The details of the methods and the results are presented in this paper.

MATERIALS AND METHODS

The subjects for this study were 21 adult males ranging in age from 27 to 39 years, except for one (Subject 19) who was 59. All but two of them (Subjects 5 and 19) had previously received 3 or more inoculations of formalin-inactivated trivalent (Salk) vaccine. Subjects 1, 5 and 12 had previously had poliovirus infection and Subject 1 had also taken one oral dose of live, attenuated trivalent (Cox) vaccine. All the subjects were free of proteinuria by the heat and sulfosalicylic acid tests. A specimen of serum from venous blood was obtained from each subject and the overnight output of urine was collected without preservative on the same day. In a few instances the urine was collected over a 24-hour period. A booster dose of one or another kind of poliovirus vaccine was given to seven of the subjects and serum and urine were again collected 1 to 7 weeks later; two of these seven subjects also received a second booster of a different kind of vaccine.[1]

Preparation of urine concentrates. Each overnight specimen (or 24-hour collection) of urine was cleared of insoluble debris by centrifuging at 2,000 rpm. The cleared urine was dialyzed for 48 hours against running tap water at 4 to 8° C and then subjected to pervaporation either at 4° C or at room temperature, followed by desiccation from the frozen state *in vacuo* (lyophilization). The resulting buff-colored or white powder was reconstituted to about 5 ml with 5 per cent glucose buffered to pH 7.0 with 7.5 per cent sodium bicarbonate and containing antibiotics (see below); this was rendered bacteria-free by Seitz filtration. Approximately 100-fold concentration of the urine was thus achieved. In several instances ammonium sulfate was added to the urine to a concentration of 50 per cent; the resulting precipitate was redissolved in 0.85 per cent sodium chloride and processed like untreated urine.

Tissue cultures. Cultures of monkey (rhesus) kidney (MKTC)[2] were maintained in Eagle's medium (11) to which 5 per cent inactivated horse serum and antibiotics[3] were added; this medium was not changed during the 7 days over which the cultures were observed in the neutralization and immuno-inactivation tests.

A culture of HEp2 cells[2] was maintained in this

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[1] The Salk-type vaccine was obtained from Wyeth Laboratories, Inc.; the type 3 oral attenuated vaccine was furnished by Dr. A. B. Sabin and a "purified and concentrated, inactivated vaccine" (Purifax) was supplied by Dr. M. R. Hilleman of Merck Sharp & Dohme Research Laboratories, West Point, Pa.

[2] Obtained from Microbiological Associates (MBA), Bethesda, Md.

[3] All tissue culture media contained penicillin, streptomycin, and nystatin in concentrations of 250 U, 250 μg, and 100 U per ml, respectively.

laboratory. This tissue was grown in Blake bottles in Eagle's medium plus 10 per cent calf serum. The cells were subcultured every 2 or 3 days by harvesting with 0.025 per cent trypsin in Hanks' balanced salt solution (BSS), centrifuging at 1,200 rpm for 10 minutes, and resuspending the cell pellet in twice the original volume of growth medium.

Plaque cultures were prepared in 25-ml screw-capped plastic flasks,[4] each of which contained 3 ml of cell suspension (1:150 suspension of packed cells in growth medium). The cells usually formed confluent sheets in 72 hours. Plaque counts of virus were made by a modification of the method of Holland and McLaren (12). After the sheet formed, the growth medium was decanted and the cells allowed to incubate for 1 hour at 37° C with 0.1 ml of dilutions of virus or with 0.2 ml of virus-serum or virus-urine concentrate mixtures, the tissue in the flasks being kept moist during this time by the addition of 1.0 ml of BSS. After this adsorption period, all fluid was poured off of the cell culture and replaced by 1 ml of 1 per cent Noble agar (Difco) in doubly distilled water which had been kept fluid at 42° C, and 1 ml of a medium containing twice-concentrated Earle's salt solution, 10 per cent chick serum, 0.4 per cent bovine plasma albumin, and 0.4 per cent yeast extract [5] kept at 37° C. The flasks were then incubated at 37° C for 72 hours, after which the semisolid agar was allowed to slide off gently, and the poliovirus plaques on the sheet of cells were developed by staining for 1 minute with 1 per cent crystal violet in 20 per cent ethanol in doubly distilled water. Excess dye was washed off with several milliliters of physiologic saline.

Polioviruses. The strains of poliovirus used in the neutralization tests were originally isolated in this laboratory from cases that occurred in 1959. Type 1, strain 372, was isolated from a rectal swab of a 1.5 year old boy with aseptic meningitis in primary culture of human amnion cells. Type 3, strain 283, was isolated in MKTC from a rectal swab of a 21 year old man with paralytic poliomyelitis. Both strains were passed several times in MKTC, after which a 20 ml pool of each was prepared by harvesting a number of cultures inoculated at one time. Each pool was assayed for infectivity in MKTC, using 3 tubes per 10-fold dilution, and the virus reidentified by the neutralization test as recommended by the Committee on Enteroviruses (13). The pools contained from 10^5 to 10^7 TCD_{50} per ml [6]; they were kept frozen at −20° C and remained stable throughout these studies.

