Enteric Adenoviruses: Detection, Replication, and Significance

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Adenoviruses can be demonstrated readily in the stools of pediatric gastroenteritis patients by electron microscopy or counterimmunoelectrophoresis, but in 45% of these cases the virus will not grow in cell culture. Indirect immunofluorescence microscopy can be used to detect nongrowing strains of adenovirus; these strains have a unique single-cell fluorescence pattern. Hematoxylin and eosin staining reveals adenovirus-like inclusion bodies in the same distribution as fluorescent cells. Pretreatment of nongrowing adenovirus with convalescent-phase patient serum neutralized its ability to infect the cell culture and produce fluorescent cells. Indirect immunofluorescence microscopy compared favorably with electron microscopy in demonstrating virus in the stools of patients.

Adenoviruses were among the first viral agents associated with acute nonbacterial infectious gastroenteritis (2, 6). Adenovirus serotypes 9, 12, 13, 18, and 25 through 28 were first isolated from feces (16). These agents also have been found in patients with intussusception (11). More recent studies employing electron microscopy (EM) have revealed adenovirus in the stools of two fatal cases of gastroenteritis (15) as well as in numerous hospital ward outbreaks (3, 7, 9, 13). The observation was made in these studies that a variable number of stool specimens contained adenovirus particles by EM, yet these adenoviruses failed to replicate in cell culture. Our approach in this study has been to examine cell cultures infected with stool adenovirus by indirect immunofluorescence microscopy (IIF). We hoped to determine whether a virus-cell interaction occurred which led to an abortive infection and whether IIF could be used as a diagnostic test for the nonreplicating enteric adenoviruses.

MATERIALS AND METHODS

Stool specimens were collected from infants admitted to The Hospital for Sick Children with acute gastroenteritis. These specimens were examined by negative-contrast EM for the presence of particles with viral morphology (9). Stools containing adenovirus were suspended in Earles lactalbumin-yeast extract cell culture medium which contained mycostatin at 50 U/ml and kanamycin at 50 μg/ml. This suspension was centrifuged at 5,000 × g at 4°C for 10 min. The supernatant fluid was centrifuged at 100,000 × g at 4°C for 90 min and the resulting pellet was resuspended in 2 ml of Earles lactalbumin-yeast extract medium and stored at −20°C.

A continuous line of human amnion cells HAE-70 (4) was grown on 15-mm circular cover slips in Costar 24-well culture dishes, after which the growth medium was removed. Each monolayer was then inoculated with 0.1 ml of stool suspension and held at 37°C for 1 h. After removal of the inoculum by suction, 2 ml of Eagle minimal essential medium was added to each well, and the cultures were incubated at 37°C in a 5% CO2 atmosphere for 48 h. After incubation, the cover slips were washed with phosphate-buffered saline and fixed in acetone at −20°C for 10 min. They were reacted with guinea pig antiserum to adenovirus type 5 for 45 min at 37°C, washed, and then reacted with rabbit anti-guinea pig antiserum conjugated to fluorescein, followed by counter staining with 0.001% Evans blue. Guinea pig antiserum to adenovirus type 5, prepared as outlined previously, was arbitrarily selected (8). Fluorescein-conjugated antiserum to guinea pig globulin was purchased from Hyland Laboratories, Inc., Costa Mesa, Calif.

Stool-derived nongrowing adenovirus preparations were inoculated onto monolayers of HAE-70 cells and superinfected with simian virus 40 (SV40) by the method of Reich et al. (10).

RESULTS

Retrospective analysis of virus detection by EM. In the 4 years from 1975 to the end of 1978 our laboratory identified virus particles by EM in 2,606 stool samples from patients with acute gastroenteritis. These agents included rotaviruses, astroviruses, caliciviruses, picorna- or parv-like viruses, a 30-nm particle we have referred to as “mini-reovirus,” and adenoviruses. Of these, 392 (15%) were adenoviruses.

