PATHOLOGY OF EXFOLIATED OROPHARYNGEAL EPITHELIAL CELLS INFECTED WITH WILD-TYPE ADENOVIRUS

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Adenovirus disease was diagnosed in 2 hr by staining exfoliated infected oropharyngeal cells with fluorescein-tagged rabbit antisera to adenovirus types 4 and 7. The method was as sensitive as the standard virus isolation procedures, but serological cross reactions were observed. Viral antigens were detected in both the nucleus and cytoplasm of infected cells. Infection was accompanied by the outpouring of large numbers of polymorphonuclear leukocytes and smaller numbers of mononuclear cells. The method provides a model for the study of the cellular response to viral upper respiratory disease. (U)
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<td>ROLE</td>
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<td>Adenovirus</td>
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Pathology of Exfoliated Oropharyngeal Epithelial Cells Infected with Wild-Type Adenovirus

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Adenovirus disease was diagnosed in 2 hr by staining exfoliated infected oropharyngeal cells with fluorescein-tagged rabbit antisera to adenovirus types 4 and 7. The method was as sensitive as the standard virus isolation procedures, but serological cross reactions were observed. Viral antigens were detected in both the nucleus and cytoplasm of infected cells. Infection was accompanied by the outpouring of large numbers of polymorphonuclear leukocytes and smaller numbers of mononuclear cells. The method provides a model for the study of the cellular response to viral upper respiratory disease.

MATERIALS AND METHODS

The methods for preparation and conjugation of antisera have been described in detail (12). The fluorescent-antibody method described in this report shortens the diagnosis time from a minimum of 7 days to 2 hr. The method is not expected to replace the usual virus isolation techniques. However, it may find a use when there is particular need for a rapid diagnosis in special situations: when chemotherapeutic agents are used, or when a rapid shift in virus type has occurred, or as a spot check in a population where the standard virus isolation methods are not available such as in small communities or at military bases having no virus laboratory.
Adenovirus complement-fixing antibodies were detected in acute and convalescent sera from Marine Corps trainees by the micromethod of Sever using 4 to 6 units of antigen and 2 units of complement (16).

Fluorescent-antibody conjugates. Globulins were prepared from the hyperimmune rabbit serum by precipitation with cold 3.32% (NH₄)₂SO₄ (6). Ammonium sulfate was removed by dialysis against 0.15 M sodium chloride. Total protein, albumin, and globulin were assayed by the biuret method.

Purified globulin suspended in 0.15 M sodium chloride was diluted in 0.5 M pH 3.0 carbonate-bicarbonate buffer so as to contain 10 mg of protein globulin/ml of solution. Fluorescein isothiocyanate isomer I (FITC, BioQuest, Division of Becton, Dickinson, and Company) was added to the globulin suspension at a ratio of 0.02 mg of FITC/mg of protein globulin. After incubation of the reaction-mixture, excess unconjugated FITC was removed by dialysis.

Fluorescent-antibody conjugates cross-reacted with other adenovirus types in vitro unless they were first absorbed with HeLa-M cells at a concentration of 4 ml of packed cells/3 ml of FITC-conjugated antiserum as was done. Preincubation of antigen with unconjugated specific antisera blocked specific staining by fluorescein-tagged antisera.

Fluorescein-tagged antisera were titrated in the usual fashion by the direct fluorescent-antibody staining method. Target antigens were HeLa-M cells infected with the appropriate adenovirus type. Infected cells were removed to cover slips, stained, and mounted on microscope slides in Elvanol (Du Pont) (12). Stained cells were examined for fluorescence on a Zeptopan research microscope using a 3.0-mm BG 12 exciter filter and a GG 9 or GG 13 barrier filter.

Trainees. A total of 57 Marine Corps trainees were studied with oropharyngeal scrapings and anesthesia. The group on which studies were essentially complete included four trainees hospitalized for acute respiratory disease and eight trainees seen as outpatients for acute respiratory disease (ARD) (Table I).

Trainees were studied between 13 and 23 March 1972 during the final stages of an adenovirus type 4 epidemic. From 6 March until 31 March 1972, 27 trainees were admitted to the hospital for acute respiratory disease and adenovirus type 4 was isolated from 15 of these men. During the same period of time, 155 trainees were seen at the dispensary for ARD, and adenovirus type 4 was isolated from 33 of these men. The Virology Division isolated adenovirus type 7 from only one man during the period from 13-23 March.

