POLIOMYELITIS IN THE CYNOMOLGUS MONKEY

IV. Further Observations on Exposures Confined to the Stomach and Intestines, with Notes on the Fecal Excretion of Virus*

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In an earlier publication (1) we showed that non-traumatic exposure of the stomach and intestines to poliomyelitis virus (*Per* strain) without simultaneous exposure of the oropharynx and upper esophagus failed to induce infection in a series of 26 *cynomolgus* monkeys; while the same strain administered by simple feeding, which involved simultaneous exposure of both the pharyngeal and gastrointestinal levels, had been shown by Sabin and Ward (2) to induce infection in 40 per cent of 15 tests. These observations strongly suggested that the gastrointestinal mucosa, as compared with the oropharyngeal, was relatively impervious to poliomyelitis virus, casting doubt on the validty of the concept that the gastrointestinal tract is the usual portal of entry in this disease.

This earlier study was an exploration which we desired not only to repeat with another strain of virus, preferably recently isolated, but also to amplify and expand. In particular we were anxious to observe the appearance and persistence of virus in the stools which is one of the conspicuous features of human poliomyelitis and which has not as yet been adequately explained. In the first study, probably because of the inadequacy of the technique then available for virus recovery from stools, we failed to detect virus in the stools of any of the treated animals tested. We also desired to discover whether intestinal exposure alone, without infection, would set up a state of resistance to later intracerebral inoculation. It will be recalled that in the previous study (3), during which a series of other exposures to virus followed the gastrointestinal, 4 of 5 of the surviving animals displayed a limited resistance to later intracerebral inoculation.

The present experiments concern 18 *cynomolgus* monkeys fed by capsule by the same method as was used in the earlier study, and the results will be related to those of simple feeding of the same strain of virus, which we are reporting elsewhere (4).

EXPERIMENTAL METHODS

Arrangement of Animals.—All monkeys used in this experiment were *M. irus* (*cynomolgus*) from the Philippine Islands, received on Oct. 7, 1946. On Oct. 23, 22 normal, healthy monkeys

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were separated into 3 groups of 6 each for the experiment, and one group of 4 as controls. The animals of each group were in adjacent cages, containing 2 each, but the groups were separated by a distance of at least 3 feet.

Preparation of Virus.—The *Cam* strain, isolated in this laboratory in July, 1945, from stools of a mild paralytic human case of poliomyelitis, was used throughout, in a mixture of the first five passages. This strain has produced infection, frequently in mild form, in 87 per cent of *rhesus* and 94 per cent of *cynomolgus* monkeys inoculated intracerebrally. Of 6 monkeys fed this strain mixed with the regular diet, 3, or 50 per cent, succumbed to the disease (4). The variability of clinical manifestations has been reminiscent of that seen in outbreaks of the human disease, some of the infections being non-paralytic, some monoplegic, a few bulbar, and only rare ones, fulminant.

A mixture of histopatholgically proved, severely involved segments of spinal cord from 10 paralyzed *rhesus* monkeys sacrificed 5 to 15 months previously was used as virus. 45 gm. of tissue, which had been stored on dry ice, was emulsified in a Waring blendor with 70 cc. of cottonseed oil. The same suspension was used throughout, being stored on dry ice between feeding periods. Enough suspension for a single feeding period was thawed when needed, and kept in the refrigerator until used.

Preparation and Administration of Capsules Containing Virus.—The required number of number 4 gelatin capsules for one feeding only were filled with well mixed suspension and immediately coated with a heavy layer of crisco.[1] There was no visible contamination of the outside of the capsules while filling or coating. Three capsules were placed in a specially constructed curved metal cannula, which was then placed in the esophagus of an unanesthetized monkey. The capsules were forced out by means of a closely fitting metal obturator. In a few instances, unopened capsules returned to the mouth as a result of gagging, but were immediately reintroduced into the esophagus. No subsequent regurgitation was observed. Monkeys were given capsules before the morning meal on 3 successive days and received approximately 1.2 gm. of cord (wet weight) in this period. The dates of feeding and the interval between feedings in those animals exposed more than once are stated below.

