JOURNAL OF CLINICAL MICROBIOLOGY, July 1980, p. 95-100
0095-1137/80/05-0095/06$02.00/0
Vol. 12, No. 1

Direct Identification of Enteric Adenovirus, a Candidate New Serotype, Associated with Infantile Gastroenteritis

M. E. JOHANSSON,[1] INGRID UHNOO,[1] A. H. KIDD,[2] C. R. MADELEY,[2] AND G. WADELL[3]*

Department of Virology, National Bacteriological Laboratory,[1] and Department of Virology, Karolinska Institutet,[3] S-105 21 Stockholm, Sweden, and Department of Infectious Diseases, University of Glasgow, Ruchill Hospital, Glasgow G20 9NB, U.K.[2]

Two enzyme-linked immunosorbent assay techniques for detection of adenovirus in stools were developed. The first, which is group-specific, detects the 35 established adenovirus types and, in addition, enteric adenoviruses associated with infantile gastroenteritis. The second technique, which is type-specific, selectively detects enteric adenovirus. The efficiency of these techniques was assayed on nine coded stool specimens from Glasgow children. Eight of nine were classified as adenovirus by the group-specific enzyme-linked immunosorbent assay. The six enteric adenovirus specimens were antigenically distinct from each of the 35 established adenovirus types but not from each other. They are suggested to represent a new adenovirus serotype which appears to be associated with gastroenteritis without clear-cut respiratory symptoms.

It has been possible to isolate adenoviruses from stools for many years. Some serotypes are isolated almost exclusively from fecal rather than respiratory specimens (16). With the application of direct electron microscopy (EM) to examination of stool extracts, it has become clear that adenoviruses can be shed in the stools of children and yet not be detected by isolation in tissue cultures. This phenomenon was first described by Flewett et al. (2) and White and Stancliffe (24), but it has been confirmed widely. These strains might be new serotypes, or strains of one or more of the 35 established serotypes that are more difficult to isolate for some unknown reason. Since these viruses cannot be cultured, typing by a conventional neutralization test cannot be done.

It is possible to differentiate between adenovirus serotypes on the basis of polypeptide composition (21). However, the amount of virus required for analysis limits this application with clinical material. Immune EM is also theoretically possible for typing adenovirus, but cross-reactions between serotypes have been reported (18, 20). We have previously described an immunoelectroosmophoresis technique for detection of enteric adenoviruses (5). In this paper we report on the more sensitive enzyme-linked immunosorbent assay (ELISA) used to identify adenovirus in stool extracts, and we show that the enteric adenoviruses constitute at least one new serotype.

PATIENTS AND METHODS

Patients. Stool specimens from nine Glasgow children and one Helsinki child were selected. Each contained adenoviruses detectable by EM. The adenoviruses from the Helsinki child could not be cultured, and purified extracts were used in preparing reagents as described below. The patient was a 5-year-old diabetic boy with severe diarrhea. Details of the nine Glasgow children are given below.

Patient I. Patient I was a 4-week-old boy admitted with diarrhea and vomiting. Adenoviruses which could not be cultured were detected by EM in single specimens taken on days 2, 4, and 7 after admission. The first two specimens were associated with watery stools and vomiting. The specimen from day 2 was studied.

Patient II. Patient II was a boy of 8 weeks admitted with acute respiratory distress. Adenoviruses which could not be cultured were detected in five specimens taken on days 24, 26, 29, 30, and 32 after admission. There were frequent or watery stools on days 26, 28, and 30. The specimen from day 30 was tested.

Patient III. Patient III was a normal 11-week-old boy at home. A stool specimen contained adenoviruses which could not be cultured. Gastrointestinal symptoms started 4 days after the specimen was taken.

Patient IV. Patient IV was a 13-week-old boy admitted to the hospital with diarrhea and upper respiratory tract infection. Adenoviruses were detected by EM on days 14, 15, and 17 after admission, and a type 31 strain was isolated from the stool taken on day 17. Frequent or watery stools were noted from day 14 to day 17. The specimen from day 17 was tested.

