Detection of Poliovirus in Feces of Chimpanzees During Late Convalescence by Means of Freon 113.* (27439)

HOWARD A. HOWE†

Bureau of Laboratories, Maryland State Department of Health, Baltimore

This preliminary report is concerned with recovery of type 1 poliovirus from previously negative feces in 3 of 4 chimpanzees during late convalesence from full blown alimentary infections induced by feeding of the Mahoney strain. Virus was demonstrated by applying the method of Gessler and his co-workers(1, 2) to fecal specimens which had been collected 7 to 41 days after the last isolation and were considered virus-free by ordinary procedures.

Materials and methods. The chimpanzees here described (P56, P104, P110, P111) had experienced a primary infection following oral ingestion of a virulent, wild, type 1 poliovirus (Mahoney strain). The inoculum was an aliquot of a large frozen pool of rhesus monkey spinal cords which in 1959 contained approximately 64,000 $TCID_{50}$ per ml. Each animal was fed 5 ml of this pool mixed with 1% sucrose from a luer syringe, P56 in 1955, P104 in 1956, and the remaining animals in 1957. Feces, tonsillar swabs, and sera were collected daily after the feeding for 10 days, then 3 times weekly until the 30th day. Feces and blood were also collected on the 42nd and 59th days following virus ingestion. Specimens were processed, assayed, and typed for the inoculated virus by methods previously described(3,4). Briefly the assaying of feces for virus consisted in inoculating 0.5 ml of the clarified ether treated specimen at a dilution of 1:9 in modified Hanks' saline and in successive 2-fold dilutions into each of 2 Kimble tubes containing monolayers of trypsinized monkey kidney cells in 2 ml of the medium. This maintenance fluid known as "medium 5", was composed of Hanks' balanced saline plus 0.5% lactalbumen hydrolysate, .003% phenol red, with 100 μg of streptomycin and 100 units of penicillin per ml. The kidney cells had been previously grown out in the same medium enriched by addition of 2% calf serum. Prior to inoculation the tubes were washed for 2 hours on a revolving drum in "medium 5" which was then renewed to receive the inoculum of 0.5 ml. Incubation was maintained at 37°C. Forty-eight hours later the tubes were returned to the complete growth medium which was renewed every 3 or 4 days. Cultures were observed daily through the microscope until the 10th day, when they were usually discarded if no cytopathogenic effect had been noted. This procedure to a large extent avoided the intrusion of indigenous simian viruses.

Typing of virus isolates was carried out after one tissue culture passage to determine whether the strain in question showed the usual growth characteristics of poliomyelitis. The typing itself was also done in MK monolayers with 2 tubes for each antiserum and 2 for the virus control. Standard typing sera in a dilution of 1:32 were mixed in equal quantities with the unknown isolate which was not titrated, but usually diluted to 1:125. The serum-virus mixture was incubated at room temperature for 2 hours and 0.5 ml of the resultant product was then inoculated into the designated tubes which contained 2 ml of "medium 5". These cultures were incubated at 37°C and observed daily through the microscope for cytopathogenic effects. To be considered "protective" an antiserum was required to inhibit visible evidence of viral activity for 7 days. Unless the virus control was destroyed within 24 hours the dose was usually regarded as insufficient and the test

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was repeated with a lower dilution of the unknown. Heterotypic sera usually delayed destruction of the cultures for 36-48 hours, depending, apparently, upon the titer of the unknown agent.

The identical fecal specimens which had failed to yield virus by the above methods in 1959-60 but had been stored at –30°C were assayed again 1-2 years later utilizing a technic devised by Gessler and others(1,2). This procedure, which involved homogenization of the specimen with Freon 112, had originally been developed to purify viruses in tissue suspensions by the precipitation of extraneous protein. It was elaborated and adapted by Hummeler and Ketler(5) and by Ketler and others(6) to dissociate the antigen-antibody complex of a known quantity of poliovirus neutralized by rabbit antisera. Their technic consisted of homogenizing the specimen from 2 to 90 minutes with Freon 113 (trifluorotrichloroethane) in a ratio of either 1 to 1 or 1 to 2 in an iced container. This was done with a Serval Omnimixer operating at top speed. The resulting mixture was then centrifuged at 2000 rpm for 10 minutes and the supernate used for virus assay. After 5 minutes of homogenization a large proportion of the previously neutralized virus was found to be infective.