Intracerebral Challenge.—The animals of each group were tested for possible refractoriness by intracerebral (intrathalamic) inoculation approximately 5 weeks after the last feeding for the particular group. Fourth passage *Cam* virus was used for the purpose, in 20 per cent concentration for group I, and 10 per cent concentration for groups II and III. The 4 control animals were inoculated intracerebrally at the same time and with the same amounts as group III, which also served as a test of the activity of the virus.

RESULTS

Group I. One Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 monkeys (C1–10, C1–13, C1–14, C1–15, C1–16, C1–27) were given 3 capsules each day. Nov. 11, 1946: C1–14 became paralyzed and was sacrificed. Dec. 2, 1946: the remaining 5 animals were challenged by intrathalamic inoculation of 0.8 cc. of 20 per cent *Cam* strain (4th passage). Dec. 8–12, 1946: all 5 became paralyzed and were sacrificed.

Protocol, C1–14.—Oct. 30, 31, Nov. 1, 1946: 3 capsules daily were administered, as above noted. Nov. 4–9, 1946: no symptoms were noted. Virus was recovered from pooled stools of the entire group up to Nov. 3, 2 days after feeding, and from stools of C1–14 pooled with C1–15, collected during the period Nov. 4–9. Stools from all other animals were negative during the latter period. Nov. 10, 1946: the temperature was 103.0°, about a degree above normal for the animal. No clinical symptoms were observed. Nov. 11, 1946: the tempera-

[1] Crisco is stated by the manufacturer, Proctor and Gamble, Cincinnati, to be "a pure vegetable shortening."

ture was 103.3°. There were slight tremor, slight overbreathing, easy fatigability, and paralysis of the left arm. No bulbar symptoms were noted. The animal was sacrificed immediately for histological study. Colon contents were removed and found to contain virus. It thus seems fairly certain that virus recovered from the stool pool from C1–14 and C1–15 came from C1–14.

Microscopic Examination.—The olfactory bulbs and tertiary olfactory centers were normal throughout. Minimal PvI and PrI[2] were found in the hypothalamus, but only PvI in the thalamus. Lesions in the midbrain were confined to the reticular formation and the right mesencephalic nucleus of the fifth cranial nerve. Comparable lesions were found in the locus coeruleus on the same side. In the pons, involvement of the reticular formation increased in intensity from minimal rostrally to moderate caudally. The main sensory nucleus of the fifth nerve showed minimal lesions on the left side only, while the nucleus of the spinal tract of the fifth nerve was questionably involved on the left and moderately so on the right. Lesions in the right spinal V nucleus were more marked than elsewhere in the pons, with the exception of the reticular formation. Minimal lesions were found in both vestibular and both motor VII nuclei. In the medulla, moderate involvement was found in the reticular formation and the left hypoglossal nucleus, minimal in the olive, and none elsewhere.

Scattered lesions of varying severity were found throughout the spinal cord, more marked in the cervical regions. Several levels were free of lesions excepting an occasional small PvI. The sympathetic columns showed no involvement of nerve cells at any level; at T_1 there were a few scattered infiltrating microglial cells, and at T_{11} there was a small PvI with PrI medial to the column with a few infiltrating cells in the column itself apparently extending from the adjacent lesion.

Of the peripheral ganglia of the cranial nerves, only the Gasserians were significantly involved. Moderate to marked PrI with marked neuronophagia was found on both sides. It should be noted in this connection that the central sensory connections of the V nerve were only minimally involved on the left side and moderately so on the right where a large well isolated focus was found in the spinal V nucleus in the pons. The greater severity of lesions in the peripheral component of the fifth cranial nerve system suggests to us that lesions in the Gasserian ganglia were of centripetal origin. Other cranial nerve ganglia contained only minimal infiltrative foci of questionable significance, or none whatsoever.

The celiac ganglion showed a few infiltrative lesions but no neuronophagia. One superior cervical sympathetic ganglion showed marked lesions with probable neuronophagia; the other, only minor lesions. The stellate, thoracic sympathetic, lumbar sympathetic, and spinal ganglia were not studied.