Tests for antibody. Neutralizing antibody to polioviruses was measured in the usual manner as previously described (14) or by the immuno-inactivation (IA) modification of Gard (15). In the latter procedure the virus and serum, or virus and urine concentrate, were incubated at 37° C for 6 hours instead of at 20° C for 30 minutes. Plaque reduction combined with immunoinactivation (PRIA) was also used primarily with the urine concentrates, and the levels of neutralizing antibody were calculated at 20, 50, and 80 per cent reduction in plaque-forming units (PFU). Virus controls usually contained about 50 PFU per flask. Appropriate controls of virus, cells, normal urine,[7] and normal rabbit serum were included in each test in a ratio of 1 to 10.

Comparison of biologic properties of the urine concentrates with those of serum. A specimen of urine concentrate and one of serum, each of which had previously been shown to neutralize type 3 poliovirus to about the same titer, were subjected simultaneously to each of the following procedures, after which the neutralizing titer was again determined by the IA test: *1)* incubation at 37° C for 30 minutes with an equal volume (0.5 ml) of 0.05 per cent trypsin in BSS; *2)* treatment with an equal volume (0.5 ml) of 0.9 M potassium periodate in BSS for 8 hours at 4° C followed by addition of 1.0 ml of 10 per cent glycerine to neutralize the excess periodate; *3)* incubation at 37° C with an equal volume (0.5 ml) of a filtrate of *Vibrio cholerae* containing receptor-destroying enzyme (RDE, neuraminidase); and *4)* incubation at 56° C and at 65° C for 30 minutes.

Also, *5)* serum and a urine concentrate known to neutralize types 1 and 3 polioviruses in high titer, were each mixed with an equal volume of serum from a rabbit that had received repeated intramuscular injections of a pool of normal human serum.[8] They were also similarly absorbed with serum of a rabbit that had received multiple injections of human γ-globulin (AHGG). The mixtures were refrigerated at 4° C overnight and the resulting fine precipitates were removed by centrifugation at 1,200 rpm for 15 minutes (16). Normal rabbit serum and the other usual controls of virus and cells were also tested simultaneously with the supernates, using both type 1 and type 3 polioviruses.

Finally, *6)* the kinetics of neutralization of type 3 poliovirus was determined in a serum (IA titer 1:320) and a urine concentrate (IA titer 1:160). A stock virus ($10^{6.5}$ TCD_{50} per ml) was used; 0.3 ml of each specimen was mixed with 0.6 ml of BSS and 0.1 ml of the virus suspension and incubated at 37° C for 6 hours. Aliquots of 0.1 ml of each mixture were removed at appropriate intervals, placed in separate 5-ml screw-capped vials containing 0.9 ml of cold (4° C) BSS and stored promptly at −20° C. Similar aliquots of the control suspension of virus in BSS were treated in the same manner. All of the vials were subsequently thawed and 0.1-ml aliquots were assayed for viral content using 10 MKTC tubes for each 10-fold dilution. The vials were immediately refrozen and the contents of some of them were subse-

[4] No. 3004 (formerly TCF25), Falcon Plastics Co., 550 W. 83rd St., Los Angeles 45, Calif.

[5] Supplied by Armour Pharmaceutical Co., Kankakee, Ill.

[6] TCD_{50} is the amount of virus which produces cytopathic effects in 50 per cent of inoculated culture tubes.

[7] Concentrated urine from a person lacking specific IA antibody in serum.

[8] This rabbit serum was prepared and supplied by Dr. Manuel E. Kaplan of the Hematology Division, Thorndike Memorial Laboratory.

quently reassayed at different times with results that were in close agreement.

RESULTS

Methods of concentrating urine.

Urine concentrates prepared by the various methods employed here yielded very similar results. This was demonstrated clearly with urines collected from Subject 1 on successive days and concentrated by different methods. One specimen, prepared by pervaporation at 4° C and then lyophilized, yielded an IA titer of 1:32 for type 3 poliovirus; a second specimen subjected to pervaporation at room temperature before lyophilization, and a third prepared by precipitation with 50 per cent ammonium sulfate, each gave an IA titer of 1:64 with same virus. For convenience, pervaporation at room temperature to a volume of about 100 ml followed by lyophilization was adopted for routine use (10, 17).

Some biologic properties of the urine concentrate

Effect of heat, trypsin, periodate, and RDE. The effects of several procedures on the poliovirus-neutralizing activity of one of the urine concentrates and of one specimen of serum are compared in Table I. The neutralizing activity of both the serum and the urine was destroyed by exposure to 65° C for 30 minutes, but it was virtually unaffected by treatment with trypsin, potassium periodate, or cholera vibrio filtrate. An ammonium sulfate precipitate of one of the urine concentrates that was active in the IA test was subjected to electrophoresis and it was shown to migrate as a γ-globulin.