Our efforts were rather crude by comparison since the only available homogenizing device was a Waring blendor and rotor jars having wells at the bottom which were designed to contain 50 ml of fluid. In most instances the volumes of clarified fecal suspensions remaining at our disposal after the assays of the preceding years were rarely more than 2-5 ml of a 1:9 dilution. Furthermore it was necessary to set aside enough material to serve as a control for the Freon treatment. Consequently specimens which had formerly been negative at a dilution of 1:9 suffered further dilution which ranged from 2- to 8-fold. Homogenization of equal parts of stool suspension and Freon was considered optimal, but in several cases it was necessary to use 3 parts of Freon to 1 of stool in order to cover the rotor blades of the blendor cup. Refrigeration of the cups seemed particularly vital because of the appreciable heat developed locally by the bearings of the rotor unit, but this was difficult to achieve. The cups were prechilled to –30°C and during operation were surrounded by a plastic bag filled with cracked ice. It is impossible to hazard a guess as to the temperature of the Freon-stool mixtures during the 5 minutes of homogenization. Centrifugation at 3,000 rpm for 20 minutes and inoculation were carried out promptly, as suggested by the authors. The viral assays differed from those of previous years in that the monkey kidney monolayers were purchased from the Microbiological Associates and were maintained in medium 199 of Morgan and Parker(7), either with or without 1% normal calf serum in the manner previously described. Dilutions were also made with 199.

Results. Despite the technical difficulties just recounted and the small number of kidney monolayers available, the findings seemed sufficiently impressive to warrant this note. Table I summarizes these data together with the observations made in 1959-60.

In the earlier studies virus titrations of stools from alternate days following inoculation showed uninterrupted elimination of type 1 virus in all of the animals which reached a maximum between the 7th and 11th days (Table I). These levels were maintained for about 2 days, after which there was a gradual decline of virus titer at a rate varying with each animal. Virus was no longer demonstrable in individual chimpanzees after intervals ranging from the 18th to the 30th days after feeding, nor was it found in later specimens from the 28th to 59th days.

To rule out possible deleterious effects of Freon, a stool from P104 (20th day) which had titered 1:1,000 early in 1960 was withdrawn from storage and treated with Freon 113. After this operation the titer was found to be essentially unchanged (>1:800, Table I, see also ref. 2,5,6). As Table I indicates, further experiments with Freon 113 yielded poliovirus in 3 of the 4 animals from feces which had been collected 7 to 41 days after the first negative test, and were themselves considered negative by the methods then employed. Two of the isolates were type 1 and the 3rd has not to date been typed. In one

TABLE I. Summary of Assays for Poliovirus in Chimpanzee Feces Processed by Conventional Methods and with Freon 113.

			Result of virus assay							
			Routine method				Freon method			
	Fecal collection		1959-60	1961			1961			
Animal No.	Day from feeding	Day from last pos.	Titer	T.C. inf. ratio	Passage	Typing	T.C. inf. ratio	Passage	Typing	Antibody
P 110	11		16,000							32
	18		8							2,000
	30		<8‡							1,000
	42	24	<8	0/3			0/3			*ca.* 1,000
	59	41	<8	0/3			1/3	+	I	750
P 104*	12		2,000							16
	20		1,000				>800†	+		500
	24		<8	0/3			0/3			
	35	15	<8	1/3	+	I	1/3	+	I	1,000
P 56	11		2,000							<4
	21		8							16
	25		<8							16
	28	7	<8	0/6			2/6	+		32
P 111	7		32,000							<4
	11		8,000							500
	18		<16							8,000
	30	19	<8							8,000
	38	27	<8	0/3			0/3			
	42	31	<8	0/3			0/3			1,500

* P 101 was paralyzed on the 12th day.
† The only titration attempted.
‡ No virus was demonstrable at 1:8 and it is concluded that such specimens were negative within the range of the test.

instance (P104, 35th day) a stool which was previously negative yielded virus before Freon treatment as well as after. The failure to isolate virus from 2 stool specimens of P111 was probably due to technical difficulties, which were many, and is of much less significance than the positive findings.

