Detection of Adenovirus by Enzyme-Linked Immunosorbent Assay

MAURICE W. HARMON,* STEPHANIE DRAKE, AND JULIUS A. KASEL

Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

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A solid-phase direct enzyme-linked immunosorbent assay (ELISA) was developed for the detection of adenovirus antigen in extracts of infected cells by using anti-hexon serum. Results with simulated clinical specimens consisting of normal nasal wash specimens seeded with varying concentrations of adenovirus type 5 showed that antigen could be detected in extracts of HEp-2 cell cultures inoculated with $10^{2.5}$ 50% tissue culture infective doses (TCID₅₀) and $10^{1.5}$ TCID₅₀ after 2 and 4 days of incubation, respectively. Fifty-three clinical nasal wash specimens containing adenovirus type 5 (stored for 5 years at -70°C) were used to evaluate antigen detection by ELISA in HEp-2 cell extracts and by manifestation of cytopathic effect in human embryonic kidney cells. After 2 days of incubation, 62% were positive by ELISA, whereas none was positive for cytopathic effect. After 4 days of incubation, 76% were ELISA positive and 47% were positive for cytopathic effect. The results according to infectivity titers indicated that clinical specimens containing $10^{3.0}$ TCID₅₀ or greater were all positive by ELISA after 2 days of incubation in HEp-2 cells, and by 4 days all but one specimen containing $10^{2.0}$ TCID₅₀ or greater were ELISA positive. ELISA and immunofluorescent methods for antigen detection were compared using 24 of the 53 clinical specimens containing adenovirus type 5. Nearly equivalent sensitivities were demonstrated. These results suggest that ELISA may provide an alternative method of detecting and identifying adenoviral infections in humans.

Recovery of adenoviruses in tissue culture is considered one of the primary methods for detecting these agents in clinical specimens. Although this method is sensitive, the time interval between inoculation and manifestation of cytopathic effect (CPE) can be long and variable. The rapidity with which CPE appears is generally attributable to the concentration of infectious virus or the type of adenovirus in the clinical specimen (8, 12). In specimens with small quantities of virus, CPE may be delayed for up to 28 days (6).

Once a virus is detected by CPE, complement fixation and immunofluorescence (IFA) tests are the serological methods most commonly employed to identify an isolate as belonging to the adenovirus family (7). Both procedures are based on the reaction of soluble hexon subunits which are synthesized in excess during the virus replication cycle. Adenovirus family identification by these procedures requires the use of only a single antiserum prepared against the family-reactive antigenic determinant carried by the hexon subunit (5). However, to obtain a sufficient concentration of antigen for detection by the complement fixation test, CPE needs to

progress until approximately 75% of the tissue culture is involved. This requirement often incurs a delay in identification. The IFA test requires less antigen, but a diagnostic interpretation by this method is sometimes based upon subjective observation of only a few infected cells.

The availability of alternative methods to detect and identify small amounts of adenoviral antigen prior to CPE development would substantially accelerate viral diagnosis. This report describes the applicability of the enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection and identification of adenovirus antigen in extracts of infected cells.

MATERIALS AND METHODS

Antiserum. Adenovirus type 5 (Ad5) hexon subunits were purified as previously described (11). Antiserum for the ELISA was produced in guinea pigs by footpad inoculation of 40 μg of hexon antigen in complete Freund adjuvant, followed by a booster dose, consisting of 10 μg in incomplete Freund adjuvant, on day 28. Animals were bled by cardiac puncture 34 days after the booster inoculation. Globulins were precipitated from the antiserum with 18% (wt/vol) sodium sulfate, washed twice each in 18% and 12% sodium

sulfate, and redissolved in phosphate-buffered saline (PBS). After dialysis against PBS, the protein concentration was determined by absorbance at 280 nm. The conditions for conjugating alkaline phosphatase to globulins were described by Engvall and Perlmann (4). The source of alkaline phosphatase was calf intestine (Sigma type VII; Sigma Chemical Co., St. Louis, Mo.), and the specific activity of the enzyme preparation used was 1,140 units per mg of protein.

Before use in immunofluorescent tests, antiserum was heated at 56°C for 30 min and absorbed with 15% baby beef brain. Fluorescein-conjugated rabbit anti-guinea pig 7S immunoglobulin G (Hyland Laboratories, Costa Mesa, Calif.) was used as the second antibody.