TABLE I

Effect of various procedures on neutralizing titers of serum and urine concentrates *

Procedure	Titer of immuno-inactivation of type 3 poliovirus†	
	Serum	Urine concentrate
Control (56° for 30 min)	320	160
Exposure to:		
65° for 30 min	<10	<10
Trypsin	160	96
Filtrate of cholera vibrio	160	96
Potassium periodate	160	96

* The serum was from Subject 9 who had been immunized with an inactivated (Salk) vaccine; the urine was from Subject 1 who had a natural infection and also had been vaccinated successively with inactivated and live attenuated vaccine.

† The titers are expressed as reciprocals of the endpoint dilution; a 2-fold difference in titer is considered to be within the experimental error.

TABLE II

Effect of single absorption with antihuman serum (AHS) and with antihuman γ-globulin (AHGG)*

Material tested†	Poliovirus	Control titer‡	Titer‡ after absorption with	
			AHS	AHGG
Serum	Type 1	512	80§	
	Type 3	>1,024	320§	
Urine concentrate	Type 1	64	<4	<4
	Type 3	>128	16	<4

* Serum of rabbit obtained after repeated intramuscular injections of pooled normal human serum.
† From Subject 1.
‡ Reciprocal of endpoint dilution in immuno-inactivation test.
§ Same titer obtained with equal parts of rabbit serum used undiluted or diluted 1:10.

Effect of absorption with AHS and AHGG. The IA titers of a serum and a urine concentrate from Subject 1 before and after a single absorption with antihuman serum (AHS) and AHGG are given in Table II. Absorption with AHS reduced the IA neutralizing titer of the serum against type 1 poliovirus from 1:512 to 1:80 and against type 3 from >1:1,024 to 1:320. The corresponding reductions in IA neutralizing titers of the urine concentrate were from 1:64 to <1:4 for type 1 and from >1:128 to 1:16 for type 3 poliovirus. Absorption with AHGG was even more effective in reducing the IA titers. The poliovirus-neutralizing activity of serum and urine concentrates was thus similarly affected by these absorptions.

Kinetics of the neutralization. The results of the study of the kinetics of the neutralization of type 3 poliovirus by a serum and a urine concentrate over a period of 4 hours are shown in Figure 1. The titer of the virus was not affected by the incubation with BSS during the 4 hour period. The curve depicting the changes in titer of type 3 poliovirus at successive intervals during incubation with the urine concentrate is consistent with inactivation by antibody, the first portion showing the early rapid inactivation to be that of a first-order reaction (18); the curve is similar in character to that resulting from incubation of the virus with serum.

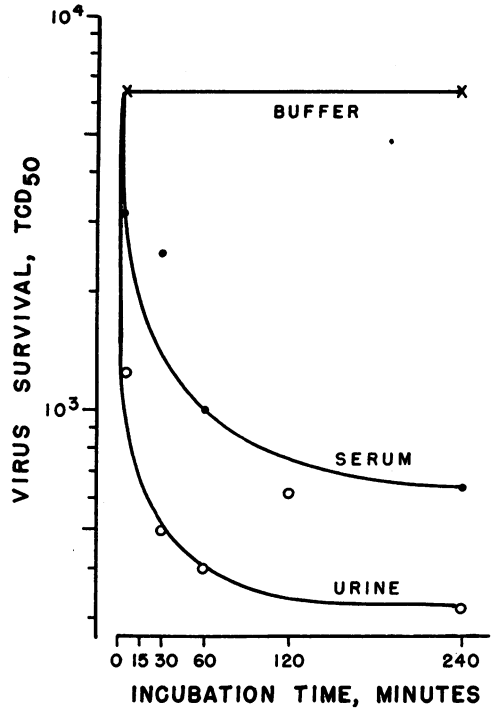

FIG. 1. KINETICS OF NEUTRALIZATION OF TYPE 3 POLIOVIRUS BY SERUM AND URINE.

Plaque-reduction endpoints. Neutralizing antibody was measured in both serum and urine by IA. In the urine and in occasional sera that were negative by the IA test, the plaque-reduction method was combined with IA. Such PRIA tests were performed and evaluated in 29 urine concentrates from the 21 subjects and calculated at 20, 50, and 80 per cent reduction in PFU. The results are listed in Table III. At the 80 per cent level of reduction in PFU, neutralization was demonstrated against type 1 poliovirus in 8 of the urine concentrates and in only 4 of them against type 3 poliovirus. (Only 28 specimens were tested with the type 3 strain.) At the 50 per cent level, the number that neutralized these viruses increased to 11 and 7 for type 1 and type 1 and type 3, respectively, whereas at the 20 per cent level of reduction in PFU the corresponding numbers in which neutralization was demonstrable increased to 15 and 8. In individual specimens in which neutralization was demonstrated by this method, the titer generally, but not always increased when a lower percentage reduction in PFU was used for the endpoint. For comparisons with antibody in serum, the 50 per cent

