Diagnosis of Fastidious Enteric Adenoviruses 40 and 41 in Stool Specimens

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Thirty-five stool specimens, collected over a 14-week period from pediatric gastroenteritis patients and shown to contain adenovirus by electron microscopy, were inoculated onto 293 and HeLa cells. Virus isolates were characterized by serum neutralization and restriction endonuclease cleavage analysis of viral DNA from infected cells. Adenovirus was isolated upon primary inoculation of 293 cells from all 35 specimens shown to contain adenovirus by electron microscopy. Fastidious adenoviruses 40 and 41 (Ad40 and Ad41) were found in 17 (49%) of the stool specimens, and 4 of these specimens contained a conventional species (Ad1, Ad1, Ad18, Ad31) as well as Ad40. This was first manifest by the observation that four of the isolates which initially grew only in 293 cells acquired the capacity to grow in HeLa cells upon subsequent passage. In each case, the conventional species was undetectable by DNA analysis in the original inoculum but was selected in 293 cells and became the only one detectable by the second passage. Four other specimens, containing Ad1 or Ad31 alone, failed to grow initially in HeLa cells but did grow in 293 cells. The results of this study demonstrate therefore that (i) 293 cells are more sensitive than HeLa cells for the isolation of conventional as well as fastidious enteric adenovirus species and (ii) identification of viruses from patient specimens should involve minimal passage of the virus in cell culture, as a single passage can result in misdiagnosis of the virus associated with the infection.

Adenoviruses 40 and 41 (Ad40 and Ad41) are referred to as fastidious enteric adenoviruses because they can be readily detected by direct electron microscopic (EM) examination of stool specimens from patients with gastroenteritis but are refractory to isolation in cell cultures currently used for the propagation of adenoviruses (KB, HeLa, HEp-2, human amnion, human embryonic kidney). However, these viruses do replicate and produce infectious progeny in 293 cells (15), a continuous line of human embryonic kidney cells transformed with Ad5 DNA (6).

Before the use of 293 cells for virus isolation from stool specimens, adenovirus was isolated in this laboratory from less than 50% of the specimens positive for adenovirus by EM (13, 14). A study was then initiated to answer two questions: (i) whether the isolation rate from stool specimens positive for adenovirus by EM could be increased by using 293 cells and (ii) whether Ad40 and Ad41 could be easily distinguished from conventional adenovirus species by their ability to grow in 293 but not in HeLa cells.

MATERIALS AND METHODS

Collection of specimens. The stool specimens used in this study were among those submitted to the virology laboratory at The Hospital for Sick Children for routine diagnosis from early March to mid-June 1982. All specimens selected for the study were from children with diarrhea and were shown to contain adenovirus by direct EM examination. With one exception, the age of the patients ranged from 2 weeks to 2 years, with a mean age of 7 months. One of the Ad40 isolates was obtained from a 7-year-old boy with an ileostomy.

Cells and virus. The 293 cells were obtained from F. Graham, McMaster University, at passage 43 and were used in this study between passages 60 and 80. The cells were grown in minimal essential medium (AutoPow: Flow Laboratories, Inc.) supplemented with 10% fetal bovine serum, penicillin (300 μg/ml), and streptomycin (250 μg/ml). Cells were subcultured with a trypsin-EDTA solution containing 500 μg of trypsin per ml (1:250), 140 mM NaCl, 5.4 mM KCl, 6 mM glucose, 6.9 mM NaHCO3, 0.5 mM EDTA, and 0.0002% phenol red.

For the primary isolation of virus from stool specimens, cells were seeded in 24-well tissue culture plates so that the cells were subconfluent at the time of inoculation, within 2 to 3 days after being seeded. Cells infected for the purpose of extracting viral DNA for restriction endonuclease analysis were seeded in 60-mm petri plates and infected the day after seeding.

Stool specimens were suspended in a minimal volume of minimal essential medium with a Vortex mixer and clarified by centrifugation in an Eppendorf centrifuge at 12,000 × g for 5 min. For routine isolation, cells were inoculated with a 1/10 dilution of the supernatant fluid.

