Biological Properties of Norwalk Agent of Acute Infectious Nonbacterial Gastroenteritis (36508)

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Acute infectious nonbacterial gastroenteritis is a self-limited disease which attacks a broad cross-section of the population, both in an institutional setting and in the home (1–3). Clinical manifestations of illness last 24–48 hr, and include diarrhea, vomiting, low grade fever, nausea, abdominal cramps, and malaise (4, 5).

Despite intensive efforts, an etiologic agent of this syndrome has not been cultivated *in vitro* (6, 7). Recently, we described transmission of the disease to volunteers, through two serial passages, by the oral administration of a stool filtrate from a secondary case which occurred during an institutional outbreak in Norwalk, OH (8). Clinical features of the induced disease were similar to those which were seen in the outbreak. Recovery was spontaneous and complete, without complications.

Although the Norwalk agent produced gastroenteritis in volunteers, it could not be detected in a wide variety of *in vitro* systems, including human fetal intestinal organ culture. Thus, in order to define certain important distinguishing properties of the agent, it was necessary to continue studies in volunteers, using man as the experimental host. Administration of infectious material was considered to be justifiable because of the benign nature of the experimental illness in healthy adult volunteers.

Materials and Methods. Inocula. The inocula containing the Norwalk agent were derived from an adult who became ill during a well-defined, typical outbreak of acute nonbacterial gastroenteritis which occurred in Norwalk, OH, and which was described by Adler and Zickl (9). A rectal swab specimen from an acutely ill secondary case was diluted 1:100 in veal infusion broth, filtered through a 1200 nm Millipore membrane, and administered to volunteers. The Norwalk specimens were provided through the kindness of Dr. M. Hatch of NCDC, Atlanta, GA and Dr. J. Adler of Channing Laboratory, Boston, MA. Diarrheal stool, from a volunteer who became ill after oral administration of this inoculum, was then made up to a 2% suspension in veal infusion broth and filtered through a 1200 nm Millipore membrane. The resulting filtrate, which represented the first passage of the Norwalk agent in volunteers, was used as the standard inoculum in each of the studies to be described, except for the experiments in which heat stability of the Norwalk agent and the development of homologous immunity were investigated. In the latter studies, a second passage suspension of diarrheal stool was used as well; this material was derived from a volunteer who developed gastroenteritis after receiving the first passage inoculum. Both the first and second passage inocula were shown to be free of known bacterial, fungal, and viral agents by the standard safety test techniques previously described (10). Administration of the two inocula by a variety of routes into suckling and adult mice, adult rabbits, guinea pigs, and rhesus monkeys failed to produce disease.

Volunteers. Individuals who participated in

these studies were from the normal volunteer program of the NIH Clinical Center or were inmate volunteers at the Maryland House of Correction, Jessup, MD. Informed consent was obtained, and close clinical supervision was provided as previously described (8). The inoculum, which consisted of 5 ml of diluted stool filtrate, was further diluted in 30 ml of tap water and administered orally to volunteers, who had been given 2 g of $NaHCO_3$ by mouth, 5 min previously.

Criteria for illness. Following administration of the agent, fever, transient leukocytosis, and a variety of symptoms, including malaise, headache, and anorexia, developed with varying frequency and intensity, but only those subjects who had diarrhea and/or vomiting, in addition to the other signs and symptoms, were considered to have been ill. The clinical spectrum of disease produced by the Norwalk agent has been described previously (8).

Ultrafiltration, ether stability, acid stability, and resistance to heat (60° for 30 min). Standard methods were used for these evaluations (11–13).

Resistance to reinfection. Volunteers who became ill after receiving the first and second passage stool filtrates were rechallenged orally with the same inoculum, 6–14 weeks later. The criteria for illness were the same as those employed in the initial challenge.