TABLE III

Titers of poliovirus-neutralizing activity in urine concentrates determined at three levels of reduction in number of plaque-forming units

	Titer of poliovirus neutralization in urine (reciprocal)					
	Type 1			Type 3		
Subject*	20%†	50%	80%	20%	50%	80%
1	640	160	80	320	320	320
2	<4	<4	<4	<4	<4	<4
3	16	16	8	>64	>64	>64
4	<4	<4	<4	<4	<4	<4
5	>32	>32	>32	<4	<4	<4
	64	16	4	<4	<4	<4
	<4	<4	<4	<4	<4	<4
6	<4	<4	<4	<4	<4	<4
	<4	<4	<4	16	4	<4
	32	16	8	32	16	8
7	<4	<4	<4	8	8	<4
8	16	<4	<4	<4	<4	<4
9	32	32	16	<4	<4	<4
10	4	4	4	<4	<4	<4
	32	4	<4	<4	<4	<4
11	<4	<4	<4			
12	<4	<4	<4	<4	<4	<4
13	<4	<4	<4	4	<4	<4
	4	<4	<4	<4	<4	<4
14	<4	<4	<4	<4	<4	<4
15	<4	<4	<4	16	8	8
	<4	<4	<4	<4	<4	<4
16	<4	<4	<4	<4	<4	<4
	4	4	<4	<4	<4	<4
17	<4	<4	<4	<4	<4	<4
18	8	<4	<4	8	8	<4
19	16	<4	<4	<4	<4	<4
20	16	8	<4	<4	<4	<4
21	4	4	4	<4	<4	<4

* Multiple specimens from 6 of the subjects correspond to those listed in Table V.

† Per cent reduction in number of plaque-forming units.

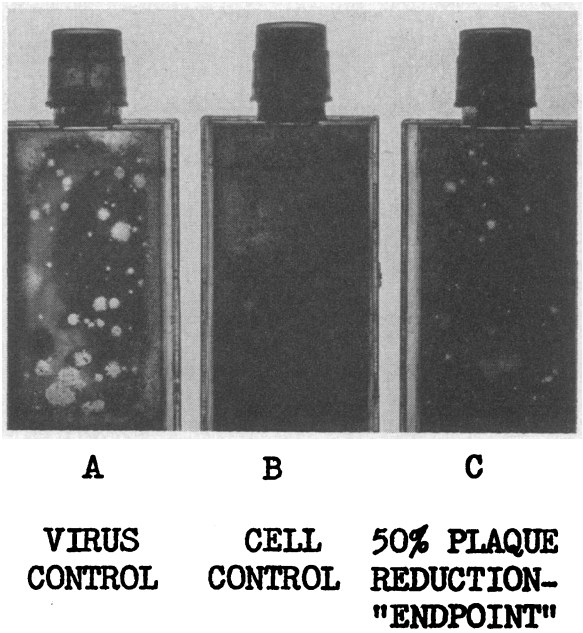

FIG. 2. TISSUE CULTURE FLASKS SHOWING APPROXIMATELY 50 PER CENT REDUCTION IN NUMBER OF PLAQUE-FORMING UNITS OF TYPE 3 POLIOVIRUS BY A URINE CONCENTRATE.

plaque-reduction endpoint was chosen. Because simultaneous replicates varied by as much as 10 per cent, reliance was not placed on the 20 per cent reduction endpoint. Other workers have employed either 80 or 50 per cent reduction for endpoints (19, 20). Typical plaque bottles illustrating approximately 50 per cent reduction in PFU of poliovirus are shown in Figure 2.

Occurrence of poliovirus antibody in serum and urine concentrates

Single observations in 21 subjects. The neutralizing titers for types 1 and 3 poliovirus obtained by IA in single specimens of serum and in corresponding urine concentrates of the 21 subjects of this study are listed in Table IV. PRIA titers evaluated at the 50 per cent level of reduction in PFU are also listed. Similar data obtained before and after administration of booster doses of poliovirus vaccines to 7 of these subjects are presented in Table V. Neutralizing activity was not detected in any of the urine concentrates by either test in the absence of antibody in the corresponding serum, except on one occasion in Subject 13 before a booster dose of vaccine when there was a 20 per cent reduction of PFU of type 3 poliovirus with a 1 : 4 dilution of the urine concentrate and no IA in 1 : 10 dilution of the corresponding serum; the same specimen of urine concentrate failed to produce 50 or 80 per cent reduction in PFU in the same test.

