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Detection of Adenovirus in Patient Specimens by Indirect Immune Electron Microscopy

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Immune electron microscopic procedures for the detection of adenovirus type 7 directly in throat swab specimens from patients are described. Nineteen of 25 throat swab samples, known to be positive for adenovirus type 7 by isolation of virus from tissue culture, were shown to contain aggregates of adenovirions coated with antibody. Sensitivity tests of the immune electron microscopic method showed that as few as 16 to 32 tissue culture infective doses of virus could be detected by the direct immune electron microscopic technique. It was also demonstrated that aggregation of virus-antibody complexes could be further enhanced by use of anti-immunoglobulin G sera (indirect immune electron microscopic procedures). These results demonstrate that examination of patient specimen by immune electron microscopic procedures is a feasible and rapid method for adenovirus detection and suggest that it could be applied as a routine laboratory procedure for the diagnosis of other virus infections.

Procedures have been described for the visualization and identification of viral morphological particles by immune electron microscopy (IEM) (1). Several investigators have been able to utilize this technique to identify virions in tissues or secretions taken directly from patients (2, 3). In a recent report, it was demonstrated that adenovirus isolates from tissue culture could be typed serologically by use of IEM (7). As yet it has not been shown that this technique is feasible as a routine method for the laboratory diagnosis of viral infections. This capability would decrease the time and expense of viral diagnosis and would justify the cost of an electron microscope laboratory. But before IEM can be accepted as a routine diagnostic procedure, it must be proven that it has sufficient sensitivity and reliability for practical use.

One of the major obstacles is to capture a sufficient number of morphological particles from the patient's specimen to insure their visualization in the electron microscope.

The following report describes the enhancement of virus detection by an indirect serological method, whereby the virus-antibody complexes were further aggregated by anti-immune globulin sera (anti-immunoglobulin G [IgG]). This indirect IEM method was used to rapidly identify adenoviruses in throat swab specimens taken from patients with respiratory disease. (This study was done in connection with Research Project MR41.05.02-AAAA, Bureau of Medicine and Surgery, Navy Department, Washington, D. C.)

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MATERIALS AND METHODS

Viral material. Tissue cultures. Prototype strains of adenovirus type 7 (AD-7) were propagated in HeLa cell cultures. The virus infectivity titer, as measured by micro tissue culture technique (4), was approximately 10^4 tissue culture infective doses ($TCID_{50}$) per ml. This stock virus was used in experiments to test the sensitivity of the IEM by either the direct or indirect technique.

Patient specimens. Navy recruits with symptoms of acute respiratory disease were sampled for virus by swabbing the oropharyngeal surface. Swabs were immersed in a diluent of veal infusion broth supplemented with 0.5% bovine serum albumin. All samples had been shown to contain AD-7 by isolation of the virus in HeLa cells (6). These positive specimens had been frozen for over 1 year before use in these experiments.

Serological material. Adenovirus immune sera. A pool of human adenovirus immune sera (anti-AD-7) was employed as the primary virus-aggregating antibody. The neutralizing antibody titer of the pool, as measured by type-specific microassay (4), was approximately 400 neutralizing antibody units per ml when challenged with 100 $TCID_{50}$ of AD-7.

Anti-IgG sera. Human IgG (Cohn's fraction II) was diluted in phosphate-buffered saline (0.1 M, pH 7.2) to a concentration of 10 μg/ml. Equal volumes of the diluted antigen and Freund complete adjuvant were mixed and injected intramuscularly into rabbits at multiple sites (2.5 ml/site). Three weeks later, the animals were re-injected intravenously with 2.5 ml of

diluted antigen. This injection was repeated 1 week later, and the animals were bled the following week. Tests for the optimal combining proportion (maximal precipitate formation) of the antigen (anti-AD-7) and antibody (anti-IgG) were made by chess board titration.

Direct IEM. The methods described by Almeida and Waterson (1) were employed for the direct IEM technique. One-half milliliter of stock virus was mixed with an equal volume of anti-AD-7 sera diluted 1:20 (10 neutralizing antibody units). The mixture was incubated at 37 C for 1 h and then kept at 6 C for 16 to 20 h. Then, the mixture was centrifuged at 12,000 × *g* for 30 min in a Sorvall-RC2 centrifuge (SS34 rotor). The supernatant fluid was discarded, and the pellet was suspended in 0.1 ml of distilled water. One drop of the suspension was mixed with a drop of 3% potassium phosphotungstic acid (pH 6.0), and this mixture was applied to a 300-mesh, carbonized, Formvar-coated grid with the aid of a droplet nebulizer to insure uniform dispersion. The specimens were examined with an EMU-3G electron microscope at an acceleration voltage of 50 kV.