The type strains of Ad40 (Dugan) and Ad41 (Tak) were obtained from the American Type Culture Collection. The cells were prepared in 293 cells.

Preparation of viral DNA. Virus preparations (clinical specimens and prototype strains) were inoculated onto 1-day-old subconfluent monolayers of 293 cells in 60-mm petri dishes. When the cytopathic effect (CPE) was well developed, cells were scraped into the culture fluid with a rubber policeman, transferred to a centrifuge tube, and pelleted at 75 × g for 5 min. The cell pellet was suspended in 0.3 ml of 20 mM Tris (pH 7.5)-10 mM EDTA and transferred to a siliconized microcentrifuge tube (1.5-ml capacity). Viral DNA was isolated by a modification of the Hirt procedure (7) as follows. Sodium dodecyl sulfate was added to a concentration of 0.6% along with protease K (Boehringer-Mannheim Biochemicals) (final concentration, 250 μg/ml). After incubation for 1 h at 37°C, 5 M NaCl was added to a final concentration of 1 M. and the preparation was held at 4°C overnight. The microcentrifuge tube was placed in an International ultracentrifuge SB283 bucket and spun at 17,000 × g for 30 min to pellet the high-molecular-weight cellular DNA. The supernatant fluid was placed into a

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second siliconized microcentrifuge tube and extracted once with phenol saturated with Tris-hydrochloride (pH 8.1) and once with chloroform-isooamyl alcohol (24:1). After precipitation with ethanol for 1 h at −70°C, the DNA was collected by centrifugation at 12,000 × g in an Eppendorf microcentrifuge. The pellet was suspended in 10 mM Tris-hydrochloride (pH 7.4)–10 mM MgCl₂ and digested with RNase (25 μg/ml) for 1 h at 37°C, and the DNA was precipitated with ethanol. After drying, the DNA pellet was suspended in 10 mM Tris-hydrochloride (pH 7.4) and stored at −20°C.

**Restriction enzyme digestion.** Restriction enzymes HindIII and Smal were purchased from Boehringer-Mannheim. The digestion conditions were those described by Bethesda Research Laboratories, Inc. An HindIII digest of λ DNA was purchased from Bethesda Research Laboratories.

**Gel electrophoresis.** Samples were electrophoresed through 5% polyacrylamide gels (polyacrylamide-bisacrylamide, 30:0.8) in 0.5× TBE (pH 8.3) (1× TBE is 89 mM Tris plus 90 mM boric acid plus 2.5 mM disodium EDTA). Electrophoresis was at constant voltage for ca. 1.000 V-h.

**Staining of gels.** Polyacrylamide gels were stained with silver, using the photochemical method described by Beidler et al. (1).

**Neutralization tests.** The adenovirus isolates were classified according to species in microneutralization tests with 293 cells. Specific antiserum against Ad1 through Ad7, Ad8 through Ad31, and Ad32 through Ad39 were obtained from Microbiological Associates, the National Institutes of Health, and the Centers for Disease Control, respectively. Antiserum against Ad40 and Ad41 were a generous gift from G. Wadell, Umeå, Sweden.

**RESULTS**

**Classification of the basis of CPE.** Of the 35 specimens examined, all induced CPE in 293 cells (293⁺), whereas only 17 induced CPE in HeLa cells (HeLa⁺) upon primary inoculation (Fig. 1). Progeny virus from the cells in which CPE was induced was then passaged as follows. Cells were suspended in the culture fluid and transferred to a tube, and the virus was released by freezing and thawing. The suspension was clarified by centrifugation at 75 × g for 10 min and used to inoculate fresh 293 and HeLa cells.

Four distinct growth patterns in 293 and HeLa cells were recognized. Of the 17 specimens which were HeLa⁺ upon primary inoculation, 14 were successfully passaged to HeLa cells (group I), whereas 3 were not (group II). Two of these three specimens did, however, initiate infection when passaged from HeLa to 293 cells. Of the 18 specimens which induced CPE only in 293 cells and not in HeLa cells, i.e., the prospective fastidious enteric adenoviruses, 11 could not be passaged from 293 cells to HeLa cells (group III), whereas 7 which were 293⁺ HeLa⁺ on primary inoculation did produce CPE in HeLa cells after passage through 293 cells (group IV).

