

Isolation and Identification of Enteric Adenoviruses

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Thirty-four out of 64 faecal samples with adenovirus particles, as seen by electron microscopy, were found to contain adenovirus 40 or 41 by direct isolation and neutralization in Chang's conjunctival cells, mostly within one week. (Ad40 and 41 candidate viruses are serologically related.) 6 other adenovirus species were isolated; 6 samples gave equivocal results, and 18 were negative. A genus-specific ELISA with an anti-hexon coat yielded positive results in 40 out of 55 samples; the test failed to identify adenovirus antigen in 10 out of 17 specimens, which were found negative by culture. All of them were negative by immunofluorescence of inoculated Chang cell cultures. Hence the failures are probably due to insufficient amount of virus in the samples. The predominance of only two adenovirus species associated with gastroenteritis in children and the ease of cultivating and identifying them should help to elucidate their etiological significance.

Key words: adenovirus, enteric adenoviruses, adenoviruses 40/41, identification, ELISA, immunofluorescence

INTRODUCTION

The use of electron microscopy (EM) has focussed attention on various viruses as possible enteric pathogens in young children. Adenoviruses are secondary in frequency only to rotaviruses in faecal excretions [Brandt et al, 1979]. Most of the adenoviruses visualized by EM cannot be cultivated in cell cultures by conventional means; these will be referred to as "enteric adenoviruses" (EA) in this report. Johansson et al [1980] were able to recognize antigenically related adenoviruses by a type-specific ELISA in eight out of nine EM-positive faecal specimens. Kidd and Madeley [1981] passaged some of the EA in Chang's conjunctival cells with CPE; again most of them appeared to be

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identical by neutralization (SN) with an antiserum prepared against an enteric adenovirus strain. Hence, most of the EA appear to be related antigenically; they had been provisionally called adenovirus 38 (Ad38) candidate virus [De Jong et al, 1981]; the number had to be changed later. It turned out, however, that these fastidious adenovirus strains represent two clusters of strains which, although serologically closely related, exhibit great differences in their DNA structure [Wadell et al, 1980, in press]. Since it is difficult to distinguish the two variants by serological methods, they are being referred to as Ad40/41, with the knowledge that this complex includes (at least) two viruses. Even so, if Ad40/41 can be identified in cell cultures, it is a step forward to indicate the etiological role of EA for gastroenteritis in children, which is as yet unproven.

The possibility of growing at least some of the enteric adenoviruses in Chang's conjunctival cells prompted us to attempt a serological identification by SN directly from those faecal specimens, which contained adenovirus particles. Indeed, identification was achieved in a considerable proportion of specimens, whereas no growth was obtained in 28%. The reason for this failure might be quantitative or qualitative in nature. We attempted to answer this question by demonstrating genus-specific adenovirus antigen either by immunofluorescence of inoculated cell cultures or by an ELISA.

MATERIALS AND METHODS

Cell Cultures

Chang's conjunctival cells (Chang cells), obtained from Flow Laboratories, were grown with Leibovitz medium + 5% foetal bovine serum; the maintenance medium contained 2% foetal bovine serum. After inoculation of the culture tubes, they were incubated at 33°C [Kidd and Madeley, 1981]. HeLa cell cultures, strain Saarbrücken, were incubated at 37°C with Eagle's MEM + 4% lactalbumin hydrolysate + 2% calf serum as maintenance medium. In both types of cultures, the medium was changed twice weekly.

Faecal Samples

Faecal samples containing adenovirus particles by EM were obtained from 64 infants and small children with diarrhoea.

Electron Microscopy

The 10% stool suspensions in Eagle's MEM were prepared for electron microscopy either by ultracentrifugation with an airfuge (Beckman Instruments) with 50.000 rpm [Gelderblom et al, 1978] directly onto the grid or with the agar diffusion technique [Kelen et al, 1970, Baumeister et al, 1976].

After staining with 6% phosphotungstic acid, the preparations were ready for electron microscopy (Siemens Elmiscop 101 or Zeiss EM 9S2; voltage 80 or 60 KV, respectively). In addition to rotaviruses, coronaviruses, astroviruses, adenovirus particles could be visualized in the faecal samples and identified by their typical morphology; adenovirus-positive stool samples were sent from Muenster to Homburg by express mail.

Identification

A rabbit antiserum (R.90), prepared by immunization with a filtered fecal extract containing EA particles of Ad40 type, was kindly provided by Dr. Alistair H. Kidd, London. This serum had a homologous SN titer of 1:640. It neutralized Ad41 strains

with nearly the same titer, but did not neutralize human adenoviruses 1 to 39 in a 1:10 dilution.

Either 0.1 ml of R.90 diluted 1:20 or Hanks' BSS, respectively, was mixed with 0.3 ml of undiluted faecal suspension. After incubation for 1 hr at 37°C, 0.3 ml of both samples were inoculated into one Chang culture tube each. The cultures were observed for CPE at least every other day until 12 days. Identification as Ad40/41 was considered positive, if the CPE in the culture inoculated with virus-serum mixture remained negative or appeared ≥ 2 days after that in the virus control tube. One HeLa culture tube was inoculated with each specimen and observed for 30 days.