Administration of nasopharyngeal washings. Nasopharyngeal washings were collected with saline from a volunteer who was acutely ill, 48 and 72 hr after administration of the first passage stool filtrate. These washings were pooled, diluted 1:100 in veal infusion broth, and filtered through a 1200 nm Millipore filter. Streptomycin (1000 μg/ml) and tetracycline (33 μg/ml) were added to the inoculum. After safety tests failed to reveal the presence of a known agent, the nasal washings were administered by the oral route.

Organ cultures. Human fetal intestinal organ cultures were prepared and maintained as described previously (14). One set of cultures was inoculated with the original rectal swab fluid specimen from the Norwalk outbreak, and 4 serial passages were performed in organ culture. The organ cultures were washed twice with 1.0 ml of medium at 12 hr and twice at 24 hr after inoculation. At the end of the fourth passage, the final dilution of the original inoculum was 10^{-13}. Another set of cultures was initiated with first passage stool filtrate and 5 serial passages were performed, with a final dilution of the original inoculum of 10^{-14}. The final harvests were filtered through a 1200 nm Millipore membrane and tested for adventitious agents; none were found. The fourth and fifth passage organ culture harvests were administered orally to volunteers as above.

Results. Serial passage of Norwalk agent. The first passage stool filtrate induced disease in 7 of 9 volunteers to whom it was administered, as described in a previous report (8). A stool filtrate derived from one of those ill volunteers induced disease in 2 of 3 volunteers who received it. Thus, the Norwalk agent induced disease through 3 serial passages in volunteers. The frequency of disease remained high throughout these passages, and the clinical spectrum of illness remained essentially unchanged. However, the incubation period appeared slightly shortened during the third passage; initially the incubation period was 18 to 48 hr, but during the third passage it was 16–24 hr.

Titer of the Norwalk agent. The first passage stool filtrate produced disease in a majority of unselected volunteers (Table I). After a $10^{-2.7}$ dilution, the first passage material also produced disease, but after a $10^{-4.7}$ dilution this effect was not observed. The second passage stool filtrate produced disease in 7 of 19 unselected volunteers. A 10^{-2} dilution did not produce disease in 4 volunteers to whom it was administered.

Estimated size. The agent in first passage filtrate apparently passed through gradocol membranes with an average pore size of 110 nm and 60 nm, but not through the 20 nm membrane. Definite illness developed in a small proportion of volunteers who received the 110 nm or 60 nm filtrate (Table I), suggesting that the agent has a diameter less

TABLE I. Properties of Norwalk Agent.

Material tested	Property studied	Treatment	Response of volunteers (no. ill/no. tested)
First passage Norwalk agent	Titer	Diluted 1:5	14/21
		1:500	2/4
		1:50000	0/4
Second passage Norwalk agent		Undiluted	7/19
		Diluted 1:100	0/4
First passage Norwalk agent—diluted 1:5	Size	Filtration through gradocol membrane (nm)	
		(a) 110	2/9
		(b) 60	1/9
		(c) 20	0/4
	Ether stability	Exposure to 20% ether for 24 hr 4°	3/5
	Acid stability	Exposure to pH 2.7 for 3 hr at room temp.	2/3
	Heat stability	Heated 60° for 30 min	0/4
Second passage Norwalk agent, undiluted	Heat stability	Heated 60° for 30 min	4/17

than 66 nm and likely less than 36 nm (11). The 110, 60, and 20 nm membranes prevented the passage of intestinal bacteria (at a concentration of 10^7/ml) and of adenovirus 7. Type 1 poliovirus (Lsc-1) with an initial titer of $10^{6.5}$ $TCID_{50}$/ml was reduced 100-fold by filtration through the 110 nm membrane and 3000-fold by filtration through the 60 nm membrane. No poliovirus was detected in the filtrate which passed through the 20 nm membrane.

Ether treatment. Sixty percent of 5 volunteers who received the ether-treated Norwalk inoculum developed the typical clinical syndrome. Identical treatment of herpes simplex virus reduced its titer 30,000-fold or greater, while the infectious titer of poliovirus remained unchanged after ether treatment.