Discussion. The pathology of poliomyelitis is so nearly identical in the chimpanzee and in man that immune reactions are probably similar in the two species. The isolations achieved by these experiments, though few, point strongly to the prolonged persistence of virus in the alimentary tract, but in a form which is not readily detected by ordinary methods of virus assay. Since serum neutralizing antibody had reached levels nearly maximal for each animal, one naturally thinks of masking by combination between virus and antibody. Credence is lent to this idea by the fact that homogenization with Freon 113 has been used to dissociate *in vitro* combinations of virus and antibody (5,6). Neutralizing substances have been described in the stools of convalescents(8,9) and these observations are complimented by the finding that fecal virus titers in the chimpanzee and in man vary inversely with serum neutralizing antibody levels(3,10). This may reflect diminished virus propagation, or increased neutralization of the virus which reaches the lumen of the alimentary tract. Or both of these mechanisms may be operative. It is hoped that the method of unmasking virus with Freon will shed more light on the equilibrium which has previously been postulated between virus and antibody following active infection(11).

Summary. In chimpanzees during late convalescence from infection with poliovirus, the feces have yielded homotypic virus after treatment with Freon 113 when other methods of virus detection had failed.

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Natural Occurrence of *Leptospira icterohaemorrhagiae* in an Opossum. (27440)

L. B. EVANS, G. E. WOOD, V. FLYGER, A. D. ALEXANDER, R. H. YAGER, AND H. L. RUBIN

WHO/FAO Leptospirosis Reference Laboratory, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C. and Natural Resources Institute, University of Maryland, Annapolis, Md.

Shortly after *Leptospira icterohaemorrhagiae* was shown to be the cause of Weil's disease(1), the Norway rat was found to be the principal host carrier(2). The strain was subsequently found sporadically in infections in domestic animals and in low incidence in few other rodent hosts. In the past decade, there has been a broader recognition of the host distribution of pathogenic leptospiras, particularly in larger mammalian wildlife. In addition to rats, mice, dogs, cattle and swine, *L. icterohaemorrhagiae* has been isolated from skunks, foxes, mongooses, raccoons, muskrats and nutria(2,3,4,5,6).

This paper reports the natural occurrence of *L. icterohaemorrhagiae* in opossums. This study also afforded the opportunity to reexamine the serological interrelationship of several members of the *icterohaemorrhagiae* serogroup.

Materials and methods. Animals were live-trapped on a military reservation at Aberdeen, Md. from 5 May 1961 to 18 July 1961. Animals were killed by exposure to chloroform and carbon dioxide (Dry Ice) in a closed 20-gallon can. Heart blood was obtained for serologic studies. Bladder urine and a 10% suspension of triturated kidney, as well as one in 10 dilutions thereof were cultured in Fletcher's semisolid medium. The employed diluent was a phosphate-buffered (pH 7.4) physiological salt solution. Each preparation was cultured in 4 tubes of medium employing minimal inocula; *e.g.,* 1 drop per 5 ml medium.

All cultures were incubated at 28°-30°C and were examined for growth at 2-week intervals by darkground microscopy. All cultures negative after 6 weeks incubation were discarded.

Serums were examined for leptospiral agglutinins by the microscopic-agglutination technic with live antigens. The following 18 screening antigens were used: *L. andamana, L. butembo, L. celledoni, L. bataviae, L. pomona, L. djasiman, L. hyos, L. autumnalis, L. ballum, L. canicola, L. icterohaemorrhagiae, L. pyrogenes, L. alexi, L. grippotyphosa, L. borincana, L. wolffi, L. javanica* and *L. australis.*

For the preliminary culture typing of the isolates, rabbit antiserums of known serotypes were used. The procedures for the microagglutination and agglutinin-adsorption tests, and the method for preparing antiserums have been described(7).

Results and conclusion. During the specified interval, 37 opossums were studied. Isolates were cultured from the kidney and/or urine of 2 opossums (strains Op-3 and Op-6). Both of these animals were serologically positive, one had a titer of 1:25 versus *L. icterohaemorrhagiae,* the other a titer of 1:100 versus *L. ballum.* Three additional animals had