A Type 3 Attenuated Poliovirus Genetically Stable after Human Intestinal Passage.* (26769)

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The type 3 strains of attenuated poliovirus available fail to maintain their genetic markers after passage through the human intestinal tract(1-5). Although no epidemiological evidence has been adduced so far for reversion of these strains to agents virulent for man(6,7), the fact that *in vitro* markers may change to those frequently indistinguishable from virulent virus makes it more difficult to monitor the live virus vaccination program. As changes in genetic character of type 3 strains are occasionally accompanied by an increase in pathogenicity for monkeys, public health authorities have expressed concern for the safety of contacts of subjects fed the live attenuated type 3 strains presently available (3,8).

The reproductive capacity of type 3 viruses isolated from feces of vaccinated subjects in tissue culture systems incubated at 40°C (rct/40°C marker)† has been used most frequently as a test of vaccine strain stability, since a fair correlation has been established between the rct/40°C marker and other markers, including pathogenicity for monkeys(3). In Tables I and II, respectively, are summarized the rct/40°C characteristics of virus strains representing the first human intestinal passage of the 2 attenuated type 3 strains that have been used most widely in mass immunization programs: Leon KP-34(9) and W-Fox(10). Both strains grow relatively poorly in tissue culture systems kept at 40°C and are therefore classified as rct/40°C–; however, when fed to man, both strains tend to induce excretion of viruses which were found to replicate equally well at 37°C and 40°C and are therefore classified as rct/40°C+.

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† Reproductive capacity at temperature 40°C. Determined by dual titration of a virus at 40°C in comparison with control titration at 37°C.

As shown in Table II, results of 4 studies of the Leon KP-34 indicated that a majority of subjects fed this strain excreted rct/40°C+ virus. Similarly, it has been found that children fed W-Fox strain (Table I), in 16 out of 16 cases excreted strains which were rct/40°C+.

Because of the instability of these viruses, an attempt was made to develop an attenuated type 3 strain which would remain genetically unaltered after passage through the human intestinal tract.

Materials and methods. Tissue culture systems. Freshly harvested explants of Rhesus monkey kidney tissue were used throughout the study. Cultures were prepared from either one million kidney cells dispersed in petri dishes or 200,000 cells in culture tubes which had been incubated at 37°C in growth medium consisting of Eagle's medium in Earle's solution with calf serum to a 10% concentration. After 6 days' incubation in presence of 4% CO_2 in air inflow, the growth medium was replaced by maintenance medium consisting of Eagle's medium in Earle's solution without serum. At the same time the cultures were exposed to type 3 virus preparations. Cultures in tubes were used for virus passage purposes and for titration of virus preparations at different temperatures of incubation, whereas plates were used for cloning the virus at different passage levels and for titration of stools at several temperatures. For passage in tube culture, 3 tissue culture tubes were exposed to 0.1 ml of undiluted virus from the previous passage. After one hour's incubation at room temperature (25°C) the virus was removed, the tubes washed once, and fresh maintenance medium added. The tubes were incubated at 23 or 37°C until 50% cellular degeneration was noted, or until cells in the non-infected control tubes began to degenerate. This latter period varied between 12 and 16 days at the

TABLE I. Reproductive Capacity at 37°C and 40°C of Fecal Virus Isolates from Infants Either Fed $10^{5.5}$ TCD_{50} of the W-Fox Strain or Infected by Contact.

Subject	No. days post-vacc. virus isolated	Material tested	$Log_{10}TCD_{50}$ 37°C	$Log_{10}TCD_{50}$ 40°C	$Log_{10}\frac{TCD_{50}37°C*}{TCD_{50}40°C}$
W-Fox	—	Vaccine	7.7	4.7	3.0
H-24	—	Virulent	8.2	8.0	.2
B-157	8	St	4.4	3.7	.7
I-1	11	"	2.4	1.9	.5
I-1	7	1TC	7.5	5.7	1.8
I-6	5	St	1.9	1.4	.5
I-6	5	1TC	7.5	7.2	.3
Q-1	10	"	7.7	7.7	.0
L-1	9	"	6.7	7.2	−.5
R-1	7	"	7.5	7.2	.3
WB-110	7	St	3.9	2.2	1.7
Q-5	18	1TC	6.2	5.7	.5
I-4	9	"	7.5	7.2	.3
I-5	4	"	7.2	7.5	−.3
I-7	2	"	6.5	6.7	−.2
R-4	18	"	6.7	5.7	1.0
L-5	7	"	7.2	6.5	.7
E-5	10	"	7.7	7.2	.5
E-4	10	"	7.2	7.2	.0
B-158	5	St	3.5	2.9	.4
B-158	5	1TC	7.7	7.5	.2

* Controls: W-Fox, 3.3; H-24 (virulent), 0.2.

