

Four-Layer Radioimmunoassay for Detection of Adenovirus in Stool

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A four-layer antispecies radioimmunoassay (RIA) was developed for the detection of adenovirus in stool specimens. Polystyrene beads were used as the solid phase, anti-adenovirus guinea pig immunoglobulin (1 μg per bead) was used as the primary antibody, anti-adenovirus rabbit immunoglobulin (16 $\mu\text{g}/\text{ml}$) was used as the secondary antibody, and ¹²⁵I-labeled sheep anti-rabbit immunoglobulin was used as the indicator antibody. A highly purified, crystallized adenovirus type 2 hexon antigen was used as the immunizing antigen for the production of hyperimmune sera. The sensitivity of the test was 1 ng of hexon protein per ml. Each of the 13 stool specimens positive for adenovirus by electron microscopy was positive for adenovirus by the RIA. Of 200 nonconcentrated stool specimens negative by electron microscopy, 14 additional specimens were positive by the RIA, increasing the detection rate from 6% by electron microscopy to 13% by the RIA. A confirmatory test was done on the RIA-positive, electron microscopy-negative specimens, and the test indicated a true specific result with each specimen. A confirmatory test was also done on each specimen with a low positive counts per minute value. The specificity of the RIA was further demonstrated by the fact that a positive result was found with only 3 of 295 specimens positive by the rotavirus RIA. In two of these three specimens, adenovirus and rotavirus were also detected simultaneously by electron microscopy, and the third specimen was from a patient with serological evidence for a dual infection. The adenovirus and rotavirus RIAs are now used in a routine diagnostic laboratory, and in the 307 stool specimens tested during the first 5 months, the positive rate was 32% for rotavirus and 9.5% for adenovirus.

By electron microscopy (EM) (6), enzyme immunoassay (G. Wadell, M. Johansson, A. H. Kidd, and C. R. Madeley, Abstracts, Papers, and Posters, Eur. Assoc. Against Virus Dis. Munich, abstr. no. 16, 1979), or immunoelectro-osmophoresis (2), noncultivable adenoviruses have been detected in stool specimens collected from children with acute gastroenteritis. Recently, a four-layer antispecies radioimmunoassay (RIA) was developed in our laboratory for the detection of human rotavirus in stool specimens (7). In this test, polystyrene beads were used as the solid phase, anti-rotavirus guinea pig immunoglobulin G was used as the primary antibody, anti-rotavirus rabbit immunoglobulin G was used as the secondary antibody, and ¹²⁵I-labeled sheep anti-rabbit immunoglobulin was used as the indicator antibody.

In the present report, details of a similar RIA technique developed for the detection of adenovirus in stool specimens are presented. The technique developed has been used in a routine diagnostic laboratory, and the results of the first 5 months are also reported.

MATERIALS AND METHODS

Stool specimens. The total number of stool specimens tested in this study was 815. This material included 625 specimens from a routine diagnostic laboratory and from a rotavirus study (7). Specimens had been tested previously by the rotavirus RIA and by EM and stored at -20°C for up to 2 years. These specimens were from hospitalized patients with acute gastroenteritis, and they were mainly from children but included specimens from army trainees and other adults. The remaining 190 specimens had been collected during a prospective study of infantile gastroenteritis (T. Vesikari, M. Mäki, H. Sarkkinen, P. Arstila, and P. Halonen, Arch. Dis. Child., in press).

A 10% suspension of the specimens was made in phosphate-buffered saline (pH 7.2) and clarified by low-speed centrifugation. The supernatant fluid was used directly to make EM grids, and for the RIA it was diluted 1:2 in the RIA diluent to make the final dilution 1:20.

Adenovirus antigen. Adenovirus type 2 hexon antigen was used as the positive control antigen in the RIA test and as the immunizing antigen for the production of hyperimmune adenovirus antiserum in rabbits and guinea pigs. The method used in the purification of the adenovirus type 2 hexon antigen has

been reported in detail elsewhere (4). Briefly, adenovirus type 2 was grown in spinner cultures of KB cells. Virions and soluble antigens were harvested, and the fraction containing the soluble antigens was separated by diethylaminoethyl-cellulose. Hexons were further purified by repeated crystallization. This method yielded a hexon preparation giving a single band in polyacrylamide gel electrophoresis. The protein concentration of the preparation used in the present study was 15.0 mg/ml (5).