Indirect IEM. One-half ml of anti-AD-7 serum, diluted as described, was mixed with an equal volume of stock virus or throat swab fluid and incubated for 1 h at 37 C and then for 3 h at 6 C. The mixture was centrifuged at 12,000 × *g* for 30 min, and the pellet was suspended in 0.5 ml of phosphate-buffered saline. An equal volume of anti-IgG serum, diluted to the optimal concentration (1:10), was added, and this mixture was incubated at 6 C for 16 to 20 h. The mixture was then centrifuged at 12,000 × *g* for 30 min, and the pellet was suspended in 0.1 ml of distilled water. Staining and microscopic examination procedures were carried out as described above.

RESULTS

Sensitivity of IEM. To determine the sensitivity of the direct IEM, the stock AD-7 virus fluid, containing 10^4 $TCID_{50}$/ml, was diluted initially 1:10 and then in twofold increments. Aliquots of each dilution were mixed with diluted (1:20) anti-AD-7 sera (10 neutralizing antibody units). The mixture was treated as described for direct IEM and examined for aggregates of AD-7 virions. The degree of positivity was recorded by using an empirical range of 4+ to ± (Table 1, footnote *c*). The appearance of a typical aggregate of adenovirions coated with antibody is shown in Fig. 1.

The results of the sensitivity determinations are shown in Table 1. Immune complex aggregates of AD-7 were detected even when the stock virus was diluted 160-fold. By estimation, that dilution should have contained between 16 and 32 $TCID_{50}$ of infectious virus.

IEM enhancement by anti-IgG sera. In Table 2 the sensitivity of the direct and indirect IEM techniques for detection of virus is compared. Virus was diluted 200-fold, or beyond the concentration detectable by the direct technique (above). The primary aggregating antibody (anti-AD-7) was diluted in twofold increments (1:20 to 1:640) and mixed with an equal amount of diluted virus. Duplicate samples of virus and anti-AD-7 serum dilutions were prepared; one mixture was processed as described for the direct IEM, and the other was processed for indirect IEM by the further addition of the anti-IgG rabbit sera. Whereas no aggregates were detected by the direct IEM, aggregated virions could be seen after addition of anti-IgG sera (Table 2), although the primary antibody (anti-AD-7) had been diluted eightfold more than the minimal concentration required by the direct IEM technique.

TABLE 1. *Sensitivity of direct IEM for detection of adenovirus particles*

Adenovirus infectivity titer ($TCID_{50}$/0.5 ml)[a]	Adenovirus immune serum (NtD_{50}/0.5 ml)[b]	IEM result[c]
500	10	4+
250	10	4+
125	10	2+
62	10	2+
32	10	1+
16	10	±
8	10	−

[a] Calculated tissue culture doses after twofold serial dilutions of stock virus (10^4 $TCID_{50}$/ml) diluted 1:10.

[b] Neutralizing antibody units against AD-7 (100 $TCID_{50}$).

[c] Microscopic fields with aggregated adenovirions after a 20-min search: 4+ = 100% of fields inspected; 3+ = 75%; 2+ = 50%; 1+ = 25%; and ± = occasional aggregate.

Detection of adenovirus in throat swabs by indirect IEM. Aliquots (0.5 ml) of 25 throat swabs previously positive for AD-7 were processed for indirect IEM as described. Ten of the specimens had been found to be positive during the first passage in HeLa cells, 10 during the second passage and five required three or more passages before adenovirus cytopathology was observed. The results of indirect IEM tests for aggregates of AD-7-immune complexes are shown in Table 3. All 10 of the specimens which had been positive in the first tissue culture passage were also IEM positive. Seven of the 10 specimens positive in second tissue culture passage were IEM positive; but aggregates were visualized in only two of the five throat swabs requiring three or more passages before adenovirus cytopathology was evident. In all, 19 of the

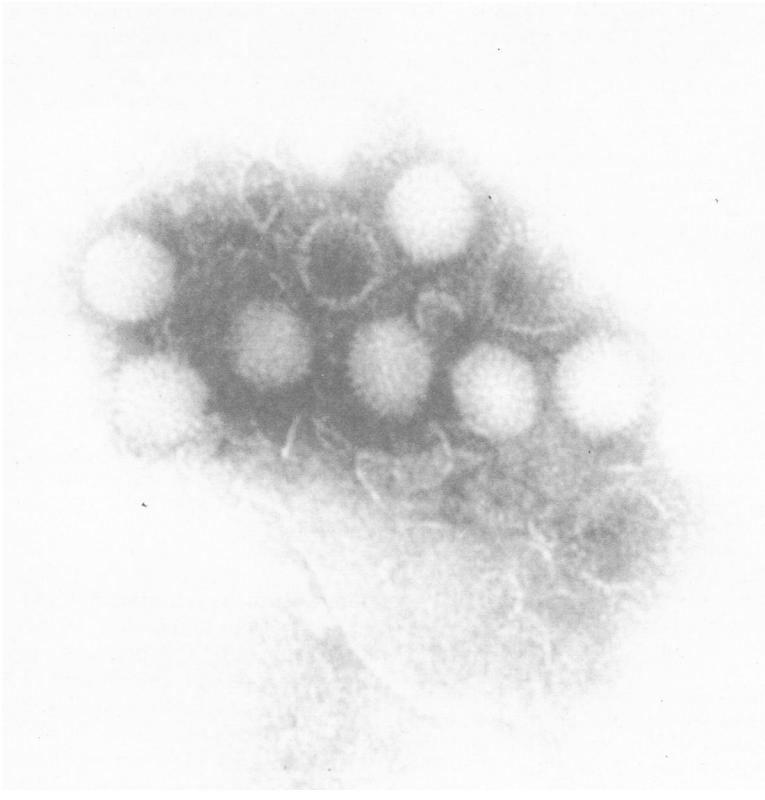

FIG. 1. *Adenovirions aggregated by immune complex with AD-7 antisera* (×200,000).