ELISA. The procedure for conducting the solid-phase ELISA was essentially that reported by Clark and Adams for the assay of plant viruses (1). The reagents used were described by Voller et al. (14). The wells of microtiter plates (Microelisa substrate plates from Dynatech Laboratories, Inc., Alexandria, Va.) were used as the solid-phase surfaces. Anti-Ad5 globulins were diluted to a concentration of 1.4 µg/ml in coating buffer (pH 9.6), and 0.2 ml was added to each well. After incubation at 37°C for 5 h, the plates were washed four times with PBS containing 0.05% polysorbate (Tween)-20 (PBS-Tween). The test sample (0.2 ml) containing Ad5 antigen was added to the wells in duplicate and incubated at 4°C for 18 h. Plates were washed four times with PBS-Tween, and 0.2 ml of anti-Ad5 globulin-alkaline phosphatase conjugate was added. After 4 h at 37°C, the plates were washed four times with PBS-Tween, and 0.2 ml of the enzyme substrate (*p*-nitrophenyl phosphate, 1 mg/ml) in 10% diethanolamine buffer (pH 9.8) was added. The substrate was Sigma 104 phosphatase substrate tablets (Sigma). After 1 h at room temperature, the enzyme reaction was terminated by the addition of 0.05 ml of 3 N NaOH. The reaction product (*p*-nitrophenol) was quantitated by measuring the absorbance at 400 nm in a spectrophotometer (Gilford Instruments, Oberlin, Ohio).

Background reactions were determined by substituting PBS-Tween or extracts of uninfected cells (where appropriate) in place of viral antigen. Three to seven controls were included in each assay. A sample was considered positive if the absorbance was greater than 3 standard deviations above the mean absorbance of the appropriate controls.

IFA. To process cell cultures for assay by the IFA procedure, maintenance medium was removed and the cells were washed once with PBS. The cultures were then scraped from the tube and placed on glass slides to air dry. Cells were fixed in cold (−20°C) acetone for 10 min, dried, and stored at −20°C until stained by the indirect technique. The primary antiserum (guinea pig anti-Ad5) was added and incubated for 30 min at 37°C. The cells were washed with PBS, and the second antibody (fluorescein-conjugated rabbit anti-guinea pig 7S immunoglobulin G) was added and incubated for 30 min at 37°C. Rodamine counterstain (Difco Laboratories, Detroit, Mich.) was included in the second antibody preparation to reduce nonspecific background staining (13). Slides were then rinsed in PBS

and distilled water and allowed to air dry. A drop of PBS-glycerol and a cover slip were added before the slides were examined through a Leitz Ortholux microscope equipped with a Ploem-type vertical illuminator. The test was considered positive if one or more specifically fluorescing cells were observed.

Test specimens. HEp-2 cell cultures used to propagate Ad5 virus for ELISA determinations were generously provided by H. Mayor, Baylor College of Medicine. The growth medium consisted of 90% Eagle minimum essential medium, 10% heat-inactivated (56°C, 30 min) fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). For maintenance of cell cultures, the concentration of fetal calf serum in the medium was reduced to 2%.

Dilutions of Ad5 virus were prepared in maintenance medium and in nasal wash specimens from uninfected adults. Nasal wash specimens were collected in veal infusion broth supplemented with 2% human serum albumin. Clinical specimens used in this study consisted of 53 nasal wash specimens from 13 adult volunteers who participated in an experimental Ad5 virus challenge trial (3). They were obtained with the same collecting medium used to prepare the simulated specimens and had been stored at −70°C for approximately 5 years. Freshly collected nasal washes from normal adult individuals were used to serve as negative controls. The above test specimens were inoculated in 0.2-ml volumes into HEp-2 tube cell cultures and incubated at 37°C on a roller drum. To extract virus antigen from infected cultures, the maintenance medium was discarded and the cells were washed once with PBS-Tween. After addition of 0.5 ml of the same solution, cell cultures were subjected to three freeze-thaw cycles and then centrifuged at $2,500 \times g$ for 20 min to collect supernatant fluid for evaluation in the ELISA procedure.

Standard laboratory preparations of H₃N₂ influenza virus, respiratory syncytial virus, parainfluenza virus type 3, rhinovirus types 1A and 3, coxsackievirus A type 21, and adenovirus types 1, 2, 3, 4, 7, 21, and 22 were diluted 10-fold in PBS-Tween and tested by ELISA without incubation in HEp-2 cells.

Primary human embryonic kidney (HEK) cells obtained from a commercial source were used to monitor undiluted clinical specimens for the time of appearance of CPE and to determine 50% tissue culture infectious dose (TCID₅₀) titers of adenovirus clinical specimens and adenovirus laboratory pools. Titers were calculated according to the method of Kärber (7). Inoculum volumes and incubation conditions were the same as those described above.

RESULTS

The sensitivity of the ELISA was determined using simulated clinical specimens containing decimal dilutions of infectious Ad5 virus. Figure 1 shows the results obtained at 2 and 4 days after inoculation of test materials into HEp-2 cells. The cell control represents the mean absorbance value of extracts from six uninfected cell cultures inoculated with 0.2 ml of normal

nasal wash specimens. After a 2-day incubation period, the extracts from two of three cell cultures inoculated with 10² TCID₅₀/0.2 ml and all those from higher virus concentrations produced a positive reaction by the ELISA. When cell extracts were assayed at 4 days, cultures inoculated with nasal wash specimens containing 10^{1.5} TCID₅₀/0.2 ml or greater synthesized sufficient hexon antigen to be detected by the test procedure.