Acid stability. Exposure of first passage stool filtrate to pH 2.7 did not inactivate the agent (Table I). Identical treatment of rhinovirus type 13 reduced its titer 100,000-fold or greater, while acid treatment had no effect on the infectious titer of poliovirus.

Heat stability. Second passage Norwalk agent heated for 30 min at 60° produced disease in 4 of 17 volunteers, while heated first passage material failed to induce symptoms in a smaller group of men. Heating of adenovirus-associated virus 1 (titer of $10^{8.0}$ $TCID_{50}$/ml) failed to reduce its infectious titer, while heating of poliovirus type 1 vaccine strain (titering $10^{6.5}$ $TCID_{50}$/ml) and Coxsackie virus A-20 (titering $10^{5.0}$ $TCID_{50}$/ml) resulted in a complete loss of infectivity. Heating of Echovirus 11 (titering $10^{7.5}$ $TCID_{50}$) reduced its infectious titer 100,000-fold.

Immunity. Illness did not develop in 6 volunteers who were rechallenged with the first and second passage stool filtrates which produced disease 6–14 weeks previously (Table II). Routine laboratory tests were within normal limits, and the patients remained asymptomatic throughout the study. In contrast, 14 of 21 men who initially were given the first passage filtrate containing the Norwalk agent, became ill, while 7 of 19 men who initially received the second passage filtrate demonstrated disease, as noted in Table I.

Search for agent in nasopharyngeal secretions. Administration of nasopharyngeal washings from a patient acutely ill with experimentally induced disease failed to produce illness in 3 volunteers.

Organ culture harvests. Administration of fourth passage material from human fetal in-

TABLE II. Further Properties of Norwalk Agent.

Property studied	Material administered	Response of volunteers (no. ill/no. tested)
Homologous immunity	First passage (1:5) and second passage (undiluted) stool filtrates, rechallenge of volunteers 6–14 weeks after original illness	0/6
Distribution of agent in man	1:100 diluted nasopharyngeal washings from acutely ill volunteers	0/3
Propagation *in vitro*	Intestinal organ culture harvests from 4th *in vitro* passage of Norwalk agent[a]	0/5
	Intestinal organ culture harvest from 5th *in vitro* passage of Norwalk agent[b]	1/4
Pathogenicity for rhesus monkeys	First and second passage stool filtrate (1:5 dilution) administered by oral route	0/10 (rhesus monkeys)

[a] Organ cultures inoculated with original Norwalk rectal swab fluid.
[b] Organ cultures inoculated with first passage stool filtrate.

testinal organ culture failed to induce disease in 5 volunteers. Fifth passage material induced disease in 1 of 4 volunteers. It was estimated that the fifth passage organ culture harvest given to volunteers represented a 10^{-14} dilution of the original 1st passage stool. In a previous study, 1 of 5 volunteers who received third organ culture passage harvest became ill, but this material represented only a 10^{-5} dilution of the original Norwalk rectal swab fluid (8).

Species specificity. Feeding of first or second passage Norwalk agent did not induce disease in rhesus monkeys (Table II). These animals failed to develop a fever or overt sign of gastrointestinal illness.

Discussion. Indirect evidence that an infectious agent was responsible for the Norwalk outbreak of gastroenteritis was provided by its epidemic spread, including secondary and tertiary cases, along with the absence of a common contaminating source (9). The serial transmission of this syndrome by oral administration of stool filtrates, now in third passage through volunteers, further supports the view that an infectious agent, rather than an inert substance alone, such as toxin, was the etiologic agent. The dilution of the inoculum which resulted from its passage through the gastrointestinal tract, and its subsequent administration to another volunteer, makes it unlikely that a material which lacks a mechanism for its own replication or at least amplification was solely responsible for the illness which was induced.

