Monoclonal Antibody Enzyme-Linked Immunosorbent Assay for Specific Identification and Typing of Subgroup F Adenoviruses

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Monoclonal antibody specific for subgroup F enteric adenoviruses (EAds) was prepared by fusing P3-NS1/Ag4-1 mouse myeloma cells with lymphocytes from BALB/c mice immunized with G1105, an adenovirus type 41 (Ad41) strain. Monoclonal 3F11/2H9, which specifically recognized Ad41, was successfully used as detector antibody in an enzyme-linked immunosorbent assay (ELISA). Additionally, previously prepared monoclonal 5D8/2C2 and 2H6/1E11, recognizing Ad40 plus Ad41 and Ad40 alone, respectively, were used to study stool and/or tissue culture specimens from 106 patients with adenovirus-positive gastroenteritis. By ELISA, 91 had EAds (22 were Ad40 and 69 were Ad41) and 15 had non-EAds. ELISA results were in concordance with restriction endonuclease results for 38 of 39 specimens, with dot blot data for 19 of 20 specimens, and with neutralization test results for 74 of 78 specimens. ELISA was at least 10-fold more sensitive than direct electron microscopy was for the detection of EAds in stool specimens.

Subgroup F enteric adenoviruses (EAds) apparently are second only to the rotaviruses as a viral cause of acute gastroenteritis in infants and young children (14). Antibodies to these viruses (adenovirus type 40 [Ad40] and Ad41) have been detected in approximately 50% of children between 6 to 8 years of age in Asia, Africa, Europe, and South America and with similar seropositive proportions in the different populations (7). EAds have been associated with outbreaks of gastroenteritis but are endemic in most countries and may be an important cause of morbidity in patients with necrotizing enterocolitis (1, 4, 5, 16). They have been associated with protracted diarrhea which may contribute to infant dehydration and malnutrition in developing countries (14). We previously reported the development of monoclonal antibodies which react with Ad40 alone and with both Ad40 and Ad41 (12). In the present study we describe the development of a monoclonal antibody specifically reacting with Ad41 only and also report the specific identification and typing by enzyme-linked immunosorbent assay (ELISA) of both Ad40 and Ad41 in stool suspensions, as well as their corresponding tissue culture isolates. The sensitivity of the ELISA for detecting EAds was also studied in comparison with that of direct electron microscopy (EM).

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MATERIALS AND METHODS

Specimens. Fecal specimens were obtained from children with acute gastroenteritis who were living in metropolitan Washington, D.C., and in Johannesburg, Republic of South Africa. Adenoviruses were diagnosed by electron microscopy, and cultivated viruses were grown by at least one passage in cell line HEK-293.

EM. EM was performed essentially as previously described (2). Briefly, diagnostic EM grids were routinely prepared by mixing an approximately 10% (vol/vol) stool suspension in deionized water with an equal volume of 1.5 to 3% sodium phosphotungstate, dropping this mixture on

Formvar-coated EM grids, and blotting them dry with filter paper after 2 min. All grids were read at $\times 36,000$ to $\times 60,000$ magnification.

Neutralization test. Neutralization testing was done in cell line HEK-293 with rabbit hyperimmune sera, a 1:640 dilution of which completely neutralized both prototype Ad40 Dugan and Ad41 Tak without reacting with various other adenovirus serotypes (1, 7).

Restriction endonuclease analysis. Fecal suspensions were extracted with Genetron 113 fluorocarbon, and the DNA was extracted by standard methods. The DNA was digested as recommended by the manufacturer with the following enzymes: *Pst*I and *Xho*I (Boehringer Mannheim Biochemicals) and *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, and *Sma*I (Bethesda Research Laboratories, Inc.). The adenoviruses were classified by restriction pattern as belonging to Ad40 (Dugan- and Hovi-X-like *Sma*I profiles), Ad41 (Tak-like *Sma*I profile), or adenovirus types other than Ad40 or Ad41 (8, 11).

Dot blot hybridization methods. Fecal samples were denatured and applied to nitrocellulose filters, and hybridization was attempted with a nick-translated probe made from a purified *Bgl*II D subfragment of the *Eco*RI B fragment of Ad41 G1105. The dot blot filters were then air dried and autoradiographed. Readily discernible EAd dots were graded qualitatively from + to +++ (strongest); doubtful or weakly positive dots were designated $\pm$ (8, 11).

Hybridoma production. Hybridoma cell lines were prepared by the fusion of mouse myeloma cells (P3-NS1/Ag4-1) with lymphocytes from female BALB/c mice immunized with Ad41 G1105 by a standard technique (9). This virus was partially purified by fluorocarbon extraction before immunization. Hybridomas were screened for antibody production by the ELISA (12). Selected hybridomas were cloned by limiting dilution with a thymocyte feeder layer. Monoclonal 3F11/2H9 reacted specifically with Ad41 Tak and Ad41 G1105 but not with representative strains of established prototype subgroups A through E (Table 1). Monoclonal 5D8/2C2 and 2H6/1E11 were obtained from the mice immunized with Ad40 G2297 (12).