Passage

Cell cultures with maximal CPE were frozen and thawed twice. Then 0.1 ml was inoculated into two new culture tubes containing 1 ml of medium. Cultures were observed for 10–12 days (Chang) or for at least 25 days (HeLa). Blind passages were not made.

Immunofluorescence

Indirect immunofluorescence to demonstrate infected cells containing the genus-specific (hexon) antigen was performed in Chang cells in Leighton tubes, which were harvested 3 days after inoculation and incubation at 37°C [Kron et al, 1974].

Enzyme-linked Immunosorbent Assay (ELISA)

A genus-specific ELISA was developed, similar to that described by Harmon et al [1979]. The coating antibody was prepared from a rabbit antiserum after immunization with purified Ad5 hexon. Hexon-specific antibodies were obtained by affinity chromatography on activated CH sepharose columns, coupled with Ad5 hexon. The immunoglobulin obtained was diluted to contain 40 ng/ml protein in carbonate buffer of pH 9.5 and adsorbed to cobalt-sterilized "immulon" plates (Dynatech M 129 B) over night at 4°C. After washing the plates three times in PBS + 0.05% Tween 20, faecal specimens, clarified by low-speed centrifugation, diluted 1:2, 1:16, and 1:128 in PBS-Tween, were inoculated into each of two cups and incubated for 1 hr at 37°C. After another washing, anti-hexon IgG from the same rabbit, labelled with peroxidase, diluted 1:500, was inoculated and incubated for another 1 hr at 37°C. After final washing, a freshly prepared solution of 10 mg orthophenylene-diamine (Merck) in 10 ml phosphate buffer (pH 6.0, Sørensen), containing 10 μ l perhydrol (Merck), was added. The enzyme reaction was stopped after 30 min at room temperature by addition of 2 N H₂SO₄; the extinctions were measured at 490 nm in a Micro ELISA Auto Reader MR 580 (Dynatech). The volume of all reagents was 0.05 ml. Samples with an extinction of at least 0.3 OD were considered positive, while negative samples had values of ≤ 0.15 .

RESULTS

Virus Identification

From the 64 faecal specimens positive by EM, 6 common adenovirus species were grown in both types of cell cultures (Table I). In 34 samples, Ad40/41 was identified by culture and SN in Chang cells. The resulting CPE involved almost the whole cell sheet in about half of the samples, whereas 25 to 75% involvement was seen in the other half. In 29 of the 34 samples, identification by SN was definite in the first attempt. In the other 5 samples, the delay of CPE by Ad40 antiserum appeared insufficient in the first

TABLE I. Virus isolation From EM-positive Faecal Specimens

Ad40/41	34
Other adenoviruses	6 ^a
No growth	18
Contamination or equivocal	6
Totals	64

^aAd1, 2, 2, 2, 3, 5.

passage. On repetition with a diminished virus concentration or with material from the first Chang passage, Ad40/41 was also identified in these cases. Furthermore, the virus "breaking through" the SN was neutralized by R.90, which excluded the possibility of a mixture of two adenoviruses. Other adenovirus antisera or normal rabbit serum did not inhibit the CPE produced by Ad40/41-containing samples. Eighteen specimens showed no CPE in either Chang or HeLa cell cultures. In 6 cases' the results were equivocal because of bacterial contamination or of overgrowth by enteroviruses.

Time of CPE

The time until the CPE appeared in Chang cells inoculated with Ad40/41-containing faecal specimens never exceeded 5 days after inoculation (Table II); the CPE was often already evident after 1–2 days. Since SN was read 2–3 days after the controls showed CPE, the whole isolation + identification procedure was mostly terminated within one week after inoculation. Five of the 6 samples with other adenoviruses produced a CPE in Chang and HeLa cell cultures between 2 and 9 days after inoculation; the last sample was slower.

Virus Passage in Chang and HeLa Cell Cultures

Of 32 Chang-positive materials 28 showed a CPE in the second passage in Chang cells. It was often either weaker or slower to appear than after inoculation of the faecal material. Further passage attempts in Chang cells were not always successful. However, one strain was passaged more than 10 times in Chang cells with a constant CPE; the virus from the 10th passage was found serologically unchanged.

In HeLa cell cultures, 15 of the faecal specimens with Ad40/41 (positive in Chang cells) produced an adenovirus-like CPE. Only 4 samples, however, showed a CPE in the second passage, and in no case in the third passage.

Comparison With Immunofluorescence

All available specimens negative by culture and eight positive specimens were tested by indirect immunofluorescence. As shown in Table III, all negative specimens were also negative by immunofluorescence, while most of the positives also showed fluorescent cells.

Comparison With ELISA

Twenty-six of 28 samples containing Ad40/41 and 7 from 17 negative samples were positive in ELISA (Table IV). In addition, adenovirus antigen could be found in 4 of 5 specimens equivocal by culture. A correlation of strong positive, weak positive, and negative specimens with the titer in our semiquantitative ELISA is evident from Table IV.