Immunization of animals. Two rabbits were immunized intradermally four times at 3-week intervals with 300 μ g of virus solution mixed with incomplete Freund adjuvant. The dose was divided into two equal volumes and was injected in two sites on the back of the rabbits. A booster dose of 75 μ g was given intravenously 3 weeks after the fourth intracutaneous dose, and the animals were exsanguinated 5 days later.

Five guinea pigs were immunized in a similar way except that about 150 μ g of antigen was used in each immunization, and the booster dose was given intramuscularly. The pool of anti-adenovirus serum from two rabbits had an RIA adenovirus antibody titer of 1:500,000, and the pool of serum from the five guinea pigs had a titer of 1:2,000,000.

RIA reagents. Rabbit and guinea pig immunoglobulin fractions were prepared from the hyperimmune adenovirus antisera and from the preimmunization sera by precipitation of serum with an 18% (wt/vol) final concentration of sodium sulfate, followed by chromatography on a Sephadex G-25 column.

The label used was iodinated sheep anti-rabbit immunoglobulin G purified by immunosorbent chromatography. Iodination was done by a chloramine-T procedure (1). The iodinated antibodies were separated from unreacted iodine by chromatography on a Sephadex G-25 column. Fractions containing the radiolabeled protein were pooled, diluted with an equal volume of phosphate-buffered saline (pH 7.2) containing 42% glycerol, 2% bovine serum albumin fraction V (Armour Pharmaceutical Company, Ltd., Eastbourne, England), and 0.2% NaN₃, and stored at 4°C until used. The specific activity of the labeled antibody was approximately 11 μ Ci/ μ g.

Diluent for the RIA test was phosphate-buffered saline (pH 7.2) containing 20% inactivated normal sheep serum, 2.0% Tween 20, and 0.1% NaN₃.

Polystyrene beads (6.4 mm in diameter; Precision Plastic Ball Co., Chicago, Ill.) were coated with anti-adenovirus guinea pig immunoglobulin by incubating the untreated beads overnight at room temperature in a dilution containing 5 μ g of immunoglobulin per ml (1 μ g per bead). Carbonate buffer (pH 9.6) was used as the diluent (8). Beads were stored in this antibody solution for 1 to 2 weeks.

RIA procedure. Stool specimens in volumes of 200 μ l, either in a single 1:20 dilution or in 10-fold dilutions beginning at 1:20, were pipetted into disposable 5-ml polystyrene tubes, and a polystyrene bead with adsorbed antibody was then added to each tube. After incubation at 37°C for 1 h, if not indicated otherwise, the stool specimens were aspirated, and the beads were washed twice with 5 ml of tap water. A 200- μ l volume of anti-adenovirus rabbit immunoglobulin (16 μ g/ml) was added to each tube, and the beads were

incubated at 37°C for 1 h. The beads were then washed as described above, and a 200- μ l volume of ¹²⁵I-labeled sheep anti-rabbit antibody was added to each tube. After another 1-h incubation at 37°C, the beads were washed, placed into clean tubes, and counted in an LKB 1280 gamma-counter with automatic deduction of equipment background. Buffer blanks and titration of a positive control antigen were included in each assay.

The assay was further standardized by diluting the iodinated anti-rabbit immunoglobulin G to a concentration that gave 5,000 cpm bound (5,000 active cpm) when a 200- μ l volume of the label was incubated with a bead adsorbed with 2 μ g of purified rabbit immunoglobulin G. The dilution of label used usually contained 100,000 to 130,000 cpm per 200 μ l. The test was considered positive if the bound radioactivity of the specimen was two times (usually 500 cpm) that of the buffer blanks.

RIA confirmatory test. A confirmatory test was performed on each specimen considered positive in the RIA test, but with only 500 to 1,000 cpm, and on the highly RIA-positive (more than 1,000 cpm), EM-negative specimens. The test was done in the same way as described above except that anti-adenovirus guinea pig serum was added before the secondary antibody. The test was performed as follows: 200- μ l volumes of the 1:20 diluted specimens were pipetted into three tubes, and a bead adsorbed with anti-adenovirus guinea pig immunoglobulin was added to each tube and incubated for 1 h at 37°C. After washing, a 200- μ l volume of anti-adenovirus guinea pig serum in a dilution of 1:500 was added to the first tube, the same volume of normal, preimmunization guinea pig serum in a dilution of 1:500 was added to the second tube, and the same volume of diluent was added to the third tube. After incubation at 37°C for 1 h, 200 μ l of anti-adenovirus rabbit immunoglobulin (32 μ g/ml) was added to each of the three tubes without removal of the previously added 200 μ l of guinea pig serum dilution, giving a total volume of 400 μ l. The tubes were then incubated for 1 h at 37°C, and the rest of the test was performed as in the assay proper.