Patient V. Patient V was a 4-month-old girl admitted with meningitis. Adenoviruses which could not be cultured were detected in stool from day 29 and in two stools from day 31 after admission. Loose stools developed on day 26 and lasted until at least day 31. One specimen from day 31 was studied.

Patient VI. Patient VI was a 7-week-old girl admitted with diarrhea. Adenoviruses which could not be cultured were detected in four specimens from days

3, 6, 7, and 9 after admission, and caliciviruses were detected by EM on days 10 and 11. Frequent or watery stools were noted on days 3, 4, and 5. The specimen from day 9 was tested.

Patient VII. Patient VII was an 8-week-old girl admitted with vomiting. Adenoviruses which could not be cultured were detected in stool specimens taken 6 days before admission, on the day of admission, and on day 2 after admission. Caliciviruses were detected by EM in a stool taken on day 3 after admission. The specimen from the day of admission was studied.

Patient VIII. Patient VIII was an 8-month-old boy admitted with diarrhea. Adenoviruses were detected by EM, and a type 18 strain was isolated from a specimen taken on the day of admission.

Patient IX. Patient IX was a normal 6-month-old girl at home. Adenoviruses were detected by EM and were also isolated from two specimens taken 9 days apart. The isolates were neutralized by antisera to both type 14 and type 16. The child had clinical measles from 1 day before until 4 days after passing the first specimen. There were no signs of gastrointestinal disorder. The second specimen was studied.

Stool suspensions. Suspensions of 10 to 20% were prepared by shaking feces in phosphate-buffered saline containing penicillin (100 U/ml) and streptomycin (50 μg/ml). The suspensions were clarified by centrifugation at 3,000 rpm. For electron microscopy these extracts were centrifuged for 1 h at 35,000 rpm in a Beckman model L5-40 centrifuge with a SW50.1 rotor. The pellets were prepared for microscopy by the method of Madeley et al. (12).

Adenovirus antigens. The prototype strains 1 to 30, originally obtained from the National Institutes of Health, Bethesda, Md., and strains 31 to 35, provided by J. C. Hierholzer, Communicable Disease Center, Atlanta, Ga., were used. Soluble adenovirus components (hexons, pentons, fibers) of the 35 established adenovirus serotypes were prepared from infected cell lysates by centrifugation on discontinuous CsCl gradients (7). Purified virion-derived antigens of adenovirus type 3 were prepared by equilibrium centrifugation of virions (22), followed by 10 cycles of freezing and thawing (14).

Antibody preparation and purification. Antisera were prepared as described below. Particular antibodies were extracted by immunoadsorption, details of which have been published elsewhere (7). Blue dextran, previously used to saturate the immunoadsorbent and prevent nonspecific protein binding, was not used because trace amounts of this substance cause nonspecific binding of protein to plastic surfaces.

(i) Group-specific antibody (human). A pool of 10 human sera, all with adenovirus antibody titers of $\geqslant$40 by the complement fixation test, was passed through an immunoadsorbent column containing soluble virus components of adenovirus types 1, 7, 15, 16, and 31 (7), representing subgroups A to D of human adenoviruses (4, 21). The column was washed with phosphate-buffered saline, and the antibodies bound to the gel were eluted by addition of 0.2 M glycine-hydrochloride buffer (pH 2.8). The eluate was immediately neutralized with 1 M K_2HPO_4 and dialyzed overnight against 0.15 M NaCl.

(ii) Group-specific antibody (rabbit). A rabbit antiserum to adenovirus type 1 virions was treated as described above. The immunization was performed as previously described (7).