Of these four groups, it appeared that group I contained conventional adenovirus species with the ability to grow in HeLa cells upon primary inoculation. Group III appeared to represent the fastidious enteric adenoviruses, which grew only in 293 cells and not in HeLa cells. The identities of group II specimens, i.e., those which induced CPE in HeLa cells only upon primary inoculation but could not be passaged to fresh HeLa cells, and group IV specimens, i.e., those which grew on HeLa cells only after passage through 293 cells, were not clear. The virus isolates in each group were therefore characterized by serum neutralization tests and DNA restriction analysis.

**Neutralization tests.** The isolates in group I were classified according to species by microneutralization tests in 293 cells. Altogether, 1 isolate was Ad2, 2 were Ad5, one was Ad18, and 10 were Ad31. The group IV specimens were also tested by microneutralization after serial passage in 293 cells. Three isolates were Ad1, one was Ad18, and three were Ad31.

**DNA restriction analysis.** The isolates in groups II, III, and IV were characterized by DNA restriction analysis. The three isolates in group II were fastidious enteric adenoviruses, identified as Ad40 by their Smal cleavage patterns (Fig. 2). By using Smal, 10 of the 11 specimens in group III were also identified as fastidious enteric adenoviruses: 2 as Ad41 and 8 as Ad40 (Fig. 2). One specimen (Fig. 2) was neither Ad40 nor Ad41 and was identified by neutralization as Ad31. The Ad40 and Ad41 isolates were neutralized by specific antiserum in microneutralization tests.

The group IV isolates were examined in an effort to account for their acquired ability to grow in HeLa cells. Duplicate plates were inoculated with the original stool

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**FIG. 1.** Identification of adenovirus isolates from stool specimens shown to be positive for adenovirus by EM. 293 and HeLa cells were incubated with clarified stool suspensions diluted 1:10 and monitored for development of CPE (+) or absence of CPE (–). Progeny virus from HeLa⁺ and 293⁺ cells was passaged to both HeLa and 293 cells. Blind passages from HeLa cells were not done. The isolates within each group are listed. The number of isolates is shown in parentheses.
It was initially thought that it might be possible to distinguish fastidious enteric adenoviruses from conventional species on the basis of their growth patterns in 293 and HeLa cells. However, this proved not to be the case. Based on the absence of CPE in HeLa cells, 18 of the specimens would have been classified as fastidious enteric adenoviruses. In fact, three of these were subsequently identified as Ad31 and one as Ad1. Moreover, three specimens which induced a CPE upon primary inoculation of HeLa cells and thus would have been classified as conventional species were identified by restriction analysis as Ad40 (group II). The fact that virus was passaged from HeLa' cells to 293 cells but not to HeLa cells in two of these cases may reflect the high concentration of virus in the original specimen, so that a sufficient amount remained to initiate infection in 293 cells after 'passage' through HeLa cells. Alternatively, HeLa cells infected at a high multiplicity of infection may release a small amount of infectious progeny sufficient to initiate productive infection in the sensitive 293 cells but not in HeLa cells. In a previous study, Kidd and Madeley (12) reported the induction of CPE in human embryonic kidney cells by some Ad40/41 (not differentiated) specimens, but the virus could not be passaged. The results of Kidd and Madeley (12), along with those reported here, are consistent with immunofluorescence studies which demonstrated that human embryonic kidney, human epithelial (HEp-2), and human amnion cells undergo abortive infection by Ad40/41 (5, 14).

In this study, it is unusual that an isolate of Ad31 was included in group III. Even after two passages in 293 cells, it did not induce CPE in HeLa cells. These results thus illustrate the value of 293 cells for the isolation of both conventional and fastidious species of adenovirus.