The distribution of lesions in this animal was compatible with and rather suggestive of entry of infection through the nasopharynx by way of the fifth cranial nerve; for entry *via* the sympathetics of the head and intestinal areas the evidence is much less suggestive but not wholly incompatible since lesions were observed in their peripheral components (ganglia) and some minor ones were found at least adjacent to their central stations (sympathetic columns of the cord) at two of the corresponding levels. While it is true that contamination of the oropharynx was carefully avoided in the original administration of virus, secondary contamination from infected stools could not be avoided, and may explain the apparent oropharyngeal portal of entry. The possibility of unobserved regurgitation of fed virus cannot be entirely excluded.

[2] PrI, parenchymal infiltration. PvI, perivascular infiltration.

Group II. Two Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 animals (C1-17, C1-23, C1-28, C1-29, C1-30, and C1-33) were given 3 capsules each day. Dec. 2, 3, 4, 1946: all animals having remained well, they were again given 3 capsules each day. Jan. 6, 1947: all 6 having remained well, their susceptibility was challenged by intrathalamic inoculation of 0.8 cc. of 10 per cent *Cam* virus (4th passage). Jan. 12-15, 1947: all 6 became paralyzed and were sacrificed.

Group III. Three Series of Feedings.—Oct. 30, 31, Nov. 1, 1946: 6 animals (C1-18, C1-19, C1-20, C1-21, C1-22, and C1-24) were given 3 capsules each day. Dec. 2, 3, 4, 1946: all having remained well, they were again given 3 capsules each day. Jan. 6, 7, 8, 1947: all having remained well, they were again given 3 capsules each day. Feb. 14, 1947: all having remained well, their susceptibility was challenged by intrathalamic inoculation of 0.8 cc. of 10 per cent *Cam* virus (4th passage). Feb. 20, 1947: C1-20 died of intercurrent causes, without observed symptoms of poliomyelitis, but showed typical lesions in the spinal cord. Feb. 21-25, 1947: the remaining 5 animals became paralyzed and were sacrificed.

Controls.—Feb. 14, 1947: 4 animals (C1-11, C1-25, C1-26, and C1-34) were housed throughout the experiment in the same room as those exposed to virus. They were inoculated intrathalamically on this date with 0.8 cc. of 10 per cent *Cam* virus, (4th passage). Feb. 21-25, 1947: all 4 became paralyzed and were sacrificed.

Stool Collection and Preparation.—Stools were collected daily, kept separate with regard to cage (2 monkeys per cage), but pooled by time interval, and stored on dry ice until prepared for inoculation.

All stools passed during the 48 hours before the first administration of virus were saved as normal controls. Feeding period stools were collected from the afternoon of the day of the first capsule feeding until 48 hours after the last; *i.e.*, until all ingested virus could reasonably be assumed to have left the alimentary tract. Thereafter, stools were collected in weekly pools from 9 a.m. Monday morning until 12 noon Saturday. An interval of from 1 to 3 days elapsed between the end of the "feeding period" collection and the beginning of the next collection.

Heavy suspensions, in distilled water, of the stools from each cage were prepared in a Waring blendor. After preliminary centrifugation for about ½ hour at 1600 R.P.M., the turbid supernate was clarified by centrifugation at 18,000 R.P.M. for 20 minutes. The clear supernates were then pooled by groups and centrifuged for 4 hours at 18,000 R.P.M. in the presence of 10 per cent normal monkey serum by the method developed in this laboratory (5). The pellets were resuspended in saline and stored on dry ice. While toxic reactions to intracerebral inoculation of material prepared by this technique are very few, they have occasionally occurred. Further high speed centrifugation at 18,000 R.P.M. for about 20 minutes after thawing and just before inoculation results in a water-clear, colorless suspension, completely devoid of toxic effects.