TABLE IV

Neutralizing antibody to polioviruses in human serum and in corresponding urine concentrates

Subject	Reciprocal of antibody titer to polioviruses*: Type 1 Serum IA	Type 1 Urine concentrate IA	Type 1 Urine concentrate PRIA	Type 3 Serum IA	Type 3 Urine concentrate IA	Type 3 Urine concentrate PRIA
1	512	40	320	>1,024	160	320
2	20	<4	<4	20	<4	<4
3	20	<4	16	80	12	>64
4	<4(>64)	<4	<4	<4	<4	<4
5	640	<4	>32	<10	<4	<4
6	<10	<4	<4	<10	<4	<4
7	>1,280	<4	<4	>1,280	<4	8
8	>1,280	<4	<4	>1,280	<4	<4
9	>1,280	<4	32	320	<4	<4
10	40	<4	4	20	<4	<4
11	20	<4	<4	>640	4	
12	<10	<4	<4	20	<4	<4
13	<10(<4)	<4	<4	<10(<4)	<4	<4
14	<10(64)	<4	<4	40	<4	<4
15	<10(<4)	<4	<4	640	2	8
16	<10	<4	<4	<10	<4	<4
17	<10(>64)	<4	<4	80	<4	<4
18	160	<4	<4	320	<4	8
19	80	<4	<4	10	<4	<4
20	>640	<4	8	320	<4	<4
21	>80†	<4	4	>80	<4	<4

* IA = immuno-inactivation; PRIA = 50% plaque reduction combined with immuno-inactivation. The values shown in parentheses represent PRIA titers of sera negative by IA.

† Result after vaccination; titer of a prevaccination serum was <4 by IA and PRIA, but corresponding urine was not available.

TABLE V

Effect of booster doses of poliovirus vaccines on titers of neutralizing antibody in sera and urine concentrates *

Subject	Previous infection or vaccination	Booster vaccine: Type	Booster vaccine: Date†	Date† of serum and urine	Reciprocal of antibody titer to polioviruses: Type 1 Serum IA	Type 1 Urine concentrate IA	Type 1 Urine concentrate PRIA	Type 3 Serum IA	Type 3 Urine concentrate IA	Type 3 Urine concentrate PRIA
5	NI			2/25	640	<4	>32	<10	<4	<4
		AL3	3/6	3/27	320	<4	<4	<4	<4	<4
		PT	5/11	6/2	>640	<4	16	160	<4	<4
				6/9			<4			<4
6	FT			2/25	<10	<4	<4	<10	<4	<4
		AL3	3/6	4/3	<10	<4	<4	10	<4	4
		PT	5/11	5/18	>80	<4	16	>80	<4	16
10	FT			2/25	40	<4	4	20	<4	<4
		FT	3/28	5/18	40	<4	4	>80	<4	<4
12	NI & FT			3/16	<10	<4	<4	20	<4	<4
		PT	4/27	5/14	160	<4	<4	>640	<4	<4
13	FT			3/16	<10	<4	<4	<10	<4	<4
		PT	5/4	5/18	80	<4	<4	>80	<4	<4
15	FT			3/16	<10	<4	<4	640	2	8
		PT	5/9	5/23	20	<4	<4	>640	<4	<4
16	FT			3/16	<10	<4	<4	<10	<4	<4
		FT	5/4	5/18	160	<4	4	80	<4	<4

* NI = natural infection. FT = formalin-inactivated, trivalent (Salk); 3 or more previous injections, but only a single injection for the booster. AL3 = live attenuated type 3 (Sabin). PT = purified (inactivated) trivalent (Purifax). IA = immuno-inactivation. PRIA = 50% plaque reduction combined with IA.

† All dates are 1961.

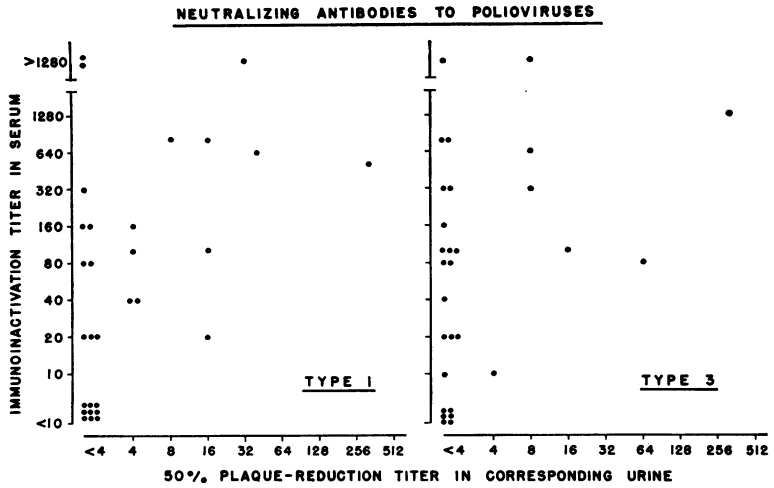

FIG. 3. CORRELATION OF TITERS OF POLIOVIRUS ANTIBODIES IN SERUM AS OBTAINED BY IMMUNO-INACTIVATION, AND IN URINE BY THE 50 PER CENT PLAQUE-REDUCTION METHOD.