(iii) Enteric adenovirus antibody (rabbit). An antiserum to the Helsinki strain of enteric adenovirus was obtained by affinity bead immunization (8). Briefly, rabbits were immunized with soluble adenovirus components coupled to cyanogen bromide-activated Sepharose beads with group-specific anti-adenovirus type 2 virion rabbit immunoglobulin G (IgG) (7). Two injections were given intramuscularly with an interval of 4 weeks. To eliminate reactivity to known adenovirus serotypes, the serum was passed through a column containing soluble virus components of adenovirus types 1, 7, 15, 16, and 31. In this case the serum constituents that did not adhere to the column containing antibodies to enteric adenovirus were collected. To remove antibodies against human IgG, the affinity bead immunization serum was passed through a column containing immobilized IgG (6).

Protein determination. Quantities of antigen and antibody were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Technical Bulletin no. 1051).

ELISA assay. A combination of the methods of Voller et al. (19) and Yolken et al. (25) was used. The wells were first coated with capture antibody and then incubated with antigen, indicator antibody, anti-IgG conjugate, and finally substrate solutions as described below.

Group-specific adenovirus antibody (human origin), diluted to 3 μg/ml in 0.1 M carbonate buffer (pH 9.6), was added to each well of a polystyrene microtitration plate (M29 AR, Dynatech Ltd.) and incubated at room temperature for 16 h. The plates were washed three times by vacuum extraction using the Dynatec microtiter apparatus. Stool suspensions or adenovirus antigens, diluted in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 and 0.02% (wt/vol) sodium azide, were added in duplicate to the pretreated antibody-coated wells. The plates were incubated at 37°C for 60 min, then washed three times. Indicator antibody, either group-specific antibody of rabbit origin or antibody to enteric adenovirus (rabbit origin) at 100 ng/ml, was added, and the plates were incubated at 37°C for 60 min. The plates were washed three times, and alkaline phosphatase-conjugated antibody against rabbit IgG (Orion Ltd., Finland) at 1:100 was added and incubated at room temperature for 3 h. The plates were then washed four times, and *p*-nitrophenyl phosphate substrate (Sigma 104, 1 mg/ml) in 0.05 M $NaHCO_3$ (pH 9.8), containing 1 mM $MgCl_2$, was added. The plates were incubated at room temperature for 30 min, and the reaction product (yellow coloration) was measured at 405 nm in a Titertek Multiscan spectrophotometer (Flow Ltd.). All reagents were added in volumes of 100 μl, and all rinses were carried out with 0.15 M saline containing 0.05% (vol/vol) Tween 20.

RESULTS

Evaluation of the ELISA. Adenovirus soluble components are produced in large excess. They carry both group- and type-specific antigenic determinants. Therefore it should be possible to use group-specific antibodies for captur-

ing of soluble viral components from all human adenovirus types.

A pool of human sera was used in the preparation of capture antibodies. Since antibodies reacting with human rotaviruses frequently occur both in human (26) and rabbit sera (5), these reagents were prepared by affinity chromatography. The absence of reaction with a stool containing a high titer of another virus (rotavirus) provides additional evidence that the test is specific.

The range of absorbance values obtained with control stools was assessed by analysis of 38 stool extracts diluted 1:10. Group-specific antibody (rabbit origin) was used as indicator antibody. Thirty-seven of the absorbance values obtained were distributed within ±2 standard deviations of the arithmetic mean. A test specimen was considered positive if it produced an absorbance value equal to or greater than 2.5 times the mean absorbance for the control stools. All 38 control stools were negative by this criterion. Purified virion-derived antigens of adenovirus type 3 at various dilutions were tested as above. Concentrations as low as 1 ng/ml gave a reaction above the cut-off level.

Using group-specific adenovirus antibody (rabbit origin) as indicator antibody in the test, preparations containing soluble virus components of the 35 established serotypes were each found to give a positive reaction (Table 1). Soluble virus components prepared from the Helsinki strain of enteric adenovirus also gave a positive reaction, whereas there was no reaction with a stool containing rotavirus (detected by EM) or control stools.