TABLE II. Time of CPE in Chang's Conjunctival Cells After Inoculating Fecal Samples Containing Ad40/41

First appearance of CPE (days)	Number of samples
≤2	18
3	10
4	4
5	2
>5	0
Totals	34

TABLE III. Indirect Immunofluorescence and Isolation of Ad40/41 in Chang Cells

Immunofluorescence	Cell culture	
	Positive	Negative
Positive	6	0
Negative	2	18

TABLE IV. ELISA and Isolation in Chang Cells

ELISA	Adenovirus Isolation					Totals
	Ad40/41	Ad40/41	neg.	? ^b	oA ^c	
	++	+ ^a				
+++ (1:128)	9	4	1	2	0	16
++ (1:16)	7	5	4	0	2	18
+ (1:2)	0	1	2	2	1	6
Negative	0	2	10	1	2	15
Totals	16	12	17	5	5	55

^aCPE present 1-2 days after inoculation (++) , or later (+).

^bContamination or equivocal.

^cOther adenoviruses.

Age Distribution

The occurrence of Ad40/41, other adenoviruses, and negative samples in children of different age (Table V) showed no significant differences between the groups.

DISCUSSION

More than half of the faecal specimens containing adenovirus particles by EM were found to yield positive cultures in Chang cells, identifiable as Ad40/41. Isolation and identification were mostly performed within a week, which is remarkably quick for adenoviruses to be grown and identified. The rate of positive Chang cultures equals that reported by Kidd and Madeley [1981], who found 28 of 67 samples positive, including

TABLE V. Age Distribution of Children

Age (months)	Ad40/41	Negative	oA	Totals
≤3	12	5	2	19
4-12	10	7	0	17
13-60	9	4	3	16
>60	1	1	0	2
	32	17	5	54

one positive subculture in Chang cells. Of 25 of their isolates 24 were serologically related (= Ad40/41), and this identification had been done with the same antiserum, although not directly from the original stool. Johansson et al [1980] recognized EA in 8 of 9 specimens by a species-specific ELISA as identical among each other and different from adenoviruses 1 to 35. Hence, Ad40/41 are obviously the predominant species associated with gastroenteritis in children. The etiological role of EA, often suggested [Richmond et al, 1979], and their epidemiology may now be studied more specifically. An antiserum against Ad40, which broadly neutralizes Ad40 and Ad41 strains in Chang cells [De Jong et al, 1982], may be prepared against the Ad40 strain Dugan [De Jong et al, 1982], or alternatively by animal immunization with a filtered stool extract containing EA particles.

Since the passage of Ad40/41 strains in Chang cells is often difficult or impossible, our method of direct isolation and identification from fecal specimens appears to be suitable for routine diagnostic from children with gastroenteritis, even without previous EM. Chang cells are well suited for cultivating other human adenoviruses as well [Kidd et al, 1982]. Chang's conjunctival cells allow some multiplication of at least some strains, as shown by passage, by immunofluorescent cell counting (data not shown), and by inhibition experiments with BUdR [Kidd and Madeley, 1981]. Cell cultures of higher susceptibility are desirable. Possibly a better growth may be obtained in Graham 293 cells [Takiff et al, 1981] or in tertiary cynomolgus kidney cells [De Jong et al, 1981, 1982], but certainly not in human embryonic kidney cells which are superior for the isolation of most other human adenoviruses. Ad40/41 appear to undergo an abortive replication cycle in HEK cells, since they may show a CPE in them, but cannot be passaged [Gary et al, 1979, Kidd and Madeley, 1981].

The failure to grow virus in Chang cells from some of the samples may have different reasons:

1. Particles of a different adenovirus type, which cannot be cultivated and which do not containing genus-specific (hexon) antigen.
2. As above, but containing genus-specific antigen.
3. As above, abortive cycle in Chang cells with antigen-producing cells, but no growth with CPE.
4. Ad40/41 present, but in small quantity; EM is more sensitive than culture in Chang cells.
5. Particles not growing, due to coating with antibody.

The first possibility appears unlikely, as the genus-specific ELISA was positive in a number of samples negative by culture (Table IV). Moreover, hexon antigen has been

found by counterimmunoelectrophoresis in nearly all faecal specimens containing adenovirus particles in EM [Gary et al, 1979, Mankikar et al, 1979]. Coating of virus particles with antibody (possibility 5) was not observed by Gary et al [1979]. The third possibility is ruled out by our finding that immunofluorescence was about equal in sensitivity as culture (Table III).

Although the second possibility cannot be strictly excluded, it appears more likely that the negative specimens do contain Ad40/41, but in amounts insufficient to be cultivated in Chang cells or to produce fluorescing cells (possibility 4). Hopefully, a more sensitive cell culture or else the application of a species-specific ELISA [Johansson et al, 1980] may help to answer this question. The genus-specific ELISA applied in this study proved to be fairly sensitive (40 of 55 positive samples). Its specificity was proved by testing several faecal samples containing either rotavirus or no virus; all of them were negative.

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