Moreover, a later specimen of urine concentrate obtained from the same subject after a booster dose of purified trivalent poliovirus vaccine failed to neutralize type 3 virus even at the 20 per cent plaque-reduction level, although the IA titer of serum obtained at the same time was $> 1:80$ for the same virus (Tables III and V).

Although high neutralizing titers in the urine concentrates were generally associated with high titers in the corresponding serum, the correlation of titers was not good, as shown in Figure 3. Some urine concentrates failed to neutralize type 1 or type 3 poliovirus in a 1:4 dilution even by PRIA, when serum obtained at the same time neutralized the virus in the IA test in a dilution of 1:1,280. On the other hand, the urine concentrate of Subject 6 obtained after a booster dose of live attenuated type 3 poliovirus vaccine, did neutralize that virus at a time when the corresponding titer of the serum was only 1:10. The demonstration of neutralizing activity in the urine, however, was not always related to recent vaccination or to administration of a booster dose of vaccine.

Comparison of PRIA and IA titers in urine concentrates. From Tables IV and V it will also be seen that the PRIA test was distinctly more sensitive as an indicator of neutralizing activity than the IA test. PRIA titers for type 1 poliovirus ranging from 1:4 to $> 1:32$ were demonstrated in 10 urine concentrates which failed to neutralize the same virus in a 1:4 dilution by the IA test. Similarly, 4 specimens that failed to neutralize type 3 poliovirus at a 1:4 dilution yielded PRIA titers of 1:4 to 1:16. In the urine concentrates which did neutralize poliovirus in the IA test, the titers of PRIA at a level of 50 per cent reduction in PFU were 2 to 8 times higher. Several sera which failed to neutralize poliovirus in the IA test (although they were obtained from subjects who had received several doses of Salk vaccine) were also tested by PRIA. Of 6 such sera, 3 were also negative by the latter test, whereas 3 neutralized type 1 poliovirus in a titer of 1:64 by PRIA (Table IV).

Effect of booster dose of vaccine. In each of the 7 subjects who were studied before and after booster doses of various poliovirus vaccines, neutralizing antibody in the serum either appeared or increased in titer to type 1 or type 3 poliovirus or to both (Table V). A trivalent, concentrated, and formalinized vaccine (Purifax), reported to be free of serologically detectable monkey kidney antigen (21), was quite active in giving rise to high titers to both types of poliovirus. In Subjects 5, 6, and 16, whose urine concentrates failed to neutralize the poliovirus before the booster dose of this vaccine, specimens obtained after the booster did neutralize type 1 poliovirus by the PRIA test. However, in view of the fact that some urine concentrates failed to neutralize poliovirus (type 1 in Subject 5 and type 3 in Subject

15) when specimens obtained previously from the same subject had shown definite neutralizing activity and in spite of the fact that the same purified vaccine had been given in the interim, the possibility of variations in urinary output of the neutralizing substance or in its recovery from the urine must be considered to account for some of the discrepancies.

Correlation of neutralizing titers in serum and urine. The gross results of the neutralizing tests of serum and corresponding urine concentrates are summarized and correlated in the two-way tables presented in Table VI. Part A of this table shows a correlation of the results of IA tests of serum and urine concentrates. Among the 30 pairs of specimens from the 21 subjects, 21 sera neutralized the type 1 poliovirus, but neutralizing activity against this virus was demonstrated by this test in only 1 of the corresponding urine concentrates. The type 3 poliovirus was neutralized by 24 of the sera and by only 4 of the corresponding urine concentrates.

The greater sensitivity of the PRIA tests as compared with IA in revealing neutralizing activity in the urine is shown in part B of Table VI, which also brings out clearly the increasing sensitivity of the PRIA test with lowering of the percentage reduction in PFU chosen for the endpoint in this test. Of the 29 pairs of specimens tested with type 1 poliovirus, 20 of the sera neutralized this virus by the IA test and the numbers of corresponding urine concentrates which showed neutralizing activity for this virus by the PRIA test were 8, 11, and 15 at the 80, 50, and 20 per cent levels of reduction in PFU, respectively. Of the 28 pairs of specimens tested with the type 3 poliovirus, 22 of the sera neutralized the virus in IA tests; the numbers of corresponding urine concentrates in which neutralizing activity was demonstrated by PRIA were 4, 7, and 7, when evaluated at endpoints of 80, 50, and 20 per cent reduction of PFU, respectively. As already noted, only 1 urine concentrate showed any neutralization when the corresponding serum failed to neutralize either poliovirus by IA; in this urine concentrate, the neutralization was demonstrable by PRIA only at the 20 per cent plaque-reduction level against type 3 poliovirus and only in 1:4 dilution. Thus, in 42 pairs of tests with type 1 and type 3 polioviruses in which IA was demonstrated in serum by IA, PRIA was demonstrated in 12 (28 per cent), 18 (41 per cent), and 22