TABLE 1. *Group diagnosis of adenoviruses assessed by the ELISA technique*

Virus	Mean reactivity[a]
Adenovirus types 1 to 35	31–64
Enteric adenovirus	42
Feces	1
Rotavirus	1
Buffer	2

[a] The mean absorbance value obtained with control stools is expressed as 1.0. The reactivity in the adenovirus-positive preparations was related to the mean value obtained from the control stools.

TABLE 2. *Homologous and heterologous reactivity of the rabbit enteric adenovirus-specific antiserum assessed by the ELISA technique*

Virus	Mean reactivity[a]
Adenovirus types 1 to 35	1–4
Enteric adenovirus	29
Feces	1
Rotavirus	1
Buffer	1

[a] The absorbance values are expressed as described in Table 1, footnote *a*.

To detect enteric adenovirus, type-specific rabbit antiserum prepared against the Helsinki strain of enteric adenovirus was used as indicator antibody. Soluble virus components of each of the 35 established adenovirus serotypes, at the same concentration as used above, gave no reaction in the test (Table 2). A stool containing rotavirus and control stools were also negative. However, soluble virus components prepared from the Helsinki strain of enteric adenovirus gave a positive reaction, and this was the only positive reaction obtained in this test. Therefore, antigens of established adenovirus serotypes were not detected on replacement of group-specific antibody with antibody to enteric adenovirus as indicator.

Stool specimens. To evaluate the sensitivity of the ELISA using stool extracts, nine coded specimens known to contain adenovirus by EM were studied. Adenovirus had been cultured from three specimens, and the other six specimens were negative by culture using primary human embryo kidney cells (Table 3). The specimens were sent from Glasgow to Stockholm and were tested under code. Separate tests were performed using as indicator antibody (i) group-specific adenovirus antibody (rabbit origin), and (ii) enteric adenovirus antibody (rabbit origin). Tenfold dilutions of the extracts were tested individually.

Group reaction. At a dilution of 10^{-1}, eight of the nine specimens gave a positive reaction in the group-specific ELISA (Fig. 1). The absorbance values obtained with seven of the eight positive specimens diminished with dilution of the extracts. The specimen from patient II gave a peak in absorbance at a dilution of 10^{-3}, although it was still positive at 10^{-1} and 10^{-2}

TABLE 3. *Techniques used for identification of adenovirus in Glasgow stool specimens*

Patient	Amount of virus particles by EM	ELISA adenovirus group reactivity	ELISA enteric adenovirus type specific	In vitro growth	Adenovirus type (neutralization test)
I	+++	+	+	−	
II	++++	+	+	−	
III	+++	+	+	−	
IV	+++	+	−	+	Ad 31
V	+++	+	+	−	
VI	+++	+	+	−	
VII	+++	+	+	−	
VIII	+++	+	−	+	Ad 18
IX	+	−	−	+	Ad 14-16

