ABSTRACT

This study concerned infections caused by minimal quantities of waterborne enteroviruses. The model system comprised young weanling swine and their homologous enteroviruses; the porcine digestive tract and its enteroviruses are like those of man, and the system affords greater reproducibility and safety than those employing humans or other primates. Subjects swallowed known numbers of viral plaque-forming units (pfu) in 5 ml of drinking water. The body was about 1000 times (600 to 750 for one virus and 1800 to 2500 for another) less likely than a tissue culture to be infected by a given quantity of enterovirus. Doses given after eating, or in four daily portions, produced similar results. No infected animal became ill, despite the reported virulence of the viruses. Chlorination reduced viral infectivity greatly, but short of total extinction, in a single trial. Two newborns were not infected by 20 pfu administered by gavage.

The potential for virus transmission through drinking water has caused concern, even though little is known of how waterborne viruses initiate infections. Levels of viruses in drinking water are likely to be low, so studies involving minimal infective doses are especially important.

Human viruses transmissible through water generally originate in the intestines, are shed in feces and may also be transmitted through food or, more likely, by person-to-person contact. The enterovirus group, comprising small, ether- and acid-stable, RNA viruses, has been a mainstay in hypothetical and experimental modeling of virus transmission through water. This is so, in part, because there are reproducible, quantitative laboratory procedures and sensitive methods of detection for enteroviruses in a variety of kinds of water samples (15). Other viruses transmitted at least occasionally through water include the agent of hepatitis A, which resembles the enteroviruses (4), and some of the viruses of gastroenteritis, for which quantitative laboratory procedures are not available (5).

Published studies of the peroral infectivity of enteroviruses for humans have been done with the oral vaccine polioviruses. The limited quantity of information, none of which was published more recently than 1967, has been reviewed repeatedly and has given rise to quite disparate conclusions (14,15,17). Problems arise from the fact that most of these studies were not designed to pertain to transmission through water and from the allegation that attenuated virus is impaired in infectivity.

The present study was intended to measure the infectivity of enteroviruses ingested in minimal doses with drinking water. The desire to work with virulent virus virtually precluded use of human subjects. Human viruses have at times been administered perorally to mice and to nonhuman primates, but neither of these combinations is a truly homologous system, so neither appeared to justify the hazard to personnel that working with virulent human agents would have entailed. The combination of mice and murine enteroviruses was rejected because the murine and human digestive tracts do not appear to be close analogs.

The model system chosen comprised swine and their homologous enteroviruses. The porcine and human digestive tracts are analogous (13); we attempted to enhance the analogy by feeding our subjects, during the experimental period, only foods produced for human consumption. The porcine enteroviruses have been extensively studied and appear to resemble their human counterparts in every respect except host range. In that rates of infection with human enteroviruses are highest among young children, we chose to work with weanling pigs approximately 4 weeks of age, which are supposed to be immunologically competent if they received colostrum at birth. Virus types not prevalent in this area were selected to minimize the chance that passive immunity to the challenge virus would be conferred by the colostrum.

MATERIALS AND METHODS

Viruses

The procedures have been described in detail elsewhere (2) and will be presented only briefly here. Porcine enterovirus types 3 (PE3) and 7
Swine data). Swine at slaughter had shown that these two types did not occur with detectable frequency in the area of Madison, Wisconsin (unpublished data). PE3 (strain ECP0-6) and PE7 (strain O56), each of which had been shown by its donor to produce polioencephalomyelitis in colostomy-deprived germfree newborn pigs, were provided by Dr. E. H. Bohl of the Ohio Agricultural Research and Development Station at Wooster and by Dr. L. Kasza, then of the U. S. Food and Drug Administration in Washington, D. C., respectively. The viruses were received as fluid from the first cell culture passage of extracts of the brains of moribund pigs and administered here without further passage, except as noted. The viruses were detected, assayed by the plaque technique and propagated in porcine cell cultures grown in 25-cm² plastic flasks of various brands.