St = Stool; 1TC = First tissue culture passage.

TABLE II. Reproductive Capacity at 40°C (ret/40°C marker) of Type 3 Leon KP-34 Attenuated Strain of Sabin after Human Intestinal Passage.

Laboratory	Tested	Properties of fecal virus. No. of specimens ret/40°C+	ret/40°C±	ret/40°C−
Sabin(1)	42	15	20	7
	27*	26	1	0
Gendon *et al.*(2)	17	5	4	8
Melnick & Benyesh-Melnick(3)	23	12	2	9
Verlinde & Wilterdink(4)	13	6	3	4
Total	122	64	30	28

* Viruses isolated from contacts.

23°C temperature of incubation. The contents of the tubes were then rapidly frozen and thawed 3 times and pooled: this material was then used as seed virus for the next tissue culture passage.

In the case of the plate cultures the virus was adsorbed for one hour, the excess removed by washing, and the monolayers covered with 5 cc of an agar overlay maintenance medium consisting of equal volumes of 2% Noble's agar and of a nutrient solution (11). On the second day post-inoculation the plates were covered with a second overlay containing neutral red and the appearance of plaques determined on the 3rd to 7th day of incubation.

Plaque passage was performed by exposing kidney monolayers to virus at terminal dilution, and by selection of one of the plaques at random, which was aspirated with a Pasteur pipette and inoculated onto other monolayer cultures if further plaque passages were desired.

Temperature control. Incubators (Assmundson Aktiebolag-Assab Bacteriological Cabinet T-200) equipped with fans to insure the most uniform temperature throughout were kept at 23, 37, and 40°C for incubation

of plate cultures. Internal thermometers were used to check the temperature which varied by ±0.1°C. Culture tubes were kept in mergotherm water baths (H. Struers Chemiske Laboratories, Denmark), maintained at 23, 37 and 40°C and equipped with an agitating apparatus to maintain a uniform temperature (variations of ±0.02°C).

Monkey virulence tests. Cynomologous monkeys weighing 2.5 to 4 kg were inoculated with 0.5 to 1 ml of virus suspensions into one or other of the cerebral hemispheres by means of a No. 24 (1″) needle. The animals were then observed daily for evidence of paresis or paralysis and after 18-21 days were sacrificed. Histological examination of the formalin-preserved brain and spinal cord tissue began with sectioning of the thalamus to demonstrate the needle tract. If the tract was found, blocks were removed from the midbrain, medulla, cervical enlargement, and lumbar enlargement, and embedded in celloidin. After being sectioned and stained with thionine, every 10th section was examined microscopically for the presence of inflammatory lesions and neuronal destruction or other histological evidence of poliomyelitis infection.

Experimental. History of passages. Fig. 1 summarizes the schedule of tissue culture passages leading to development of the new type 3 strain (the WM-III virus). Material used to initiate the present passage series represented the currently used W-Fox vaccine strain (W-Fox vaccine pools in Fig. 1). It will be recalled that originally the W-Fox virus was isolated in tissue culture from an asymptomatic child in Louisiana by Dr. John P. Fox, and found to be of low pathogenicity for monkeys(10). In this laboratory (Fig. 1) the Fox strain was passed on monkey kidney monolayers for 6 serial single plaque passages: the sixth plaque passage was made into a monkey kidney pool for seed material, and as shown in Fig. 1 was used in the course of 4 further kidney tissue culture passages for preparation of live virus vaccines employed in vaccination programs of man in the United States, the Congo, Poland, Switzerland, and Croatia(1,2,7,12,13,14).

Asymptomatic natural infection
↓
1 TCP 37°C
↓
6 PP 37°C
↓
4 TCP 37°C
(W-Fox vaccine pools)
↓
Human intestinal passage
↓
22 TCP 23°C
↓
1 PP 23°C
↓
1 TCP 23°C
↓
3 PP 23°C
↓
10 TCP 37°C
(the WM-III strain)

TCP = Serial passages in monkey kidney tissue culture tubes.

PP = Serial single plaque passages on monkey kidney tissue culture plates.

FIG. 1. Passage history of the WM-III strain.

Although W-Fox replicated relatively poorly in cultures kept at 40°C, results of comparative titration conducted at 37°C and 40°C indicated that the difference in reproductive capacity never exceeded the figure of 2.0-3.5 $\log_{10}$ TCD_{50} (Tables I and III). Usually after one human passage the virus changed to a strain which grew as well at 40°C as at 37°C (Table I).