ELISA. ELISA was performed by using microdilution

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TABLE 1. Reactivity of three hybridomas with selected strains of adenoviruses in the ELISA

Antigen	Subgroup	OD with indicated hybridoma fluid ^a		
		3F11/2H9	5D8/2C2 ^b	2H6/1E11 ^b
Ad12	A	1.5	-2	-6
Ad18	A	1	1	-7
Ad31	A	1	2	-8
Ad3	B	2.5	-6	-9
Ad7	B	1	-3	-8
Ad1	C	3.5	-4	-10
Ad2	C	2.5	-7	-8
Ad5	C	1.5	0	-7
Ad6	C	3	-2	0
Ad15	D	3	0	-1
Ad8	D	1	-4	-1
Ad4	E	0	-2	-9
Ad40 Dugan	F	1	97	88
Ad40 G22297	F	2.5	99	92
Ad41 Tak	F	28.5	121	2
Ad41 G1105	F	56.5	125	-2

^a OD blank = 0; OD $\geq$ 20 was considered positive. Actual readings have been multiplied by 100.

^b See reference 12.

plates precoated with hyperimmune rabbit antiserum to EAd (serum kindly provided by H. Takiff and S. Straus, National Institutes of Health) as previously described (10). The plates were washed with phosphate-buffered saline containing 0.05% Tween 20, and 100 μ l (1:2 dilution) of an approximately 5% (vol/vol) virus-containing stool suspension or tissue culture isolate was added and incubated for 1 h at 37°C. The plates were washed with phosphate-buffered saline-Tween 20 and incubated with 100 μ l (1:2 dilution) of the hybridoma supernatant for 1 h at 37°C. The plates were again washed with phosphate-buffered saline-Tween 20 and incubated for 1 h at 37°C with 100 μ l of goat anti-mouse immunoglobulins M and G (heavy and light chains) conjugated to alkaline phosphatase, with *p*-nitrophenyl phosphate used as a substrate. Endpoint optical density (OD) values were then measured at 400 nm. Zero-density calibrations were based on wells incubated with uninoculated tissue culture cell line 293 in Eagle minimal essential medium or wells with virus-negative stool in phosphate-buffered saline. ELISA readings were standardized by multiplying actual readings by 100. The specimens with ODs of $\geq$ 20 ($\geq$ 0.2 $\times$ 100) were considered positive. All ELISAs were done in duplicate wells, and all tests were performed at least twice. Monoclonal 5D8/2C2 was used as the initial detector antibody. Isolates which were positive for an EAd by the ELISA with this monoclonal were further determined to be either Ad40 or Ad41 by using monoclonal 2H6/1E11 and 3F11/2H9 as detector antibodies.

Characterization of antigen. The molecular identity of the antigen to which monoclonal 3F11/2H9 is directed was detected by the Western blot enzyme immunobinding procedure (12).

RESULTS

Specificity of monoclonal antibodies. We tested 48 stool suspensions and their corresponding tissue culture isolates, as well as 58 additional tissue culture isolates. In total, 106 cell culture isolates of adenovirus from gastroenteritis patients were tested by the ELISA with each of the three

TABLE 2. Identification of adenoviruses by various methods

Virus type	No. positive/no. tested by:		
	ELISA	Restriction endonuclease	Dot blot
EAd	91/106 ^a	38/39 ^b	14/15 ^c
Non-EAd	15/106	0/0	5/5

^a 22 Ad40 and 69 Ad41.

^b 9 Ad40 and 29 Ad41.

^c One specimen was equivocal.

monoclonal antibodies (Table 2). The ELISA detected virus readily in stool suspensions (OD range, 24.5 to 96.5) and detected virus in 42 of 48 tissue culture isolates (OD range, 23 to 120.2); 6 EAds failed to grow in cell line 293. Monoclonal 5D8/2C2 recognized 91 EAds and excluded 15 other adenoviruses. Monoclonal 2H6/1E11 (anti-Ad40) and 3F11/2H9 (anti-Ad41) detected 22 Ad40 and 69 Ad41 viruses in the 91 specimens with EAds, with no discrepancies. Of 39 ELISA-EAd-positive specimens tested by restriction endonuclease analysis, 38 gave typing results consisting with those of ELISA (9 Ad40 and 29 Ad41). The one discrepant result was for a specimen that was EAd positive by ELISA both as a fecal suspension and as a first-passage culture isolate in cell line HEK-293. However, the DNA was not cut by *Eco*RI or *Kpn*I in a pattern consistent with EAd DNAs and gave a doubtful ($\pm$) result by dot blot hybridization; the isolate was nonreactive by ELISA at passage 4 in cell line HEK-293. The virus was identified by neutralization as a non-EAd at passage 6 in the HEK-293 cell line, suggesting that an EAd and a non-EAd were present in the original specimen.