Swine challenge and observation

Swine were obtained from two University of Wisconsin experimental farms and three commercial farms. No farm apparently was free of porcine enteroviruses all of the time, so preexisting infections (as manifested by the presence of virus in a stool sample taken before challenge) complicated early experiments. We eventually began to obtain pigs at 2 to 8 days of age and keep them in our animal facility so that they could be completely tested for enteric virus infection before being used experimentally at approximately 4 wk of age.

The animals were housed individually during the actual experimental period, under conditions intended to preclude accidental introduction of viruses or transfer of virus from one animal to another. One control animal, not challenged with virus, was included in each experiment for each three challenged animals, unless otherwise indicated. Four, and later eight, cages were used. These were of “germfree” design and were operated under negative pressure, with all entering air drawn through four thicknesses (a total of approximately 5 cm) of glass fiber filter medium FM-004 (Owens-Corning Fiberglas, Santa Clara, California). The barriers were breached twice daily to feed and water the animals. Despite rigorous precautions, control animals were found to have gotten infected on three different occasions. Two of these three animals were found to have virus in their 14-day, but not their 7-day fecal specimens (counted from the day on which virus challenges had been administered), which suggests that lapses had occurred during feeding rather than in disinfecting the enclosures.

Animals were in their enclosures at least 3 days before challenge. Except where noted, challenge virus was administered in 5 ml per animal of Madison tap water that had been boiled previously to eliminate any residual chlorine that might have been present. The dose was delivered at the back of the tongue, and the mouth was held shut so that the animal was obliged to swallow all of it. In the first series of trials, the virus was simply thawed on the day of challenge and diluted to the desired level. Thereafter, the virus was filtered through a polycarbonate filter of 50 nm porosity (Nucleopore Corp., Pleasanton, California), to remove or break up any aggregates that might be present, in the course of dilution on the day of challenge. Previous testing of stock virus suspensions by filtration at 50 nm had not produced any decrease in plaque titer, so aggregation of the virus may not have been a problem under these conditions.

Sample collection and testing

Fecal samples for virus detection were collected from the isolators and frozen at -20 C to await testing. Feces (1-2 g) were placed in sufficient phosphate-buffered saline solution plus 2% fetal calf serum to bring the total volume to 10 ml. Tubes containing fecal samples were agitated repeatedly with a Vortex mixer, treated in ice-water in a sonic cleaning bath (20 kHz for 1.5 min), and centrifuged (20 min at 16,000 rpm) to remove fecal solids. The supernatant fluid was passed through cellulose triacetate filters (Gelman Instruments, Ann Arbor, Michigan), of 0.45 and 0.20 µm nominal porosity (the latter was sterile) and inoculated into cell cultures. A single “blind” passage was done if cytopathic effects were not seen. No disparities were detected when the identities of viruses isolated from some of the samples were verified with reference antisera. Blood samples were drawn from the anterior vena cava and tested for antiviral neutralizing antibody.

In addition to the samples taken before challenge, fecal samples were ordinarily collected on days 7 and 14 after challenge, and a further blood sample was collected on the last day of the run, which was ordinarily day 14. The criteria of infection, assuming the absence of virus and of antiviral serum antibody from before-challenge samples, were to be the presence of virus in the stool sample from day 7 or 14 or the presence of serum antibody by day 14. The antibody response was seldom definitive within 14 days, at which time the animals were ordinarily killed. The condition of the animals, and whether they had eaten their food, was ordinarily noted each time they were fed. Signs of enteroviral disease were not seen during the study.

Peroral infectivity

The experimental trials were numbered in the order in which they were performed, regardless of their purpose. The objective of many of the experiments was to determine the peroral infectious dose (PID) for virus in drinking water. The quantity of virus administered to all animals in a given experimental trial was the same. Trials 1, 2, 3, 4, 5, 6 and 8 were intended primarily to determine the PID of PE3: the animals received 1, 10, 100, 1000, 250, and 250 pfu of PE3, respectively. Trials 9, 10, 11 and 15 were intended to determine the PID of PE7; doses were 350, 1000, 250, and 250 pfu per animal, respectively. After trial 4, the number of isolator cages increased from four to eight.