In this and in previous studies, we were unable to induce disease in a variety of laboratory animals, both adult and newborn, including primates (rhesus monkeys), suggesting that the Norwalk agent has a relatively restricted host specificity for man.

From the pathophysiology of the disease and its transmission by orally administered stool filtrates, it appears likely that the agent replicates in the gastrointestinal tract. Employing human fetal intestinal organ cultures as the closest available laboratory analogue to the presumed target organ, we were able to induce disease by administering pooled harvests from the cultures, but only from the fifth passage material, and then in only one volunteer. Thus, organ culture may not be a particularly efficient system for cultivation of this agent. Successful blind serial passage of the agent is dependent on the ability of each organ culture to support the agent, and since we have as yet no direct measure of this property for each organ culture, it is possible than an insensitive culture in the chain of passage may have resulted in failure to demonstrate the agent. Thus, though current evidence suggests that *in vitro* replica-

tion of the agent may have taken place, further studies are required to prove this conclusively.

The failure of ether treatment to inactivate the infectivity of the agent, suggests that the agent lacks a lipid containing outer envelope. The agent was similarly resistant to acid treatment, at least to a pH of 2.7. This property presumably protects the agent in passage through acid gastric contents, and it suggests that it may not be necessary to alkalinize the stomach contents of volunteers prior to oral administration of the inoculum.

The Norwalk agent appeared to be relatively heat stable, since second passage material, heated to 60° for 30 min induced disease with a frequency (24%) which was not statistically significantly different from that of the unheated material (37%). Failure to produce disease in a smaller number of men by administering heated first passage agent is not clearly understood at this time, and may reflect a sampling error inherent in employing a small group of subjects unselected for preexisting immunity.

Thus, the properties of the Norwalk agent are consistent with those of a small (less than 66 nm, probably less than 36 nm), ether resistant, acid stable, relatively heat stable virus, most closely resembling the parvovirus group among known animal viruses. Definitive classification, however, must await direct biophysical characterization of the agent.

The failure to induce disease in volunteers by rechallenge with the original inoculum suggests that immunity, at least within the period of rechallenge (6–14 weeks), does develop. However, the continued high frequency of disease in volunteers first exposed experimentally to the inoculum (67–37%) suggests that natural immunity to the disease is not widespread or perhaps not long lasting.

The precise mode of spread of the disease remains unclear, though fecal-oral spread is the most likely possibility, based on analogy with other enteric infections, as well as on the results of experimental transmission of the disease. Earlier, Reimann and Price (15) were able to induce similar disease by the inhalation of throat washings while Gordon Ingraham and Korns (16) induced disease by oral administration of throat washings but not by inhalation of the same washings We were unable to induce disease by oral administration of nasopharyngeal washings from an acutely ill patient. However, because of the vomiting associated with the illness washings could only be taken at 48 and 72 hr after oral administration of the inoculum and the agent might have been present transiently at an earlier time in the nasopharynx; or the agent may have been present in lower titer than could be detected in a 1:100 dilution of nasopharyngeal washings.

Summary. Acute infectious nonbacterial gastroenteritis was induced in adult volunteers through three serial passages by oral administration of bacteria-free stool filtrates. This suggested that a replicating agent of subbacterial size was responsible for the observed disease. The biophysical properties of the agent, as assayed in volunteers, were consistent with those of a small virus, most closely related to the parvovirus group among known animal viruses. The agent appeared to have a diameter less than 66 nm and likely less than 36 nm. It appeared to lack a lipid containing envelope and was acid stable. It was stable to heating at 60° for 30 min. The agent appeared to be relatively host specific for man and conferred at least short-term immunity. Because of the high frequency of disease induced in unselected volunteers, widespread natural immunity to this agent may be absent or perhaps incomplete. Preliminary evidence suggested that the Norwalk agent replicated in an *in vitro* system, human fetal intestinal organ culture.

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