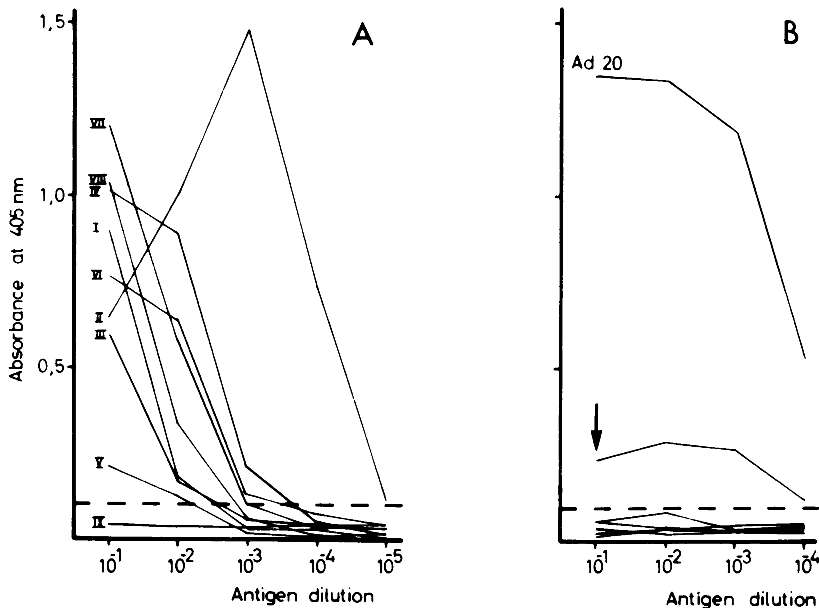

FIG. 1. *(A) Group diagnosis by ELISA of nine adenovirus specimens obtained by EM. An absorbance 2.5 times the background was chosen as the criterion for positivity. This cut-off level is indicated by the broken line. (B) Controls comprising antigens of adenovirus type 20 (Ad 20), Helsinki strain of enteric adenovirus (arrow), and five control stools.*

dilutions. The specimen that was negative by this test had the least amount of adenovirus detected by electron microscopy (Table 3).

Detection of enteric adenovirus. At a dilution of 10^{-1}, six of the nine specimens gave a positive reaction (Fig. 2). The specimen from patient II again showed a high degree of positivity with a peak at a dilution of 10^{-3}. This result is in accordance with the result of electron microscopy (Table 3) and suggests that to screen stool extracts for the presence of adenovirus, more than one dilution should be tested. Each of the six specimens of enteric adenovirus was positive in the ELISA test. Furthermore, the three specimens that were negative by ELISA when tested with antibody against enteric adenovirus yielded other adenovirus types on culture (Table 3).

DISCUSSION

The group-specific detection of adenovirus in the ELISA test depends on the reaction of group-specific antigenic determinants on adenovirus antigens with both capture and indicator antibody. The use of all 35 established adenovirus prototypes has shown that ELISA can be used as a group-specific test for adenovirus. Furthermore, this test enables detection of adenovirus group antigens in crude stool extracts, although adenovirus may not be detected by cell culture. In the present study, eight of the nine extracts that contained adenovirus by EM were positive by the group-specific ELISA. Fewer adenovirus particles were detected in the ninth specimen by EM than in the other specimens. As a consequence, the quantity of soluble antigens was probably too low to be detected by the ELISA assay. Another factor that could account for the negative group-specific ELISA result is the possible association of antibody with the group-specific adenovirus antigens in the gut, which would block capture of these antigens in the test. Enteric adenovirus infections should be expected to cause anamnestic IgG responses against group-specific adenovirus antigens. Such antibody coating has been described for rotaviruses (23).

Antibody to other viruses, if present in the test reagents, could prevent the specific recognition of adenovirus. The antibody used for coating the wells was obtained from a pool of human sera, which will have contained antibodies to other enteric viruses. However, the technique for obtaining group-specific antibodies by retrieval after adenovirus-specific reaction on an immunosorbent minimizes the possibility of capturing foreign antigens in the test, since no reactivity to rotavirus could be detected.