Peroral pathogenicity

Experiments intended to produce disease via peroral infection included trial 7 for PE3 and trial 13 for PE7. The dose of PE3 was 2.5 × 10⁶ pfu per animal of virus that had been extracted from the brain of a pig that had been inoculated intracerebrally with the virus in an attempt to enhance its virulence. Useful quantities of either virus were hard to obtain in this way (6,7), so the PE7 administered in trial 13 was an extract supernatant fluid after 30 min of centrifugation at 13000 rpm, filtered through a sterile 0.2-µm porosity polycarbonate microfilter membrane of a 10% suspension of fourteenth day feces from trial 11, at approximately 10⁵ pfu per animal.

Presence of food in stomach

Animals were ordinarily deprived of food for approximately 15 h before the virus dose was administered. Trial 12 was intended to determine whether the presence of food in the stomach would influence the peroral infectivity of PE3; the pigs received approximately half their usual breakfast, then 5 ml of water containing 1000 pfu of PE3, then the remainder of their breakfast.

Divided doses

Doses of 1000 pfu of PE3 produced infections in about one-third of the animals challenged, whereas no infections were seen to result from a single dose of 250 pfu of PE3. Trials 14 and 16 were intended to determine whether there was a minimum dose of virus below which infection could not take place. Only six animals were available for trial 14: five of these were challenged, and one served as a control. The 1000-pfu dose was divided into four; on each of four successive days, each of the five animals received 250 pfu of PE3 in 5 ml of water, and the control pig received only the water. In trial 16, six of eight animals were challenged, and two served as controls.

Chlorine disinfection

Trial 17 addressed the question of whether virus disinfected by chlorine could produce peroral infections. PE3 (1 ml containing 3.6 × 10⁶ pfu) was treated in a sonic bath, passed through a 50-nm porosity polycarbonate filter membrane, and added to 75 ml of 0.2% Clorex solution. The mixture was stirred for 5 min, and to it was added 24 ml of 0.1 M Na₂S₂O₃. This was stirred for an additional 10 min and served as the challenge virus suspension. Of seven pigs available for this experiment, five were challenged, and the other two received just water and served as controls. The dose administered, 5 ml of the chlorine-treated suspension, contained what would have been 1.8 × 10⁸ pfu of PE3 in the absence of chemical inactivation. Repeated testing of the portion which had not been administered to the pigs revealed some residual infectivity detectable without dilution but not at 10⁻³. Titration of the residual virus by the plaque technique was not successful.

Gavage challenge of newborns

Trial 18 addressed the proposition that virus would infect more efficiently if administered to newborns by stomach tube (4). Because of
the amount of individual handling that was required, trial 18 included only four pigs, two of which were challenged with virus. The animals were collected as they were born, without allowing them to nurse or to ingest anything. Challenge took place about 1½ h after the pigs were born, with 2.5 ml of sterile deionized water (to which 2% fetal calf serum had been added in an attempt to limit losses of virus by sorption to equipment surfaces) containing a total of 20 pfu of PE3. They were bottle-fed 30 min later, and initially at 2-h intervals thereafter, with bovine colostrum that had been stored frozen after it was found not to contain antibody against PE3 (f).

**RESULTS**

Preexisting infections were a major problem during early phases of this study (Table 1). After PE3 infections had been produced in a proportion of the animals challenged with 1000 pfu in trial 5, trials 6 and 8 were performed to determine whether infections with lower doses might be detected if the animals were observed for a longer period after challenge. The fecal sampling period was extended to 4 weeks from the usual 2 weeks, but no infections were shown to have resulted from a single 250 pfu dose of PE3 per animal.

Trial 9, which was the beginning of work with PE7, was the last in which animals were obtained at 3 weeks or more of age. Trial 10 was one of the very few in which each animal that was shown by fecal tests to have been infected also demonstrated a clear-cut neutralizing antibody response by the time that the experiment ended, 14 days after challenge. Trials 11 and 15 were two of the only three in the entire study in which cross-contamination led to infection of a control animal. The finding of virus only in the 14-day stool sample of each control seems to indicate that the cross-contamination occurred late and that at least two of the six animals challenged in each trial with 250 pfu of PE7 became infected as a result of the challenge.