On one occasion, however, growth of an excreted virus was impaired when the cultures were kept at 40°C and this virus strain was used to start a series of culture passages at 23°C following the technic originally suggested by Dubes and Wenner(15). During the first 2 passages no cytopathic effect was noted in tissue cultures and the material was passed blindly at 12-day intervals. On the 3rd tissue culture passage the virus produced a cytopathic effect 3-5 days after inoculation. Virus representing the 22nd tissue culture passage was plaqued on monolayers incubated at 23°C, and the clone grown for one passage in tissue culture tubes kept at 23°C (Fig. 1). Following that, 3 more plaque passages on monolayers were made at 23°C and the resulting strain passed for 10 tissue culture passages in tubes incubated this time at 37°C (Fig. 1), to provide the final material, which was named the WM-III strain.

The WM-III strain replicated equally well

TABLE III. Reproductive Capacity of WM-III in Culture Tubes Incubated at 23, 37 and 40°C.

Virus	$Log_{10}TCD_{50}$ at 23°C	37°C	40°C
W-Fox	<.0	7.7	4.7
H-24	<.0	8.2	8.0
WM-III	7.5	7.5	<.0

in cultures kept at 23 and 37°C but did not grow at 40°C (Table III). In contrast, growth of the virulent type 3 H-24 strain was unimpaired by incubation temperature of 40°C whereas the titer of the attenuated W-Fox was lower at 40°C than at 37°C; but the difference in titers, as mentioned before, did not exceed 3.0 $logs_{10}$. Both H-24 and W-Fox failed to grow at 23°C.

Infectivity of WM-III strain for man and stability after human intestinal passage. Nine infants, 4 premature and 5 full-term, at ages ranging from 3 days to 3 months were fed the WM-III strain by dropper in the amounts of $10^{4.2}$ to $10^{7.7}$ TCD_{50} (Table IV). Stools were collected twice weekly and processed as described previously(5). All infants excreted virus for one week or more. Fecal virus strains isolated 2 to 42 days after ingestion of the vaccine were titrated in cultures kept at 37 and 40°C, respectively, and on one occasion at 23°C. Virus in the original stool and after the first tissue culture passage at 37°C was used as inoculum. The results, summarized in Table IV, indicated that none of 9 feedings resulted in excretion of an rct/40°C+ virus, in contrast to the results obtained with W-Fox virus (Table I). The virus did not grow at all or grew rather poorly in cultures kept at 40°C. The rct/40°C– character was retained by the excreted WM-III strains regardless of the time interval elapsing between virus administration and its isolation from the feces. In one case

TABLE IV. Reproductive Capacity at 37 and 40°C of Fecal Virus Isolates from Infants Fed the WM-III Strains.

Subject No.	Dose fed log TCD_{50}	Virus isolated post-vacc. day	Material tested	$Log_{10}TCD_{50}$ 37°C	40°C	$Log_{10}TCD_{50}$ 37°C minus $Log_{10}TCD_{50}$ 40°C
1	7.7	6	St	4.4	<1.4	>3.0
			1TC*	8.2	5.2	3.0
		30	St	1.7	<1.5	> .2
			1TC	7.2	<2.5	>4.7
2	7.5	2	St	2.7	<1.5	>1.2
			1TC	7.7	<2.5	>5.2
		9	St	3.5	<1.5	>2.0
3	6.5	9	St	4.5	<1.5	>3.0
			1TC	7.5	3.7	3.8
		19	St	2.5	<1.5	>1.0
4	6.5	9	St	3.7	<1.5	>2.2
			1TC	7.5	<2.5	>5.0
		23	St	4.2	<1.5	>2.7
			1TC	7.2	<2.5	>4.7
5	5.5	10	St	2.5	<1.5	>1.0
6	5.5	4	St	2.2	<1.5	> .7
			1TC	6.7	<2.5	>4.2
7	5.5	18	St	2.2	<1.5	> .7
			1TC	8.2	4.2	4.0
		42	St	1.7	<1.5	> .2
			1TC	6.7	<2.5	>4.2
8	4.2	5	St	2.5	<1.5	>1.0
			1TC	7.2	<2.5	>4.7
9	4.2	5	St	5.2	<1.5	>3.7
			1TC	7.7	<2.5	>5.2

* Gave a titer of $10^{7.2}$ TCD_{50} at 23°C.

St = Stool. 1TC = First tissue culture passage in monkey kidney cells.

TABLE V. Monkey Neurovirulence (Intracerebral) of WM-III Strain and Its Human Passages in Comparison to W-Fox.