For 74 of 78 EAd-positive specimens, the results of the ELISA and the neutralization test were in agreement. For the four discrepant specimens (two Ad40 and two Ad41) in the ELISA of stools (one also rated as + and one found to be $\pm$ by EAd hybridot of stool), the corresponding tissue culture isolates of later passages of cell line HEK-293 were nonreactive in EAd-specific ELISA and non-EAd by neutralization.

Sensitivity of monoclonal antibodies. Selected stool specimens (two Ad40 and two Ad41) and their corresponding tissue culture isolates were studied in serial twofold dilutions by ELISA with the monoclonal 5D8/2C2. The ELISA detected EAd at dilutions 8- to 16-fold higher in stool suspensions than in tissue cultures of three specimens (Table 3). These four stool specimens were further studied by ELISA and EM with serial dilutions to determine the highest dilution in which adenovirus could be detected. For these

TABLE 3. Sensitivities of ELISA and direct EM for detection of EAds

Specimen no.	Reciprocal of highest specimen dilution found positive by:		
	EM of stool ^a	ELISA ^b	
		Stool	Tissue culture
1	100	2,048	128
2	100	1,024	128
3	10	512	64
4	<10	64	128

^a Tenfold dilution series used; no more than two virions were seen per five-min viewing of the endpoint stool specimen.

^b Twofold dilution series used.

specimens, ELISA was from 10- to 64-fold more sensitive than direct EM (Table 3).

Characterization of antigen. The monoclonal 3F11/2H9 reacted with the protein of a molecular size of approximately 17 kilodaltons in the Western blot immunobinding procedure. This monoclonal produced immunoglobulin G.

DISCUSSION

Identification and typing of Ad40 and Ad41 in stool specimens and in tissue cultures were done by ELISA with EAd-specific monoclonal antibodies. The ELISA results were compared with those obtained by restriction endonuclease analysis, dot blot hybridization tests, and the neutralization test. The two former tests provided the best comparison, as the results are based on the DNA genome homologies (8, 15). Restriction endonuclease enzyme analysis detects quantities of DNA in the nanogram range, whereas dot blot hybridization is capable of detecting homologous DNA in picogram quantities (8, 11, 13). The ELISA showed positive reactivity for all stool specimens which were identified by dot blot, although this does not prove comparable sensitivity. The dot blot hybridization and the neutralization tests used in this study cannot distinguish between Ad40 and Ad41.

Identification and typing of EAds by ELISA were in concordance with the dot blot hybridization and the restriction endonuclease analyses except for one specimen. For four stool suspensions which were reactive as EAds in ELISA, the corresponding late tissue culture passages were nonreactive in ELISA and were non-EAd by the neutralization test. This probably was due to the presence of mixed infection, with an EAd and a non-EAd in the original stool specimen, and an overgrowth of the non-EAd during cell culture passage. Serial passages of such mixed virus infection results in selection of non-EAds over EAds (3). One of the four stool specimens also did not show the expected correlation with restriction endonuclease analysis and gave equivocal results with dot blot hybridization, again probably due to a mixture of viruses present in the stool specimen. The equivocal ($\pm$) dot blot may also have been due to adenovirus that contains DNA segments homologous to the Ad41 Bg/IIID probe (13).

The sensitivity of the ELISA was further studied with respect to the visualization of virus by EM. ELISA was 10- to 64-fold more sensitive than was direct EM, which suggests that the ELISA probably detects concentrations of at least 10^5 adenovirions per ml. The ELISA generally detected 8- to 16-fold more virus in stool specimens than with the corresponding tissue culture isolates, indicating that stool specimens contain virus in concentrations far greater than those in cell culture.

Very recently, a different monoclonal ELISA for the detection of EAds was reported to be better than 95% sensitive compared with the isolation of virus in the 293 cell line and better than 95% specific as determined by analysis of the adenovirus genome profile (6).

Other techniques used for specific detection of subgroup F EAds require extraction of the antigen from the stool and use of a labeled probe (usually radioactive material), although a nonradioactive probe (peroxidase) has recently been described (10). Dot blot hybridization after removal of protein and lipid from specimens has been reported to increase sensitivity, but so far no comparisons with ELISA involving type-specific monoclonal antibodies have been reported. By antigenic analysis, monoclonal 3F11/2H9 reacted with the

single antigen of 17 kilodaltons, which seems to be polypeptide VII. The other two monoclonal antibodies, 5D8/2C2 and 2H6/1E11, also reacted with the single antigen of 17 kilodaltons (12). These three monoclonal antibodies seem to be directed against different antigenic determinants of the same 17-kilodalton protein and show different reactions with Ad40 and Ad41 in an ELISA.

This study demonstrates that the distinct types of subgroup F adenovirus which were originally differentiated by DNA analysis can be differentiated easily with monoclonal antibodies. The ELISA with EAd-specific monoclonal antibodies could be used for specific and sensitive detection of subgroup F EAds in fecal specimens.

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