Of particular interest were the seven specimens (group IV) which appeared to be fastidious enteric adenoviruses on the basis of their failure to induce CPE on primary inoculation of HeLa cells but which acquired the ability to grow in HeLa cells after passage through 293 cells. Two of these were identified as Ad31 and one as Ad1 in the original stool specimens. This would suggest that the infectious titer of virus in the original specimen was low, even though the number of particles was sufficient to be seen by EM and required amplification in 293 cells to cause obvious CPE in HeLa cells. The other five specimens were identified as a conventional species (Ad1, Ad1, Ad18, Ad31) in addition to Ad40. It is not uncommon to find more than one species in stool specimens, since conventional species can be shed in the stool for extended periods after respiratory or enteric infections (4, 11). In this case, Ad40 was the only virus detectable by restriction analysis of DNA from high-salt extracts of cells inoculated with the original stool specimen (Fig. 3), thus indicating that Ad40 was associated with the current episode of disease. After a single passage in 293 cells, however, the conventional species was the only one detectable (Fig. 3). Thus, identification of the isolate from the "passage 2" material, as is often done in neutralization tests, would have resulted in misdiagnosis of the virus associated with the disease. It is interesting that the conventional species was selected over Ad40 in 293 cells, which are normally permissive for Ad40. Moreover, the selection was evident within a single passage. This may simply reflect a faster growth rate of the conventional species relative to Ad40, or it may be related in some way to the host-range restriction exhibited in culture by Ad40. Experiments are currently in progress to examine these two possibilities.

Within group I, Ad31 was the predominant species (10 out of 14 isolates). This identifies Ad31 as the predominant strain

FIG. 2. DNA restriction patterns of representative isolates from groups II and III. The numbers above each track denote the adenovirus species analyzed. 40" and 41" refer to the prototype strains of Ad40 (Dugan) and Ad41 (Tak), respectively. The other isolates, including the single Ad31 isolate from group III, were from patient specimens. Minor bands (●) in two of the Ad40 isolates represent partial digestion fragments.
This study demonstrated that fastidious enteric adenoviruses could not be identified simply by their differential growth patterns in 293 and HeLa cells, since Ad40 at high concentration induced CPE in HeLa cells, and conventional species at low concentration induced CPE in 293 but not HeLa cells. A previous report on the identification of fastidious enteric adenoviruses from stool specimens relied on neutralization of the virus with species-specific (type-specific) antiserum after isolation and serial passage in susceptible cells (11). However, passage of the virus beyond primary isolation in cell culture can lead to erroneous results, as seen in this study with 4 of 17 specimens containing Ad40 or Ad41. A recent study by Wigand et al. (17) described a method for the concurrent isolation and identification of Ad40/41 from stool specimens, based on neutralization of CPE. This involved inoculation of Chang conjunctiva and HeLa cells with stool suspensions both untreated and treated with antiserum prepared against purified Ad40 virions. Ad40 and Ad41 cross-react serologically and thus were not distinguished from each other. Moreover, specimens containing a conventional adenovirus species, in addition to Ad40/41, would not be completely neutralized and hence not identified as Ad40/41.

Given that approximately half of pediatric adenovirus gastroenteritis cases are associated with Ad40 or Ad41 (16–18), there is a need for the identification of Ad40/41 directly from stool specimens. Chiba et al. (2) used DNA hybridization to identify Ad40 directly from stool specimens which were positive for adenovirus by EM. However, it is not clear whether this involved purification of virions from the stool before extraction of the DNA or whether viral DNA was extracted directly from the stool specimen. We have attempted to purify viral DNA directly from the stool specimens for restriction analysis. This was successful in only 5 of 15 cases (data not shown) and, therefore, was not reproducible. Uhnno et al. (16) refer to an enzyme immunoassay for direct identification of Ad40 and Ad41 from stool specimens. This involves the use of polyclonal antisera which have been rendered species specific (type specific) by affinity chromatography (8, 9). Such an assay deserves attention. Used with species-specific monoclonal antibodies, it has the potential to simplify and standardize the specific diagnosis of Ad40 and Ad41. Until such antibodies are widely available, the definitive identification of Ad40 and Ad41 will continue to rest on the restriction analysis of viral DNA after isolation of the virus in culture.

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