One monkey each was inoculated intracerebrally with stool concentrate from each group of experimental animals. When the result was equivocal, a second animal was inoculated with the same material. Supernate was saved from the original suspensions prepared, so that pools could be broken down to individual cages if necessary. All animals remaining free of symptoms for 4 weeks were sacrificed for microscopic examination of the brainstem for evidence of inapparent infection.

Results of Tests for Virus in the Stools

These are summarized in Table I. Virus was recovered from all groups during the feeding periods with the exception of group III at the time of the second feeding. Only one interim pool was positive, that of group I, collected

6 to 11 days following the first feeding. This group contained C1–14, the animal which developed fever the 1st day and paralysis the 2nd day after the pool was closed. Stools from each of the 3 cages housing the 6 monkeys of this group were then tested separately; the only positive result was from the cage of C1–14 and C1–15. C1–14 was sacrificed and virus recovered from its intestinal contents. C1–15 remained in the group from which stool was collected beginning the day on which C1–14 succumbed (C1–14 having been removed and the cage cleaned just before the collection began), and the pools

TABLE I

Virus in Stools after Capsule Feeding

Days of experiment when stools were collected	Group I	Group II	Group III	Controls
Control (before feeding)	0	–	–	–
1–5 (1st feeding period, 1–3)	+	+	+	–
6–11	+*	0	0	0
13–18	0	0	0	0
20–25	0	–	–	–
27–32	–	–	–	–
34–38 (2nd feeding period, 34–36)		+	0	0
41–46		0	0	0
48–53		0	?	–
55–61		–	–	–
62–68			–	–
69–73 (3rd feeding period, 69–71)			+	0
76–81			0	0
83–88			0	–

+, test positive. ?, questionable inapparent infection in animal inoculated with stool. 0, test negative. –, stool not tested.

* Virus in this pool was traced to C1-14, the animal which developed poliomyelitis (see protocol).

were negative during this and the following week. There can therefore be little doubt that the virus detected in the 6 to 11 day pool came from C1–14.

A very questionably positive test for virus was found in the animal subinoculated with stool from group III during the 2nd week after the second feeding. No clinical signs of infection were noted. Scattered PvI and rare PrI, but no neuronophagia, were found in the brainstem from the thalamus down to the upper medulla. A *cynomolgus* monkey later inoculated with the same material remained well.

DISCUSSION

Our present experiments, as well as those previously reported, show that when virus is administered in such a way as to expose only the lower alimentary

tract, avoiding contamination of the oropharynx as far as possible, poliomyelitic infection rarely supervenes. In the present series of 18 *cynomolgus* monkeys fed a total of 36 times by capsule inserted into the esophagus, only one case of poliomyelitis occurred; and in the previous series of 26 animals, each fed once by the same method, no cases occurred. Thus, in 44 animals with a total of 62 capsule-feedings, poliomyelitis resulted only once. In sharp contrast with this, is the high proportion of takes after simple oral feeding with oropharyngeal as well as gastrointestinal exposure (2, 4).

Granting that in the single case of poliomyelitis after capsule feeding and in one of the cases of simple feeding (4) entry may have occurred through the intestine (proof of which is not conclusive), the evidence substantiates our earlier conclusions that the oropharynx is a more favorable site for entry of

TABLE II

Results of Simple Feeding as Compared with Capsule Feeding

	Simple feeding			Capsule feeding		
	Per virus	*Cam* virus	Both	*Per* virus	*Cam* virus	Both
Monkeys tested	15(2)	6	21	26	18	44
Positive	6	3	9	0	1*	1
Positive, *per cent*	40	50	43	0	5.6	2.3

* Distribution of lesions in this animal suggests oropharyngeal entry rather than intestinal.

poliomyelitis than is the intestine, and that the latter is relatively impervious to the virus.