The confirmatory test indicated a specific positive result if 50% or greater inhibition of bound radioactivity was observed with beads incubated with the guinea pig anti-adenovirus serum, when compared with the beads incubated with preimmunization serum or with the diluent.

EM. Stool specimens in a 10% suspension were put on carbon-coated Formvar grids (300 mesh), negatively stained with 2% phosphotungstic acid (pH 6.5), and examined with a JEM 100 electron microscope at an instrument magnification of 30,000. At least five grid squares of fecal material were viewed from each specimen.

RESULTS

The sensitivity of the test was about 1 ng per ml of the purified viral protein, as indicated in Fig. 1. It was increased fivefold to a level of 200 pg when the volume of antigen dilutions incubated with the beads was increased 10-fold (to 2,000 μ l) and the incubation at 37°C was pro-

longed to 16 h with continuous tilting of the tubes with the beads. However, this was observed only with the highly purified hexon antigen; a similar increase in the volume and incubation time of the stool specimens produced variable results in sensitivity, apparently due to proteolytic enzymes and possible microbial growth in the specimens. The 200- μ l volume and 1-h incubation of the antibody-coated beads with the 1:20 dilution or with serial dilutions of stool specimens were used in all later experiments.

Representative results obtained on stool specimens serially diluted 10-fold are shown in Table 1. Each of the four specimens positive for adenovirus by EM was positive by the RIA up to a dilution of 1:200,000. One of the six specimens negative for adenovirus by EM was positive by the RIA up to a dilution of 1:2,000, possibly

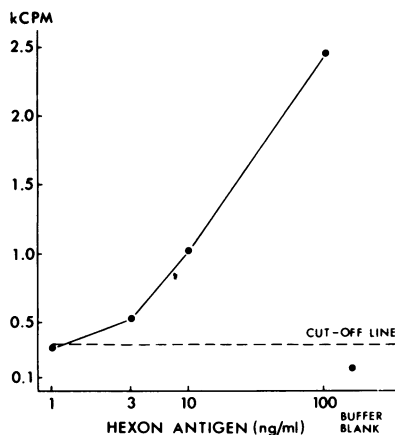


FIG. 1. Sensitivity curve of adenovirus antigen detection by the RIA. The counts per minute value of the buffer blanks is a mean of three beads, and each value in the curve is a mean of two beads. The cutoff line is two times the mean counts per minute value of the buffer blanks.

indicating a lesser amount of antigen present. Two of the six specimens were positive for rotavirus both by EM and by the RIA and negative for adenovirus by the RIA. The counts per minute values of the five positive specimens (no. 1, 2, 3, 4, 7) at a 1:20 dilution varied from about 1,800 to 3,000, whereas they were 170 or less in the same dilutions of the negative specimens. A "prozone" effect was frequently observed with positive specimens, the counts per minute value being lower in the 1:20 dilution than in the 1:200 or 1:2,000 dilution. This may be a true prozone effect or it may be caused by proteolytic enzymes or microbial growth in lower dilutions of the specimens. However, no specimen was negative in the 1:20 dilution and positive in higher dilutions. Usually the counts per minute value of the positive specimens in a 1:20 dilution was 2,000 or greater.

Each of the specimens positive by EM was also positive by the RIA, but of 200 specimens negative by EM, 14 were positive by the RIA, increasing the positive rate of 6% (13/213) by EM to 13% (27/213) by the RIA. A confirmatory test was done on each EM-negative, RIA-positive specimen, and for each of these specimens the positive result by the RIA test was shown to be specific.

A confirmatory test was also done on each specimen with a counts per minute value between 500 and 1,000. A nonspecific reaction was found in about one-third of these specimens.

Convalescent-phase stool specimens taken 4 weeks after discharge from the hospital were available from 19 patients initially positive by the adenovirus RIA. Each of the convalescent-phase stools was negative by the RIA.