The applicability of the ELISA for diagnostic purposes was evaluated with 53 nasal wash specimens collected from individuals during the course of infection with Ad5 virus. Testing of these specimens directly by ELISA indicated that only one contained a sufficient concentration of antigen to be positive by this test. Undiluted specimens were also inoculated simultaneously into HEP-2 cells for detection of antigen by ELISA and into primary HEK cells for observation of adenoviral CPE. In addition, dilutions of specimens were inoculated into HEK cells for infectivity titer determinations. Since HEK cells are considered the most sensitive for adenovirus isolation, their use for the virus titer determinations and for monitoring the time of CPE manifestation gave results that are considered to be the most sensitive using existing methodology. HEP-2 cells were used for the ELISA methodology because they gave results similar to those obtained with HEK cells and were more readily available. The results of this analysis are shown in Table 1. Extracts from 62% of the cultures inoculated with nasal wash specimens were positive by ELISA after incubation for 2 days, whereas none of the cultures showed evidence of CPE. The ELISA detected

antigen in extracts from 76% of the inoculated HEP-2 cultures after 4 days, whereas only 47% of the HEK cultures exhibited CPE. By 7 days postinoculation, virus was demonstrable by CPE in all HEK cultures. The differences in relative frequencies of positive identification by ELISA and by CPE at the same time intervals were

FIG. 1. Detection of Ad5 antigen by ELISA after inoculation of simulated clinical specimens (in triplicate) containing the indicated number of TCID₅₀ and incubation in HEP-2 cells for 2 (○—○) or 4 (●—●) days. The short horizontal lines indicate the means of the three specimens at each virus concentration. The mean background reaction produced by six uninfected cell controls inoculated with normal nasal wash specimens is indicated by the bar. The dashed line (positive cutoff) represents 3 standard deviations above the mean cell control.

TABLE 1. Detection of Ad5 in nasal wash specimens by ELISA and CPE according to infectivity titer

Log ₁₀ specimen titer ^a (TCID ₅₀ /0.2 ml)	No. of specimens	No. (%) of specimens positive on day postinoculation:					
		2		4		6	
		ELISA ^b	CPE ^c	ELISA ^b	CPE ^c	ELISA ^b	CPE ^c
0.5	9	1 (11)	0	2 (22)	0	— ^d	6 (67)
1.0	6	1 (17)	0	2 (33)	0	—	6 (100)
1.5	7	3 (43)	0	6 (86)	1 (14)	—	6 (86)
2.0	8	7 (88)	0	8 (100)	3 (38)	—	7 (88)
2.5	10	8 (80)	0	9 (90)	8 (80)	—	10 (100)
3.0–5.0	13	13 (100)	0	13 (100)	13 (100)	—	13 (100)
Cumulative total	53	33 (62) ^e	0	40 (76) ^e	25 (47)		48 (91)

^a Infectious Ad5 virus titer determined in HEK cells after incubation for 28 days.
^b Determined in extracts of HEP-2 cells inoculated with undiluted specimens (0.2 ml). Specimens were considered positive if the absorbance was 3 standard deviations above the mean of extracts of seven HEP-2 cell controls inoculated with normal nasal secretions.
^c CPE observed in HEK cells inoculated with undiluted specimens (0.2 ml).
^d —, Not tested.
^e *P* < 0.001 by chi-square analysis.

found to be highly significant in each case ($P < 0.001$). As expected, the time of appearance of detectable hexon antigen by ELISA and expression of adenoviral CPE depended upon the dose of infectious virus inoculated. The lower limit of sensitivity of the former method was approximately $10^{2.0}$ TCID₅₀ in the 4-day test and $10^{3.0}$ TCID₅₀ in the 2-day assay.

The specificity of the ELISA for the detection of adenovirus was examined by determining the reactivity of other respiratory viruses (Table 2). The detection of adenovirus serotypes other than type 5 by the assay was predictable from the use of anti-hexon serum, since that subunit contains the family-reactive determinant (5). For each of the non-adenovirus agents, the magnitude of reaction was comparable to that observed for the negative (PBS-Tween) control.

The efficiency of the ELISA for detecting Ad5 antigen in cell cultures was compared with the IFA technique. Twenty-four of the 53 clinical specimens were inoculated into duplicate HEK cell cultures and assayed for the presence of antigen after a 2-day incubation period at 37°C. The results of this comparison are shown in Table 3. Although the proportion of specimens identified by ELISA (13 of 24) tended to be higher than that observed by the IFA procedure (9 of 24), the difference was not statistically significant ($P > 0.05$). It should be noted, however, that identification of five of the nine positive cell cultures by the IFA test was based on only one to three fluorescing cells.