Two categories of adenoviruses were detected: those that replicated and caused cytopathic effects in cell culture and those that failed to replicate. A total of 216 adenoviruses grew in HAE-70 cell culture out of a total of 392 stool specimens (55%). The remaining 176 did not
grow. The latter we refer to as the nongrowing strains of adenovirus.

IIF. A total of 42 stool specimens containing nongrowing or growing adenoviruses were inoculated into HAE-70 cell cultures, and the cultures were examined by IIF after 48 h of incubation. Ten of these were growing strains and 32 were nongrowing strains. As shown in Table 1, 41 of 42 specimens produced adenovirus specific fluorescence in cell culture. The fluorescence was concentrated in the nucleus, with additional granular staining in the cytoplasm.

**Nature of fluorescence.** A distinct difference in the fluorescence pattern could be seen when stool specimens containing growing and nongrowing adenoviruses were examined by the above technique. As shown in Fig. 1a, growing strains produced clusters of adjacent fluorescent-staining cells. In contrast, nongrowing strains showed isolated cells fluorescing with no spread to adjacent cells (Fig. 1b and c). No overall difference in the pattern of fluorescence of the individual cells was seen whether they had been infected by the growing or nongrowing strains of adenovirus.

**Specificity of fluorescence.** In addition to the 42 stools with adenovirus particles, 15 other stool specimens from gastroenteritis patients that either had no virus detectable by EM or contained rotavirus or picorna- or parvo-like viruses were inoculated into cell cultures, and these were examined by IIF for the presence of adenovirus antigen. As shown in Table 1 no evidence of adenovirus antigen was detected in these cultures.

**Sensitivity.** If the above immunofluorescence assay is to be a useful alternative for rapid detection of adenoviruses that fail to grow in cell culture, its overall sensitivity must first be determined. Accordingly, serial 10-fold dilutions were made of three stool specimens containing nongrowing adenovirus that had been demonstrated by EM. Samples of these were examined by EM and inoculated into cell cultures which were then examined by IIF. Results shown in Table 2 indicate that immunofluorescence was at least as sensitive as, and possibly more sensitive than, EM for detection of these viruses.

### Table 1. Comparison of EM and IIF microscopy for detection of adenovirus in stool specimens

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. detected by</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EM</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>42</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>2</td>
</tr>
<tr>
<td>Picorna- or parvo-like virus</td>
<td>2</td>
</tr>
<tr>
<td>No virus detectable</td>
<td>11</td>
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</table>

**Manipulations of the immunofluorescence assay.** Attempts were made to enhance the sensitivity of detection of adenovirus by centrifugal inoculation of the virus. One set of serial 10-fold dilutions of three stool specimens containing nongrowing strains of adenovirus was inoculated in the conventional manner onto human amnion cell cover slip cultures; the other set of cultures was infected by centrifuging the inoculum onto cell monolayers (500 × g at 35°C for 30 min). Immunofluorescence staining was carried out after 48 h of incubation for both sets. No difference could be detected between the two sets of infected cultures in terms of enhancement of infectivity.

Attempts were made to coinfect cell cultures with adenovirus and SV40. This approach enabled human adenovirus that normally did not replicate in monkey cells to replicate (10). No increase in the numbers of fluorescent cells was evident.

Human adenoviruses are considered to grow best in primary human kidney cells (12). Such cell cultures were prepared from a neonatal kidney. No enhancement of production of fluorescent cells was evident when these cultures were infected with eight stool specimens containing non-growing adenoviruses and viewed by IIF microscopy.

To confirm that the virus antigen detected by immunofluorescence was specific, a separate system of ascertaining virus infection was used. Cells were infected as outlined above with non-growing adenoviruses. At 48 h after infection they were processed by fixing with ethanol and stained with hematoxylin and eosin. Figure 2 provides examples of typical intranuclear inclusions found in isolated cells.