The specificity of the adenovirus antigen RIA was further demonstrated by the fact that a positive result was found in only 3 of 295 specimens positive by the rotavirus RIA. In two of these three specimens, adenoviruses and rotavi-

TABLE 1. Representative results of adenovirus antigen detection by the RIA of eight stool specimens from children hospitalized with acute gastroenteritis

Specimen no.	EM results	Rotavirus RIA results	cpm at specimen dilution:				
			1:20	1:200	1:2,000	1:20,000	1:200,000
1	Adenovirus +	-	3,253	3,530	3,275	1,713	493
2	Adenovirus +	-	1,797	3,101	3,367	2,575	874
3	Adenovirus +	-	3,066	3,237	3,128	3,017	1,259
4	Adenovirus +	-	3,313	3,153	3,146	1,853	678
5	Negative	-	171	117	120	NT ^a	NT
6	Negative	-	131	149	156	NT	NT
7	Negative	-	2,369	1,397	772	223	155
8	Negative	-	157	163	170	NT	NT
9	Rotavirus +	+	149	158	134	NT	NT
10	Rotavirus +	+	129	141	159	NT	NT

^a NT, Not tested.

ruses were simultaneously detected by EM, whereas the third specimen was from a patient with an increase both in adenovirus and rotavirus complement fixation antibody titers.

The adenovirus and rotavirus antigen RIAs have been routinely used in a diagnostic laboratory since January 1979. The daily variations in the tests have been small even though several technicians and students not specifically trained in radioactive work have done the tests. The only change noticed has been a decrease in the sensitivity of the test to 10 ng of viral protein per ml, compared with the 1-ng sensitivity under the most optimal conditions during the developmental work in the research laboratory. During 5 months, 307 specimens were tested. The positive rate was 32% for the rotavirus RIA and 9.5% for the adenovirus RIA.

DISCUSSION

The results of the present study indicate that the four-layer RIA is a highly sensitive and specific method for the detection of adenovirus in stool specimens. Two times more adenovirus-positive specimens were detected by the RIA than by EM. The positive rate in the adenovirus RIA was 9.5% in unselected stool specimens sent to a diagnostic laboratory from hospitalized patients with acute gastroenteritis. This compares well with the 5% figure reported earlier in materials tested by EM (6).

The comparison of EM and the RIA was done in the present study on noncentrifuged specimens diluted 1:10 for EM and 1:20 for the RIA. Concentration of the stool specimens by ultracentrifugation, which is a standard procedure in many laboratories, may increase the sensitivity of EM but not necessarily that of the RIA, which also detects the soluble viral antigens in the specimen. With an RIA sensitivity of 1 to 10 ng of viral protein per ml and antigen titers of 1:200,000 or more in some stool specimens, the concentration of adenovirus hexon proteins in these specimens must be approximately 1 mg per g of stool.

Apparently all cultivatable and noncultivable enteric adenoviruses can be detected in stools by the anti-adenovirus hyperimmune sera used in this study. With the method of Johansson et

al. (3) for the preparation of type-specific adenovirus antisera, the test may be modified for the type-specific assay of enteric adenoviruses.

The detection of adenovirus or adenovirus proteins in stool specimens may not always be associated with the etiology of the disease. With a highly sensitive technique such as the RIA it may be possible to detect adenovirus which has been secreted from the respiratory tract or adenovirus which has been reactivated during an infection caused by some other agent. Further studies will be required to elucidate the significance of adenovirus protein excretion in stools as evidence for the etiology of the disease.

The high sensitivity of the RIA has raised hopes for the detection of adenovirus in other clinical materials, such as nose and throat secretions. Preliminary findings indicate that adenovirus antigen can be detected by the RIA in at least some respiratory specimens positive by adenovirus isolation.

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LITERATURE CITED

- Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (London)* 194:495-496.
- Jacobsson, P. Å., M. E. Johansson, and G. Wadell. 1979. Identification of an enteric adenovirus by immunoelectro-osmophoresis (IEOP) technique. *J. Med. Virol.* 3:307-312.
- Johansson, M. E., G. Wadell, P. Å. Jacobsson, and L. Svensson. 1979. Preparation of specific antisera against adenoviruses by affinity-bead-immunization (ABI). *J. Immunol. Methods* 26:141-149.
- Jörnvall, H., V. Pettersson, and L. Philipson. 1974. Studies of adenovirus type-2 hexon protein. *Eur. J. Biochem.* 48:179-192.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Middleton, P. J., M. T. Szymanski, and M. Petric. 1977. Viruses associated with acute gastroenteritis in young children. *Am. J. Dis. Child.* 131:733-737.
- Sarkkinen, H. K., P. E. Halonen, and P. P. Arstila. 1979. Comparison of four-layer radioimmunoassay and electron microscopy for detection of human rotavirus. *J. Med. Virol.* 4:255-260.
- Voller, A. 1976. Enzyme immunoassays for parasitic diseases. *Trans. R. Soc. Trop. Med. Hyg.* 70:98-106.