Isolation and Propagation of Enteric Adenoviruses in HEp-2 Cells

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Eighty-two stool samples from children with gastroenteritis in Canada, England, and Thailand which had been shown to contain adenovirus antigen (by a group-specific enzyme-linked immunosorbent assay) or adenovirus particles (by electron microscopy) or both, were tested for primary isolation of enteric adenoviruses in HEp-2 and Graham 293 cells. Graham 293 cells are known to support the replication of enteric adenovirus types (Ad40 and Ad41) on primary isolation, whereas HEp-2 cells reportedly do not. Of the 82 adenovirus isolates, 73 could be typed as Ad40 or Ad41 by type-specific monoclonal antibody enzyme-linked immunosorbent assay and by analysis of SmaI endonuclease digests. Of these 73, 30 (41%) could be isolated in HEp-2 cells, which included 43% (9/21) of those typed as Ad40 and 40% (21/52) of those typed as Ad41. On the basis of these results, the growth characteristics of adenoviruses in HEp-2 cell cultures, commonly used to distinguish enteric from nonenteric adenovirus types, are not valid for either diagnosis or epidemiological studies. For the samples studied here, use of these nondefinitive criteria would result in underestimation of the incidence of enteric adenoviruses in viral gastroenteritis.

Adenoviruses have been isolated from stools for over 20 years, but it is only in recent years that specific adenovirus types have been closely associated with gastroenteritis, types which are now designated adenovirus type 40 (Ad40) and adenovirus type 41 (Ad41). These enteric types were originally described as viruses which could be visualized in stools of patients with gastroenteritis by electron microscopy (EM) but could not be isolated in cell cultures generally used in diagnostic virology laboratories for isolation of respiratory adenoviruses (1, 7, 8, 12). Subsequently, it was found that the enteric types could be isolated and propagated in Graham 293 cells, an adenovirus type 5 (Ad5)-transformed human embryonic kidney cell line (13). In some traditional cell lines, a low-level, transient cytopathic effect (CPE) was seen with these viruses, and antigen could be detected by immunofluorescence (8, 12, 14). Also, in HeLa and HEp-2 cells some stock, cell-cultivated strains of enteric adenoviruses could be serially passaged (6). In studies with HeLa and KB cells, the reason for the abortive infection seen with the enteric adenovirus types was ascribed to an early replicative block (14).

On the basis of these studies, it has become axiomatic that detection of adenoviruses in stools by EM and failure to isolate them in cell lines known to support growth of other adenovirus types is presumptive evidence for the enteric types. Conversely, it is now assumed in epidemiological studies on adenoviruses that virus replication in HEp-2 or other conventional cell lines can be used to designate adenovirus isolates as nonenteric (5). There have been some indications, however, that enteric adenoviruses may be isolated in so-called nonpermissive cell lines. In a study by Brown et al. (2), it was found that three stool specimens which contained a virus subsequently identified as Ad40 produced a CPE upon primary isolation in HeLa cells. The isolates were able to be passaged only in Graham 293 cells.

During the course of studies undertaken to evaluate a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for enteric adenovirus diagnosis (9), it appeared that some of the enteric types could be isolated in

HEp-2 cells as well as in Graham 293 cells. This paper examines in detail the relative efficiency of enteric adenovirus isolation in each of these two cell lines. Viruses were typed as enteric or nonenteric by direct monoclonal antibody ELISA of stool samples and by analysis of DNA restriction endonuclease digests of virus isolates.

MATERIALS AND METHODS

Viruses and cells. Ad40 and Ad41 were obtained from the American Type Culture collection (ATCC), Rockville, Md. All other adenovirus serotypes were obtained from the Research Resources Branch, National Institutes of Health, Bethesda, Md., or from the ATCC. Virus isolation was done in two cell lines: Graham 293 cells and HEp-2 cells. Graham 293 cells, a continuous line of human embryonic kidney cells transformed with sheared Ad5 DNA, were obtained from the ATCC (catalog no. CRL1573) at passage 31 and used until passage 52. The cells were maintained in Eagle minimal essential medium plus 10% fetal bovine serum and passaged once a week at a subculture ratio of 1:3. HEp-2 cells were also obtained from the ATCC (catalog no. CCL23). They were received at passage 364 and used until passage 389. The HEp-2 cells were also maintained in Eagle minimal essential medium plus 10% fetal bovine serum and passaged twice a week at a subculture ratio of 1:5.