Trials 7 and 13 were designed specifically to try to induce disease (most likely involving the central nervous system) in the pigs, over and above whatever probability of disease might inhere simply from being infected with **TABLE 1. Infection of pigs by challenge with porcine enteroviruses in drinking water.**

<table>
<thead>
<tr>
<th>Porcine enterovirus</th>
<th>Experimental number</th>
<th>Number of animals</th>
<th>Controls</th>
<th>Challenged</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type, pfu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>trial number, pfu&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>10</td>
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</tbody>
</table>

<sup>a</sup>Challenge dose, in plaque-forming units.  
<sup>b</sup>Some or all animals had preexisting infections when challenged.  
<sup>c</sup>Number includes one infected control animal which was not among those intentionally challenged.

these viruses. The inoculum for trial 7 had been derived from the cerebellum and medulla of a colostrum-deprived pig that had been inoculated intracerebrally with PE3 at 1 day of age and sacrificed at 7 days of age. Challenged animals received approximately $2.5 \times 10^7$ pfu. On day 7, and again on day 14 after challenge, virus was present in the feces of each of the six challenged animals and of one control. Trial 7 was the first in which a supposedly uninoculated control became infected, and the only instance in which infection in a control was detected as early as day 7. Given the high level of virus used in this experiment, this animal may have been exposed while it was receiving its sham challenge, rather than by cross-contamination during its stay in the isolator. More important, some diarrhea and vomiting were seen on occasion in some of these animals during the 2-week observation period, but no signs specifically indicative of central nervous system involvement.

Trial 13 was performed with only five animals. Each of the four challenged animals received approximately $5 \times 10^5$ pfu of PE7 extracted from day 14 feces of trial 11 and was shedding virus on days 7 and 14 thereafter. No significant illness was noted in any of these animals during the 2-week observation period.

Trial 12 concerned the infectivity of waterborne virus administered, unlike that in other experiments, when food was present in the animals’ stomachs. The challenge dose, 1000 pfu of PE3 in 5 ml of water, was administered between halves of the pigs’ breakfast. Virus was present in the feces of one challenged pig on post-challenge day 7 and of it and another pig on post-challenge day 14. The finding of infection in two of six pigs challenged when they had food in their stomachs is identical to that obtained in trial 5, when 1000 pfu of PE3 was administered to six fasted pigs.

Only six animals were available for trial 14; five animals were each challenged with 1000 pfu of PE3, divided into quarters and administered over 4 successive days. Post-challenge days 7 and 14 were numbered from the last of the four daily dosings. Two of the five challenged animals became infected. Trial 16 was intended to be a full-scale confirmation of the results of trial 14, using two controls and six animals challenged as just described. Four of the challenged animals (three on day 7 and these plus one more on day 14) were found to have been infected.

Trial 17 comprised two controls and five animals challenged with a dose of chlorine-treated PE3 suspension which, in the absence of chlorine treatment, would have contained $1.8 \times 10^6$ pfu. One of the five challenged animals became infected. Of the four pigs used in trial 18, two were dosed by stomach tube with 20 pfu of PE3 at 1½ h of age. These and the sham-inoculated controls were reared principally on cow colostrum, as described above. There were no prechallenge fecal specimens; no virus was detected in the samples collected on days 7 and 14.
DISCUSSION

Aside from trials 1, 2, 4, 6 and 9, in which some or all animals had preexisting infections, a total of 66 animals were challenged with waterborne viruses, and 31 of these challenged animals became infected. This affords an admittedly limited data base from which to draw conclusions. Nevertheless, the present study appears to have produced two general findings which have considerable significance to public health, and particularly to drinking water safety. First, the results of the experiments with divided doses suggest that peroral infection is a possible result of ingesting as little as a single tissue culture dose of virus, and perhaps even a single viral particle. Second, the results of the peroral infectivity experiments indicate that peroral infection is an extremely improbable result of ingesting a single tissue culture dose, and especially a single viral particle. For reasons yet unknown, these enteroviruses are relatively inefficient in initiating infections per os, though this study was begun with a fair expectation that the opposite would be found.