**Evidence of infection in a patient.** In a very preliminary study, acute- and convalescent-phase sera were obtained from a patient with gastroenteritis associated with a non-growing strain of adenovirus. These were analyzed for antibody to adenovirus by a standard complement fixation test and found nonreactive. Since the patient was an infant, this observation was not unexpected (14). However, when serial dilutions of this patient's sera were incubated with samples of adenovirus extracted from his stool, and these mixtures were inoculated into cell cultures and then monitored by immunofluorescence, a significant antibody titer rise was demonstrated. The acute-phase serum suppressed appearance of fluorescent cells to a dilution of 1/16, whereas the convalescent-phase serum did so to a dilution of 1/512. The above IIF tech-
FIG. 1. IIF staining of adenovirus-infected cells. (a) HAE-70 culture infected with growing strain of adenovirus and examined by IIF after 48 h of incubation. Note cluster of adjacent fluorescent staining cells. (b) HAE-70 cell culture infected with nongrowing strain of adenovirus and examined by IIF after 48 h of incubation. Note isolated cells fluorescing with no spread to adjacent cells. (c) Higher power of (b). Note concentration of fluorescent stain in the nucleus with granular staining in the cytoplasm.
nique is therefore sufficiently sensitive to demonstrate a significant serological response in a patient suffering from adenovirus-associated gastroenteritis.

**Table 2. Comparison of sensitivity of EM and IIF microscopy for detection of adenovirus**

<table>
<thead>
<tr>
<th>Stool sample</th>
<th>Endpoint dilution</th>
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<tbody>
<tr>
<td></td>
<td>EM</td>
</tr>
<tr>
<td>1522</td>
<td>$10^{-2}$</td>
</tr>
<tr>
<td>2448</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>3343</td>
<td>$10^{-2}$</td>
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**DISCUSSION**

Adenoviruses which have been derived from stools of gastroenteritis patients and which do not replicate in cell culture have been termed enteric adenoviruses (5). Our study and those of others (13) have demonstrated that there is a limited degree of replication, though the process appears to be incomplete. A parallel phenomenon appears to occur with the human rotavirus that has been shown to replicate in a defective fashion in cell cultures (1). The replication causes a unique pattern of immunofluorescence.
in which cells adjacent to the originally infected cell do not exhibit fluorescence (J. S. Tam, unpublished data). In contrast, antigenically related strains of animal rotavirus such as calf and simian rotaviruses grow well in cell culture, and these produce an immunofluorescence pattern indicative of the spread of progeny virus to adjacent cells (17).

The limited replication of the enteric adenoviruses is also confirmed by demonstrating eosinophilic intranuclear inclusions in the inoculated cell cultures. This observation and the presence of viral antigen indicate that the replicative cycle starts but does not proceed past a certain point (probably late in infection) to yield infectious virus.

The abortive infection could not be made productive in our study either by inoculating the viruses into more sensitive primary human kidney cells or by coinfesting them with SV40. The latter is reported to make monkey cells permissive to the human adenoviruses (10).

When the value of the IIF technique was examined in terms of routine laboratory use, it was found to be at least as sensitive as EM for the detection of virus in stool specimens. Moreover, it was also nearly as efficient as EM in that it detected adenoviruses in 41 out of 42 specimens in which virus had been detected by EM. On this basis, IIF is substantially superior to counterimmunoelectrophoresis which has one-eighth the sensitivity and 90% of the efficiency of EM (8). IIF is greatly superior to the observation of adenovirus cytopathic effect since enteric adenoviruses do not produce a cytopathic effect in cell cultures.

From a clinical viewpoint, adenovirus-associated gastroenteritis accounts for about 15% of our cases of viral gastroenteritis in hospitalized patients. Our limited seroconversion study indicated that there was a significant response of the host's immune system to the enteric virus. In the past year we have documented two infant deaths from dehydration caused by severe gastroenteritis which was associated with adenovirus infection. We were able to demonstrate cells in the jejunum which contained adenovirus antigen as displayed by specific immunofluorescence. Our studies and those of others have shown enteric adenoviruses to be significant pathogens (3, 7, 9, 13). We believe that their failure to replicate in cell cultures has largely been responsible for the fact that these agents were not recognized until recent years.

ACKNOWLEDGMENT
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LITERATURE CITED