Stool samples. Stool samples were obtained from children with gastroenteritis from four sources representing three geographical areas: Canada (Toronto and Winnipeg), England, and Thailand. The Toronto stools were provided by P. Blaskovic (Ontario Ministry of Health, Toronto). The Winnipeg stools came from G. Hammond (Cadham Provincial Laboratory, Winnipeg). The stools from Canada had been stored at −70°C before and after we received them. W. D. Cubitt provided the stools from England (Central Middlesex Hospital, London). These stools were stored for several weeks at 4°C before we received them and at −70°C after we received them. The stools from Thailand were provided by P. Echeverria (Armed Forces Research Institute of Medical Sciences, Bangkok) and stored at −70°C before and after receipt.

Virus isolation. Ten percent suspensions of each raw stool were made by using sterile 0.01 M phosphate-buffered

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saline, pH 7.0, with 400 U of penicillin per ml and 400 μg of streptomycin per ml, and the suspensions were clarified by low-speed centrifugation at 2,000 × g for 10 min. The supernatant fluids were used for inoculation of cell cultures. The medium from confluent cell monolayers was removed, and 0.1 ml of virus or stool suspension was added to each well of a 24-well culture plate. Stool suspensions were adsorbed for 1 h at room temperature (20 to 22°C) on a rocking platform. The inoculum was removed, and Eagle minimal essential medium plus 2% fetal bovine serum was added. Samples were called positive when a 4+ CPE was reached. If a culture did not become positive by the time the control cell cultures deteriorated, the cells plus culture fluid were removed from the plate wells, frozen once at −70°C, and reinoculated onto a new cell monolayer as before. The same procedure was used for passage of positive cultures. If the stool suspension was toxic to the cells or if the cells were contaminated, a 1:10 dilution of the original suspension was made and used as the inoculum.

Monoclonal antibody ELISAs. Stool samples were tested as 10% (wt/vol) suspensions in phosphate-buffered saline, pH 7.0, for adenovirus group antigen, Ad40, and Ad41 by ELISA. The ELISAs for the enteric adenovirus types used monoclonal antibodies to each type as previously described by us (9, 10). For detection of adenovirus group antigen, a monoclonal antibody to adenovirus hexon group antigen (4), obtained from the ATCC, was substituted in the ELISA.

DNA restriction endonuclease analysis. Viruses isolated were typed by analysis of restriction endonuclease digests. DNA was extracted from cultivated viruses by a modified Hirt procedure described by Brown et al. (3). Isolated DNA was treated with the restriction enzyme SmaI as recommended by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and analyzed by agarose slab gel electrophoresis. The resulting bands, stained with ethidium bromide, were examined for patterns indicative of Ad40 or Ad41 (16).

RESULTS

Samples from all of the provider sites except Thailand had been examined for adenovirus particles by EM by those who provided the samples. All of the stool samples were tested for adenovirus antigen by monoclonal antibody ELISAs for adenovirus group antigen, Ad40, and Ad41. Samples positive by any of the ELISAs and by EM, when done, were the 82 samples used to determine growth characteristics in HEp-2 and Graham 293 cells. Viruses from positive cultures (4+ CPE) were typed as enteric or nonenteric adenoviruses by analysis of DNA restriction endonuclease digests. In all cases, DNA typing agreed with monoclonal antibody typing.