Virus	Dose $Log_{10}TCD_{50}$	Ratio of monkeys Paralyzed	With lesions
W-Fox	7.2	3/10	4/10
WM-III	6.5	0/5	0/5
A*	7.9	0/5	1?/5
B*	7.5	0/5	0/5

* First tissue culture passage of strains isolated from subjects fed WM-III.

in which a titration was performed at 23°C the fecal WM-III virus retained its ability to reproduce at that temperature. The 2 viruses isolated 9 days after vaccination from infants 3 and 4 were tested by a newly discovered marker, acid sensitivity(16). In this test viruses are incubated in presence of 0.35 N HCl for 3 hours at 23°C. W-Fox and WM-III are both highly susceptible to the action of acid, whereas the virulent H-24 maintains its titer in acid at the same level as at neutral pH. Excreted strains of W-Fox submitted to this test showed acid resistance in 4 cases, intermediate resistance in 2 cases, and susceptibility only in one instance. Two WM-III fecal strains which were tested, however, remained acid-susceptible.

Monkey neurovirulence test. Table V summarizes results of clinical and histologic observation of monkeys injected intracerebrally with the WM-III virus and with material representing passage of the strain through the human intestinal tract. No clinical signs of illness were noted in 5 monkeys injected with WM-III virus or in 10 monkeys inoculated with material obtained from 2 virus-fed subjects. Examination of over 150 sections of central nervous system from each monkey failed to reveal histologic changes in 14 of 15 monkeys. Only in the case of one monkey were scattered inflammatory lesions with an occasional loss of neurons observed. These results differ markedly from those obtained with the W-Fox strain, which are included in Table V for comparative purposes.

Discussion. This paper reports the development of a new type 3 attenuated poliovirus, named WM-III, which was derived from the W-Fox vaccine strain after 22 passages in tissue culture tubes, and 4 passages by plaquing and clone selection at 23°C. To insure its infectivity for the human gastrointestinal tract the final virus pool was made after 10 tube culture passages at 37°C. This material had no ability to grow at 40°C and was not neurovirulent for monkeys injected intracerebrally, in contrast to the original W-Fox strain. The results obtained with other sub-lines of W-Fox strain, as well as studies on the mechanism of cold adaptation will be reported separately(17).

The genetic stability of WM-III virus was demonstrated through its study after passage through the intestinal tract of a limited number of infants fed different quantities of the strain. The extracted virus showed poor capacity to replicate in tissue cultures incubated at 40°C (rct/40°C– character), which in 2 cases tested, retained their acid sensitivity. In addition, fecal viruses isolated from 2 vaccinated cases were found to be non-pathogenic for monkeys injected intracerebrally. Stability in relation to 3 markers makes the WM-III strain quite different from the 2 other attenuated type 3 strains investigated in the same way, as pointed out in Tables I and II(1-5). In addition to changes in *in vitro* markers, the excreted viruses derived from feeding of Leon KP-34 and W-Fox were found to be more virulent for monkeys injected intracerebrally(1-5). Melnick and Benyesh-Melnick(3), for example, reported paralysis and histologic lesions of poliomyelitis in 26 out of 52 monkeys inoculated with virus isolated from subjects fed the Leon KP-34 strain. The vaccine strain itself consistently produced asymptomatic infection.

More trials in man are required to evaluate fully the antigenicity of the WM-III strain, but the results obtained so far suggest that the high infectiousness of the W-Fox for human intestinal tract has been retained.

In view of the dissatisfaction with the stability of the available type 3 strains that has been expressed(3,8,18) and in view of a statement by the Committee on Live Poliovirus Vaccine of the Surgeon General of U.S. Public Health Service(19), and assuming

later studies confirm our results, the WM-III strain is the type 3 virus of choice for production of attenuated poliovirus vaccine.

Summary. Development of a new type 3 attenuated poliovirus (WM-III) is described. Starting with the W-Fox strain, passage and clone selection at 23°C were employed to develop a strain with improved stability after human intestinal passage. The end result was a virus which had no capacity to grow at 40°C and was not virulent for monkeys injected intracerebrally. The results of tests of fecal virus strains isolated from infants fed the WM-III strain did not show the instability in relation to laboratory markers that has been reported with other attenuated type 3 viruses.

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Studies of Inhibitory Effect of Ammonium Ions in Several Virus-Tissue Culture Systems. (26770)

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It was observed(1) that ammonium ions in trace amounts caused a very marked suppression of influenza virus replication in a dog kidney tissue culture system. This suppression was accompanied by a complete protection of the cells from the cytopathic effects of the virus. Similar results have been observed independently by Eaton and Scala (2) with both influenza and Newcastle disease virus in Krebs 2 ascites tumor cells. Since this unique inhibition may have a significant effect on many types of virus-host