The detection of enteric adenovirus depends

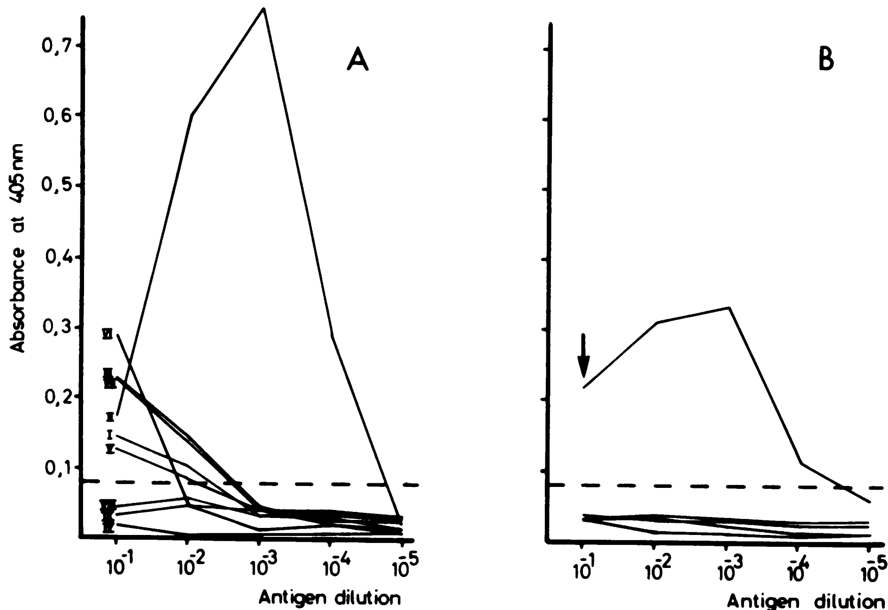

FIG. 2. *(A) Typing of enteric adenovirus by ELISA. Six of the nine adenovirus specimens were identical to the enteric adenovirus Helsinki strain when tested by type-specific antibodies. (B) Controls comprising antigens of enteric adenovirus Helsinki strain (arrow) and (below) antigens of Ad 20, Ad 7, and two control stools.*

on (i) reaction of group-specific antigenic determinants on soluble adenovirus components with capture antibody, followed after capture by (ii) reaction of type-specific determinants or presently unknown subgroup-specific determinants with indicator antibody. Although antigens of all established adenovirus serotypes were tested, positive results were confined to tests on specimens containing adenovirus that could not be cultured. Therefore, a close antigenic relationship exists between the six Glasgow enteric adenovirus strains and the Helsinki strain. Furthermore, we could detect no difference between the genomes of these strains. Four of the Glasgow strains that yielded sufficient amount of DNA to be analyzed by DNA restriction had restriction patterns identical to each other and to the Helsinki strains (G. Wadell, M.-L. Hammarskjöld, G. Winberg, T. Varsanyi, and G. Sundell, Ann. N.Y. Acad. Sci., in press).

Adenoviruses that cannot be cultured have been recognized in stools from children with gastroenteritis (1-3, 5, 9, 10, 12, 13, 15, 17). In the present study, four of the six children who shed enteric adenovirus had diarrhea, vomiting, or both on the day the specimen was taken. The finding of another virus (calicivirus [11]) in specimens from two of the children may serve as a reminder that the interplay between host and communicable agents is complex, and that caution is necessary in ascribing a pathogenic role for viruses in stools. The ELISA technique should allow the degree of relatedness of enteric adenovirus from different sources to be determined. Furthermore, the frequency of shedding of these viruses in asymptomatic children could be determined by ELISA.

ACKNOWLEDGMENT

Tapani Hovi, Helsinki, kindly provided stool specimens containing the enteric adenovirus Helsinki strain.

LITERATURE CITED

1. **Appleton, H., M. Buckley, M. H. Robertson, and B. T. Thom.** 1978. A search for faecal viruses in new-born and other infants. J. Hyg. **31**:279-283.
2. **Flewett, T. H., A. S. Bryden, H. Davies, and C. A. Morris.** 1975. Epidemic viral enteritis in a long-stay children's ward. Lancet **i**:4-5.
3. **Gary, G. W., J. C. Hierholzer, and R. E. Black.** 1979. Characteristics of noncultivable adenoviruses associated with diarrhea in infants: a new subgroup of human adenoviruses. J. Clin. Microbiol. **10**:96-103.
4. **Green, M., J. K. Mackey, W. S. M. Wold, and P. Rigden.** 1979. Thirty-one human adenovirus serotypes (Ad 1 - Ad 31) from five groups (A-E) based upon DNA genome homologies. Virology **93**:481-492.
5. **Jacobsson, P. Å., M. E. Johansson, and G. Wadell.** 1979. Identification of an enteric adenovirus by immunoelectroosmophoresis (IEOP) technique. J. Med. Virol. **3**:307-312.
6. **Johansson, M. E., and J. Å. Espmark.** 1978. Elimination of interspecies reactive anti-IgG antibodies by affinity chromatography. J. Immunol. Methods **21**:285-293.
7. **Johansson, M. E., and G. Wadell.** 1978. Preparation of sera for typing of adenovirus infections by immunoflu-

orescence. J. Immunol. Methods **19**:259-267.