The efficiency with which virus infects per os and in cell cultures is most readily compared by means of the Poisson formula, applications of which have been well presented by Sobsey (16). Point estimates of the PID, calculated on the basis that the probability of not becoming infected is $e^{-m}$ (where $e$ is the base of the natural logarithm and $m$ is the number of PIDs ingested), range from 1800 to 2500 pfu for PE3 and from 600 to 750 pfu for PE7. Because we did not count virus particles in this study, it is hard to be sure of the reason that PE7 appears to be more efficient than PE3; it could as well be that particles of PE7 are less efficient in initiating plaques in cell culture as that they are more efficient in initiating infections in vivo. Particle counts are virtually impossible to include in studies such as this so it may well be that those who have reported producing infections with fewer tissue culture doses have simply had less efficient tissue culture assay systems.

The lack of perceptible illness in animals infected with these virulent viruses was unexpected. Neither very large doses nor attempts further to enhance the virulence of the viruses by intracerebral passage resulted in disease. Possibly some additional insult that would help to compromise the host’s resistance needed to be superimposed upon that of virus infection, as has again been reported recently for parenteral infections potentiating paralytic poliomyelitis in humans (18). This problem merits further study; it is important to know whether some quantity of virus, greater than that which produces infection, is really needed to cause disease.

The relevance to human health of a model using swine and their homologous enteroviruses is virtually impossible to prove directly. Analogous experiments in which humans are challenged perorally with virulent, homologous enteroviruses are unlikely to be done. Administering human enteroviruses to nonhuman primates would not afford a homologous combination of host and virus, and little seems to be known of the in vivo properties of simian agents that might be used as homologous agents with nonhuman primates. The murine digestive tract is probably not a close analog of the human tract; peroral infection experiments with mice have employed human (i.e., heterologous) enteroviruses (9,10) and are not directly pertinent to the question of human infections with minimal quantities of waterborne enteroviruses. The experimental system comprising swine and the porcine enteroviruses is, quite simply, the best available for this purpose.

Individual variations among pigs, in the absence of detectable antibody against the challenge virus, seemed to have little influence on the efficiency with which ingested virus initiated infection; peroral infection at marginal doses was apparently quite random. Neither did the presence of food in the stomach have a perceptible effect upon the efficiency of virus infection; this suggested that the PID might be the same for virus in food as in water. The ability of small quantities of virus to infect was apparently less enhanced by extremely early exposure to virus, by colostrum deprivation, and by gavage challenge, than had been reported in studies by others working with humans (14).

The irrevocability of chlorine inactivation merits further study. The dose per animal in trial 17, had it not been for the chlorine treatment, was $1.8 \times 10^6$ pfu per pig. If virus that was inactivated (in the sense that it could no longer infect a cell culture) by chlorine were still infectious per os as has been suggested (11), all five of the challenged pigs should have been infected. Instead, one of the five became infected, which suggested that a fraction of PID was present in the inoculum. A trace of residual infectivity was detected, but this level of virus would not have been expected to produce 20% infections if a larger number of animals had been challenged with it. It does not seem reasonable to attribute this result to the presence of aggregates in the suspension, since the suspension had been passed through a 50-nm filter membrane just before the chlorine treatment.

It is possible that an isolated enterovirus infection, and perhaps illness, might occur in a consumer of drinking water contaminated at levels far below what one could reasonably hope to detect by means of the most powerful concentration methods available (12,13). And, this hypothetical infection might give rise to others by direct (contact) transmission. However, contact transmission appears to be the most prevalent (and perhaps least understood) mode of transfer of enterovirus infections (5). An infected member of a community is probably a greater threat to his contacts than to those whom his infection could be transmitted only via the water route.

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INFECTION BY WATERBORNE VIRUSES

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REFERENCES