TABLE 2. *Enhancing effect of anti-IgG for detection of aggregated adenovirus by IEM*

Adenovirus titer ($TCID_{50}$/0.5 ml)[a]	Adenovirus immune serum (NtD_{50}/0.5 ml)[b]	IEM result[c]	
		Without anti-IgG	With anti-IgG[d]
25	10.00	—	3+
25	5.00	—	3+
25	2.50	—	1+
25	1.25	—	1+
25	<1.00	—	—
25	<<1.00	—	—

[a] Calculated dosage after 1:200 dilution of virus pool (10^4/ml).

[b] Neutralizing antibody units against AD-7 (two-fold serial dilutions).

[c] Extent of adenovirus aggregation; compare Table 1, footnote *c*.—, No data.

[d] One-half milliliter of optimally diluted (1:10) anti-IgG sera.

25 (76%) throat swabs positive for adenovirus by virus isolation were found to be positive by indirect IEM.

DISCUSSION

The adenovirus is an exellent model to determine the sensitivity and reliability of IEM procedures. This virus is easily recognized morphologically and is quite stable to adverse biophysical and biochemical conditions. Although they are readily isolated in tissue cultures, the final infectivity titers are usually low.

The proportion of infectious to noninfectious particles which occur during replication is unpredictable and may depend on the host cell employed. The high frequency of empty adenovirion capsids often seen in electron micrographs indicates that many particles are defective. This suggests that the sensitivity of the IEM technique may be greater than that indicated by a comparison of IEM results with infectivity titers.

The results of this investigation demonstrated that immune aggregates of AD-7 could be quickly detected (within 24 h) by a direct examination of throat swabs from patients. This method would be useful for diagnostic purposes, assuming that a laboratory has access to an

TABLE 3. *Detection of adenovirus by indirect IEM in throat swab samples positive by tissue culture isolation*

Diagnostic method	No. of positive throat swabs		
Adenovirus isolation in tissue culture	10 (Passage-1)[a]	10 (Passage-2)[a]	5 (Passage-3)[a]
Adenovirions aggregated and visualized by IEM	10 (Passage-1)[a]	7 (Passage-2)[a]	2 (Passage-3)[a]

[a] Passage first showing adenovirus cytopathic effect.

electron microscope. The experimental studies with stock AD-7 virus showed that as little as 16 to 32 $TCID_{50}$ of virus could be detected by the direct IEM technique and studies with the indirect method suggest that the sensitivity of the technique could be improved by addition of anti-IgG sera.

Rosenbaum et al. previously reported that recognition of adenovirus particles in thin sections of tissue culture could hasten the diagnosis of infections (5). The IEM method described here is even more rapid and less expensive since it obviates the need for tissue cultures, the expertise in ultramicrotomy, and expensive equipment.

Whereas the reliability of the method was not extensively investigated, the results of the throat swab studies, in which more than three-fourths of the 25 specimens were found to be positive by indirect IEM, indicated reasonable comparability with the longer tissue culture method. Furthermore, of the six specimens where no aggregates were found, three required multiple tissue culture passages before adenovirus cytopathology was detected. This indicates that the amount of virus present in those swabs was probably minimal and might not have been recovered in a re-isolation attempt.

We emphasize, however, that special care should be taken in the procurement of the initial specimen from the patient. Too often this is a haphazard affair carried out by untrained or disinterested personnel. In our investigation no special effort was made to obtain the best possible specimen from the patient, or in handling the swab to derive the optimum material. In the future we would recommend that a combination of a vigorous swabbing followed by a gargle with a larger volume (20 to 30 ml) of sampling diluent (e.g., sterile saline) might improve recovery of virus particles. Also, the small amount of time and effort required to concentrate the virus by centrifugation, ultrafiltration, or chromatographic methods would be worthwhile enhancing the success of the IEM technique.

Recently, Vassall and Ray reported that the IEM serotyping of adenovirus could be accomplished as soon as tissue culture cytopathology was evident, by using intersecting pools of adenovirus immune sera (7). Direct application of such procedures to a patient's specimen would further shorten the time required to identify these viruses.

We do not suggest that tissue culture can be eliminated entirely from the virus diagnostic laboratory, but rather that it might be applied more selectively as a back-up system if further identification or specimen work-up is required. On the other hand, where viruses are noncytolytic or have fastidious tissue or organ culture requirements, the IEM technique would be useful, if not indispensable.

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