The number of enteric and nonenteric adenovirus isolates obtained in the two cell lines used is given for each provider site in Table 1. It was found that many of the samples from all of the sites contained enteric adenoviruses that could be isolated in HEp-2 cells. This included 43% (9/21) of those typed as Ad40 and 40% (21/52) of those typed as Ad41. Thus, there were no apparent differences in the ability of either Ad40 or Ad41 to be isolated in HEp-2 cells.

The overall isolation rates for the three provider sites in each of the cell lines are summarized in Table 2. Although Graham 293 cells were more efficient for isolating enteric adenoviruses from clinical stool samples, 41% (30/73) could be isolated in HEp-2 cells. Also, three of nine nonenteric adenovirus types were isolated only in Graham 293 cells. To determine whether any of the enteric adenoviruses isolated

TABLE 1. Distribution of enteric and nonenteric adenovirus isolates

Provider site(s) and adenovirus type	No. of isolates cultured in:	
	Graham 293 cells	HEp-2 and Graham 293 cells
Canada (Toronto and Winnipeg)		
Ad40	10	8
Ad41	17	5
Nonenteric	2	3
England (London)		
Ad41	8	12
Nonenteric	1	3
Thailand (Bangkok)		
Ad40	2	1
Ad41	6	4

in HEp-2 cells could be passaged, 15 of the 30 positive samples were randomly selected and reinoculated onto HEp-2 cell monolayers. Of the 15 samples, 9 gave a 4+ CPE after passage. By comparison, all of the samples could be passaged in 293 cells. Analysis of the bands obtained after SmaI digestion of viral DNA and agarose gel electrophoresis showed no detectable differences between the Ad40 or Ad41 isolates which grew in HEp-2 cells and those which grew only in Graham 293 cells.

DISCUSSION

In studies which have used specific immunoreagents for diagnosis, both enteric adenovirus types have clearly been shown to be major causes of gastroenteritis in children, perhaps second in importance only to rotaviruses among those viruses studied (15). Consequently, studies on the occurrence of enteric adenoviruses continue to be of interest. However, because of reports indicating that enteric adenovirus types are highly fastidious, many studies use or recommend the cultivation characteristics of adenoviruses in HEp-2, combined with EM or adenovirus group antigen tests, as the sole means to distinguish enteric adenoviruses from nonenteric adenoviruses (5, 11). The results we present demonstrate that many enteric adenovirus isolates go unrecognized if these criteria are used. Also, Graham 293 cells were more sensitive than HEp-2 cells for isolation of three of the nonenteric adenoviruses, which could result in false-positive identification of enteric adenoviruses by cell growth characteristics. A similar finding has also been reported for HeLa cells (2).

Isolation of enteric adenoviruses in HEp-2 cells was found not to be dependent on the geographical source of the specimens or the specific enteric adenovirus type. There

TABLE 2. Summary of adenovirus isolations from stools

Adenovirus type	No. of isolates cultured in:		% Cultured in HEp-2 cells (no. cultured/total)[a]
	Graham 293 cells	HEp-2 and Graham 293 cells	
Enteric	43	30	41 (30/73)
Nonenteric	3	6	67 (6/9)

[a] Of all 82 enteric and nonenteric adenovirus isolates, 36 (44%) were cultured in HEp-2 cells.

were no detectable differences in the bands obtained after *Sma*I digestion in the enteric adenoviruses which grew in HEp-2 cells and those which did not. Also, there was no correlation between the magnitudes of ELISA values in the original stool preparation and subsequent cell cultivation characteristics (data not shown). Thus, we have no definitive explanation as to why some enteric adenoviruses can be isolated in HEp-2 cells and others cannot. It is conceivable that the source of HEp-2 cells used and their passage level affect their susceptibility to infection with Ad40 and Ad41.

On the basis of our findings for the samples tested in this study, we conclude that (i) enteric adenoviruses can be frequently isolated from stool samples in HEp-2 cell cultures, and (ii) definitive, type-specific tests should be used to diagnose enteric adenovirus infections and to determine the role of enteric adenoviruses in epidemiological studies of viral gastroenteritis.

ACKNOWLEDGMENTS

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