8. **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.
9. **Kapikian, A. Z., H. W. Kim, R. G. Wyatt, W. L. Cline, J. O. Arrobio, C. D. Brandt, W. J. Rodriguez, D. A. Sack, R. M. Chanock, and R. H. Parrott.** 1976. Human reovirus-like agents as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. N. Engl. J. Med. **294**:965-972.
10. **Maass, G., H. G. Baumeister, and N. Freitag.** 1977. Viren als Ursache der akuten Gastroenteritis bei Säuglingen und Kleinkindern. Münch. Med. Wochenschr. **119**:1029-1034.
11. **Madeley, C. R., and B. P. Cosgrove.** 1976. Caliciviruses in man. Lancet **i**:199-200.
12. **Madeley, C. R., B. P. Cosgrove, E. J. Bell, and R. J. Fallon.** 1977. Stool viruses in babies in Glasgow. 1. Hospital admissions with diarrhea. J. Hyg. **78**:261-273.
13. **Middleton, P. J., M. T. Szymanski, and M. Petric.** 1976. Viruses associated with acute gastroenteritis in young children. Am. J. Dis. Child. **131**:733-737.
14. **Prage, L., U. Pettersson, S. Höglund, K. Lonberg-Holm, and L. Philipson.** 1970. Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion. Virology **42**:341-358.
15. **Richmond, S. J., E. O. Caul, S. M. Dunn, C. R. Ashley, S. K. R. Clarke, and N. R. Seymour.** 1979. An outbreak of gastroenteritis in young children caused by adenoviruses. Lancet **i**:1178-1180.
16. **Rosen, L., J. F. Hovis, and J. A. Bell.** 1962. Observations of some little-known adenoviruses. Proc. Soc. Exp. Biol. Med. **111**:166-171.
17. **Schoub, B. D., H. J. Koornhof, G. Lecatsas, O. W. Prozesky, I. Freiman, E. Hartman, and H. Kassel.** 1975. Viruses in acute summer gastroenteritis in black infants. Lancet **i**:1093-1094.
18. **Vasall, J. H., and C. G. Ray.** 1974. Serotyping of adenoviruses using immune electron microscopy. Appl. Microbiol. **28**:623-627.
19. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506-512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.
20. **Wadell, G.** 1972. Sensitization and neutralization of adenovirus by specific sera against capsid subunits. J. Immunol. **108**:622-632.
21. **Wadell, G.** 1979. Classification of human adenoviruses by SDS-polyacrylamide gel electrophoresis of structural polypeptides. Intervirology **11**:47-57.
22. **Wadell, G., M.-L. Hammarskjöld, and T. Varsanyi.** 1973. Incomplete virus particles of adenovirus type 16. J. Gen. Virol. **20**:287-302.
23. **Watanabe, H., I. D. Gust, and I. H. Holmes.** 1978. Human rotavirus and its antibody: their coexistence in feces of infants. J. Clin. Microbiol. **7**:405-409.
24. **White, G. B. B., and D. Stancliffe.** 1975. Viruses and gastroenteritis. Lancet **ii**:703.
25. **Yolken, R., R. G. Wyatt, and A. Z. Kapikian.** 1977. ELISA for rotavirus. Lancet **ii**:819.
26. **Yolken, R. H., R. G. Wyatt, H. W. Kim, A. Z. Kapikian, and R. M. Chanock.** 1978. Immunological response to infection with human reovirus-like agent: measurement of anti-human reovirus-like agent immunoglobulin G and M levels by the method of enzyme-linked immunosorbent assay. J. Immunol. Methods **19**: 540-546.