This conclusion has, we believe, an anatomical basis which would apply to man as well as to the lower primates. If, as we have elsewhere suggested (6), poliomyelitic infection enters the body through the superficial fibers (telodendria) of the regional nerve supply where virus is deposited, the reason for the failure of virus in the intestinal canal to cause infection may be looked for in the anatomical arrangements of its terminal nerve fibers. The absence of sensation for touch and pain excepting in the mesentery (7) (Vater-Pacinian receptors) indicates that there are no exteroceptive endings in the epithelium; and the histological studies of Cajal (8) and of Oshima (9) show clearly that such nerve fibers of any kind as approach the epithelial surface penetrate no farther than to the subepithelial level; they would, therefore, appear to be protected from direct contact with intestinal contents and so with virus in the contents, unless the integrity of the epithelial layer were broken. Although further study of the point is desirable it appears that in the pharynx and mouth the telodendria of regional nerves, at least of the trigeminal (10), actually do penetrate between the epithelial cells to the surface and thus are in a position to afford direct contact with virus lodged on the surface. If this is correct,

the relative ease with which infection can enter through the oropharyngeal surfaces obtains an anatomical explanation. The normal oropharynx would, so far as its superficial nerves are concerned, present an open door to poliomyelitis virus but the same would not be true of the lower alimentary tract, where trauma or ulceration of the surface would presumably be required for entry.

Another mode of invasion through the surface, by lymphatic absorption, deserves consideration. Since Flexner and Clark's (11) original observations it has been known that in poliomyelitis, virus is not uncommonly present in the tonsils and sometimes in other regional lymph nodes, including the cervical and mesenteric (12). The possibility therefore exists that virus might make its primary entry by the lymphatics and after deposit in lymph nodes make contact with nerve elements to produce the initial infection. The present experiments argue against such a theory, since lymphatic absorption is probably much greater in the intestine than elsewhere in the body, whereas infection *via* the intestine occurred no more than twice despite heavy exposures.

The conditions under which virus appeared in the stools, and then disappeared or persisted deserve attention. It appeared quite regularly during the immediate periods after feeding, and, with a single exception, disappeared thereafter. Obviously, the intestinal mucosa was in all instances thoroughly exposed and the virus had an excellent opportunity to implant itself upon and to multiply in the intestinal epithelial cells if they are in fact potential hosts to the virus, as is suggested by the comments of Evans and Green (13). Nevertheless, the only instance in which virus was recovered from the stools after the immediate feeding period occurred in the animal that developed manifest poliomyelitis. The inference is therefore at least tentatively permissible that intestinal epithelium is not a suitable host for this particular virus, and that it appears in the stools of subjects of the disease by a process of excretion from infected nerve tissue. Preliminary observations on the mechanism of excretion of poliomyelitis virus, pointing in that direction have already been published by us (14), and others will, it is expected, appear later on.

Tests for resistance to poliomyelitis virus following gastrointestinal exposure without infection were uniformly negative. The method of intracerebral inoculation with large doses of virus is obviously the severest possible test, and would fail to reveal such lesser degrees of immunity as might conceivably have occurred.

SUMMARY

36 exposures of the stomach and intestines of 18 *cynomolgus* monkeys to large doses of poliomyelitis virus by a method designed to avoid simultaneous exposure of the oropharynx and upper esophagus induced poliomyelitis in only one instance.

These observations are to be compared with a previous study in which ex-

posures of the entire alimentary tract including oropharynx and upper esophagus by simple feeding of comparable amounts of the same strain resulted in poliomyelitis in half of the test animals.

In the capsule-fed animals virus regularly appeared in the stools during and immediately after the feeding periods but disappeared thereafter excepting in the single case of poliomyelitis, in which it persisted.

No evidence of resistance to subsequent intracerebral inoculation was observed in the uninfected capsule-fed animals.

CONCLUSIONS

1. The gastrointestinal mucosa appears to be relatively impermeable to poliomyelitis virus. On the experimental evidence as well as on anatomical grounds we regard the gastrointestinal mucosa as an infrequent and exceptional portal of entry.

2. The experiments suggest that persistence of virus in the stools in poliomyelitis is due to excretion, presumably originating in infected nervous tissue, rather than to multiplication of the agent on intestinal epithelium.

3. Exposure of the gastrointestinal epithelium without resulting infection failed to produce immunity to intracerebral inoculation.

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