TABLE VI

Correlation of poliovirus antibody demonstrated in serum and in corresponding urine concentrates

A. *Results obtained with immuno-inactivation*

Antibody in serum	Neutralizing activity in corresponding urine concentrates: Type 1 No. tested	Type 1 Present	Type 1 Absent	Type 3 No. tested	Type 3 Present	Type 3 Absent
Present	21	1	20	24	4	20
Absent	9	0	9	6	0	6
Total	30	1	29	30	4	26

B. *Results obtained in urine concentrates by plaque-reduction immuno-inactivation*

Poliovirus	Antibody in serum	Neutralizing activity in corresponding urine concentrates: No. tested	80% Reduction Present	80% Reduction Absent	50% Reduction Present	50% Reduction Absent	20% Reduction Present	20% Reduction Absent
Type 1	Present	20	8	12	11	9	15	5
	Absent	9	0	9	0	9	0	9
	Total	29	8	21	11	18	15	14
Type 3	Present	22	4	18	7	15	7	15
	Absent	6	0	6	0	6	1	5
	Total	28	4	24	7	21	8	20

(52 per cent) of the corresponding urine concentrates at 80, 50, and 20 per cent plaque-reduction endpoints, respectively. The IA titers in serum and PRIA titers of corresponding urine concentrates at 50 per cent plaque-reduction endpoint are also correlated in Figure 3.

Among the 21 subjects included in this study, poliovirus-neutralizing antibody was demonstrated in the urine concentrates of 13 subjects—in 9 for type 1, in 7 for type 3, and in 3 for both types.[9] This antibody was found in the urine only after the booster dose in 2 of these subjects —in 1 of them against both types, and in the other only against the type 1 poliovirus.

DISCUSSION

Agglutinating antibodies to cholera vibrio and to typhoid bacilli have been demonstrated in the unconcentrated urine of normal volunteers immunized with the corresponding vaccine; the agglutinins to *V. cholerae* were also demonstrated in globulins isolated from the urine (22). Leptospira agglutinins have been found in unconcentrated urine during the acute and convalescent stages of Weil's disease; the urines of a few of these patients were free of protein by the sulfosalicylic acid test (23). Salmonella antibodies have been demonstrated in carriers of this organism, most of whom had urinary schistosomiasis and gross proteinuria (24, 25), but the finding of flagellar antibodies in urine of normal immunized individuals could not be confirmed (24). A recent paper from this laboratory reported the demonstration of precipitating antibody to diptheria toxoid in the concentrated urine of normal individuals after immunization with this antigen, and to type-specific pneumococcal polysaccharides in the concentrated urines of patients convalescent from pneumonia due to pneumococci of the homologous type (17). The authors are unaware of any reports of the finding of antiviral antibodies in urine.

The data presented here indicate that the poliovirus-neutralizing activity demonstrated in the urine concentrates that were studied had the following characteristics in common with neutralizing antibody to polioviruses found in the serum. It was nondialyzable, precipitated by 50 per cent ammonium sulfate, resisted heating for 30 minutes at 56° C but was destroyed by heating at 65° C for 30 minutes. It resisted treatment with trypsin, periodate, and RDE (*C. vibrio* filtrate, neuraminidase), was type-specific, appeared in some subjects after a booster dose of vaccine, and was reduced in titer or eliminated by absorption with AHS and AHGG. Moreover, the kinetic curve of the neutralization reaction of poliovirus with urine concentrate was similar to that of serum antibody (Figure 1) and differed from that usually attributed to other types of viral inhibitors such as interferons (26), anticellular sera (27), or polysaccharides (28), which interfere with multiplication of the virus within cells but do not have a direct effect on the virus itself. Furthermore, the ammonium sulfate precipitate of the urine concentrate migrated electrophoretically like serum γ-globulin. This neutralizing substance of the urine thus has the essential characteristics of antibody (29). Further studies on the biochemical and physical characterization of this and other urinary antibodies are in progress.[10]

The plaque-reduction method proved particularly useful when combined with immuno-inactivation to demonstrate small amounts of neutralizing antibody in serum, and particularly in urine concentrates. The PRIA test was evaluated at 20, 50, and 80 per cent levels of reduction in PFU before the 50 per cent level was selected for routine use. As was to be expected, the 20 per cent reduction endpoint permitted the detection of neutralizing antibody more frequently than when 50 or 80 per cent levels were used. At the 50 per cent level, antibody could not be detected in the urine in the absence of antibody of the homologous type in the serum; this was also true for the 20 per cent reduction endpoint except for a single specimen of urine concentrate, and this may have been a spurious result.

The plaque-reduction method has proved useful in other studies of neutralizing antibodies to ECHO 4 virus (19), polioviruses (8), variola

[9] Calculated at the 50 per cent plaque-reduction endpoint. PRIA at the 20 per cent endpoint was demonstrated in urine of 4 additional subjects against type 1 and in 1 additional subject against type 3 poliovirus.