DISCUSSION

In the present investigation, the results obtained with clinical specimens by using ELISA

TABLE 2. *Specificity of the adenovirus ELISA*

Virus Antigen	Titer (log ₁₀ TCID ₅₀ /0.2 ml)	Absorbance at 400 nm
Ad1	5.3	2.408
Ad2	5.3	2.016
Ad3	5.8	2.575
Ad4	— ^a	2.856
Ad5	5.6	2.854
Ad7	5.5	2.316
Ad21	—	3.125
Ad22	—	2.483
Influenza A (H ₂ N ₂)	6.5	0.086
Respiratory syncytial virus	4.0	0.097
Parainfluenzavirus type 3	5.6 ^b	0.099
Rhinovirus type 1A	4.5	0.083
Rhinovirus type 3	5.3 ^b	0.083
Coxsackievirus A type 21	6.3	0.074
None (PBS-Tween)		0.098

^a —, Not done.

^b Titer expressed as plaque-forming units per 0.2 ml.

TABLE 3. *Detection of Ad5 by ELISA and IFA in extracts of HEK cell cultures inoculated with nasal wash specimens*

IFA results ^a	No. of specimens	ELISA results ^b	
		Positive	Negative
Positive	9	8	1
Negative	15	5	10
Total	24	13	11

^a The test was considered positive if one or more specifically fluorescing cells were observed.

^b Specimens were considered positive if the absorbance was 3 standard deviations above the mean of six HEK cell controls inoculated with normal nasal wash specimens.

demonstrated the utility of this immunological technique as a diagnostic tool. It offers the advantages of rapidity, sensitivity, and objectivity, while being relatively inexpensive to perform. Incorporation of the ELISA for adenovirus detection into a viral diagnostic laboratory routine would be a simple matter. As specimens are received, they could be tested directly for adenovirus antigen by ELISA, and, if positive, this test would provide an immediate diagnosis. If negative, the specimens could be inoculated into two HEp-2 and two HEK cell cultures. The inoculated HEK cultures would be followed by microscopic observation for evidence of CPE, followed by identification of IFA or complement fixation tests at the time of CPE detection. The inoculated HEp-2 cultures would be tested by ELISA on days 2 and 4 postinoculation. A positive result by ELISA on HEp-2 cell extracts would provide both early detection and identification of the isolate. Our data indicate that virus antigen was detected (and identified) by ELISA in extracts from the majority (62%) of HEp-2 cell cultures 2 days after inoculation with clinical specimens. By comparison, the mean day of onset of CPE in HEK cells was 4.7 days postinoculation. Thus, the ELISA reduced the time necessary for detection of the majority of virus isolates by 2.7 days.

In its present form the ELISA test would best serve a diagnostic laboratory that had a high incidence of adenovirus isolates, such as one serving military recruits or an ophthalmology service. However, this methodology may be more utilizable when ELISA tests for other respiratory virus antigens are developed, since more than one virus type may be detected and identified using the same cell culture extract.

An increase in sensitivity, which would allow for the detection of lower concentrations of viral antigen, would also improve the utility of the ELISA. One method of increasing sensitivity might be the use of polystyrene beads as the

solid phase, as reported for the detection of herpes simplex virus antigen (10), rather than microtiter wells. In addition, an increase in sensitivity may be expected if the test is changed from a direct to an indirect test as described for the detection of *Escherichia coli* heat-labile enterotoxin by Yolken and his associates (16).

For convenience purposes, performance of the ELISA included an overnight incubation step and technically added 1 day to the identification time. However, the total time can be reduced to 7 h without any loss of sensitivity. The essential changes include a reduction in the antigen incubation step from 18 h at 4°C to 3 h at 37°C and reduction of the conjugate incubation step from 4 h at 37°C to 3 h at 37°C. Both procedures resulted in the detection of antigen in a stock Ad5 virus pool at a 1:6,000 dilution and loss of detectability at a 1:10,000 dilution. The 7-h test is a more rapid identification procedure than complement fixation and is essentially comparable to the time required for IFA.

Evidence was also presented demonstrating the specificity of the ELISA for the adenovirus family antigen. No evidence of cross-reactions was observed with any of the other respiratory virus antigens tested. The use of purified antigen for the production of antiserum most likely contributed to the specificity of the assay.

The solid-phase ELISA has been shown to be comparable in sensitivity to solid-phase radioimmunoassays for the detection of rotavirus (17), hepatitis B surface antigen (15), and hepatitis A (9). The ELISA possesses several advantages relevant to the clinical laboratory situation. The reagents pose no radiation hazard to personnel and are stable for long periods of time. The results can be read visually or quantitatively with the aid of a spectrophotometer. In addition, the ELISA lends itself to automation (2).

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