[10] In collaboration with Dr. Ezio Merler, Children's Hospital Medical Center, Boston, Mass. After submission of this paper, viral-neutralizing activity was shown to reside in a fraction of urinary γ-globulin having a $S_{20,w}$ of 1.5 or less (38).

(20), and eastern equine encephalomyelitis virus (30), and permitted the demonstration and titration of such antibodies when they could not be measured by the usual tube methods. Itoh and Melnick (19) estimated that this method increased the neutralizing titers 50-fold. With similar techniques Barnett, Nasou, Utz and Baron (31) were able to demonstrate antibodies to 8 enteroviruses in the serum of 2 patients with congenital agammaglobulinemia, and postulated this as a possible explanation for the ability of such patients to react normally to viruses whereas they are repeatedly infected by bacteria. In a recent study with Coxsackie A viruses in mice in this laboratory (32), and in a few tests (not shown in the tables) in this study, the IA test generally yielded neutralizing titers 2 to 4 times higher than those obtained by the conventional tube method. In the present study the plaque-reduction method permitted increases of 10- to 25-fold in titer over that obtained by IA. PRIA thus yielded 20 to 100 times higher neutralizing titers than would have been obtained by the tube method.

In 3 of the subjects studied, neutralizing antibody was demonstrable in serum only by the PRIA method; each of these individuals had received multiple doses of vaccine. Such a finding may explain the apparently greater protection against serious poliovirus infection afforded by Salk vaccination than might be expected on the basis of the results of antibody studies done in the usual way in vaccinated individuals. This also suggests the possibility that small amounts of poliovirus-neutralizing antibody similar to those detected in the concentrated urines in this study may inhibit the occurrence or prevent the recognition of viruria.

No simple explanation has been found for the wide discrepancies between the relative titers of antibody in urine and in the corresponding serum, or in urine concentrates obtained at different times from the same individual, particularly for the failure to demonstrate neutralizing activity in the urine concentrates of some of the subjects who had high titers in the serum. Although the amount of protein excreted in the urine in normal subjects may vary in different individuals and also from day to day in the same individual (9), such variations are probably not sufficient to explain the discrepancies observed in the present study. The specimens of urine were all handled in essentially the same manner and the antibody once found in the concentrates was stable to repeated freezing and thawing, so that denaturation of some of the samples seems unlikely. The volume of urine and the time over which it was collected did vary considerably and the final concentrates were not standardized for their content of nitrogen or protein. It is, of course, possible that denaturation occurred before the final concentration in some specimens, or that the collection of larger volumes of urine over longer periods and a standardization of the concentrate for protein or the γ-globulin content might have resulted in the demonstration of antibody in the urine of some subjects in whom this was not done in spite of the presence of high titers of homologous antibody in the serum. Repeated assays of the same urine concentrates gave reproducible results within the error of the methods.

Although there was no close correlation between the titer of poliovirus antibody in the urine and that of the antibody to the homologous type in the serum, the failure to demonstrate antibody in urine concentrates in the absence of such antibody in the serum and the appearance of antibody in the urine of some individuals a week or more after booster doses of vaccine, which also resulted in the appearance or increase in titer of antibody to the same type in the serum, suggest that the urinary antibody is derived from the serum. This lends support to similar conclusions based on isotopic studies which indicate that the urinary γ-globulins are derived, at least in part, from those of the blood (33, 34). The possibility of a local origin of these antibodies, however, cannot be excluded.

In children who have had a natural infection with a poliovirus or who have been fed attenuated virus vaccine, there is relative resistance to multiplication of the homotypic virus in the alimentary tract, whereas Salk-vaccinated individuals shed poliovirus freely in their feces (35). Copro-antibodies to poliovirus have been demonstrated in feces of children with poliomyelitis (36) and "neutralizing antibodies" to Lansing strain were found in nasopharyngeal secretions of adults (37). These may conceivably be of local origin (e.g., produced in Peyer's patches or in pharyngeal

lymphoid tissue), but there is no definitive evidence for the local origin of the poliovirus antibodies in any of these excreta.

SUMMARY

Neutralizing activity for types 1 and 3 poliovirus was demonstrated in protein concentrates prepared from the urine of a number of normal subjects. The biologic characteristics of the neutralizing activity in the urine concentrates resembled those of specific antibody found in serum.

Neutralizing antibody in the urine was usually associated with high titers of the homologous antibody in the serum; it was not demonstrable in the absence of homotypic antibody in serum obtained at the same time, but also was not demonstrated in urine from some subjects who had high titers in the serum.

In some of the subjects the urinary antibody was first demonstrated 1 week or more after a booster dose of poliovirus vaccine, along with the appearance or increase in titer of the homotypic antibody in the serum.

The plaque-reduction method combined with immuno-inactivation permitted the demonstration of poliomyelitis-neutralizing antibody in small amounts which could not be demonstrated by the conventional tube assay, even when preceded by immuno-inactivation.

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