Isolation and identification of adenovirus from the infected trainees have been described (18). Oropharyngeal cells were removed by scraping with a wooden coffee stirrer (Fig. 1; Forster Mfg. Co., Wilton, Maine). Epithelial cells were smeared on cover slips, fixed for 60 sec in cold acetone, and stained by the direct method with fluorescein tagged antisera to adenovirus strains. Fluorescein-tagged antisera were used at dilutions of 1:20 to 1:30. As an alternative,

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hospital (H) or outpatient (O)</th>
<th>Adenovirus isolation</th>
<th>Adenovirus complement fixation</th>
<th>Throat scraping stained with conjugate against:</th>
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<tr>
<td>GSK</td>
<td>H Ad 7</td>
<td></td>
<td>Adenovirus type 4</td>
<td>Adenovirus antisem</td>
</tr>
<tr>
<td>CQP</td>
<td>O Ad 4</td>
<td>&lt;1:2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RJH</td>
<td>O Ad 4</td>
<td>1:8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MM</td>
<td>O Ad 4</td>
<td>N.T.</td>
<td>+</td>
<td>N.T.</td>
</tr>
<tr>
<td>JML</td>
<td>O Ad 4</td>
<td>&lt;1:2</td>
<td>-</td>
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<tr>
<td>PRD</td>
<td>H None</td>
<td>1:8</td>
<td>+</td>
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</tr>
<tr>
<td>LWW</td>
<td>H None</td>
<td>1:4</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HS</td>
<td>H None</td>
<td>1:4</td>
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<tr>
<td>LCP</td>
<td>O None</td>
<td>&lt;1:2</td>
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<tr>
<td>SMM</td>
<td>Non</td>
<td>1:32</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DEF</td>
<td>O Non</td>
<td>1:32</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NG</td>
<td>O None</td>
<td>1:8</td>
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*N.T.: Not tested.
epithelial cells were smeared on a microscope slide, fixed in ethyl alcohol, and stained by the method of Papanicolaou [13].

RESULTS
Detection of adenovirus antigens. Table 1 includes the data of the patients from whom serological and fluorescent-antibody studies were complete. Fluorescent staining detected adenovirus in cells from 10 of the 12 trainees tested, and virus was isolated from five of the trainees. In four trainees the same adenovirus type was detected by both isolation and fluorescence; however, in one instance the adenovirus type was incorrectly identified by fluorescence. Epithelial cell scrapings were recorded as negative from trainee LCP, even though a complement-fixation test indicated that adenovirus disease had been present. Adenovirus type 4 was isolated from patient .CQF on two occasions separated by a 7-day period. The scraping was positive for fluorescence only at the time of the second dispensary visit.

Two additional trainees (DEF, NG) demonstrated positive fluorescence even though there was no fourfold rise in complement-fixing antibodies and no virus was isolated. Similarly, one patient (RJS) shed virus and demonstrated fluorescent epithelial cells in the absence of a fourfold rise in antibody.

Pathology of the inflammatory response. A large number of leukocytes were admixed with the epithelial cells obtained by scraping the oropharynx. The leukocyte response consisted mainly of polymorphonuclear leukocytes, although rare mononuclear cells were also seen. Inflammatory cells were present in greatest numbers in the hospitalized trainees in whom severe and sometimes exudative pharyngitis occurred. Increased numbers of leukocytes were associated with increased destruction and alteration of epithelial cells, and an increased amount of cellular debris was present, some of which demonstrated specific staining for adenovirus antigens.

Polymorphonuclear leukocytes emitted bright nonspecific autofluorescence when the epithelial cell scrapings were examined under the fluorescence microscope (Fig. 2). Polymorphonuclear leukocytes showed bright green fluorescence regardless of the specificity of the conjugate which was applied, and fluorescence occurred in the complete absence of epithelial cell staining. It became important to differentiate on morphological grounds leukocyte with autofluorescence from epithelial cells with specifically stained intracellular adenovirus antigens. Furthermore, we could not exclude the possibility that some of the leukocytes had ingested or were infected by adenovirus antigens and were stained because they contained

![Fig. 2. Autofluorescence of polymorphonuclear leukocytes in preparations of scrapings from the infected oropharynx. This type of green fluorescence can sometimes be confused with the specific fluorescence of stained adenovirus-infected cells. Glycerine, ×600.](image-url)
such material. Studies with rhodamine-tagged antisera would have evaluated this possibility, because the leukocytes would have emitted green autofluorescence, whereas rhodamine-tagged antisera would have stained viral antigen red.

**Pathology of infected epithelial cells.** Epithelial cells scraped from the oropharynx of infected trainees demonstrated a cytopathogenic effect typical of adenovirus-infected tissue culture preparations. A striking feature was the similarity between the appearance of clusters of round cells in both the natural infection and in the infection of cell monolayers in tissue culture. Clusters were noted in both Papanicolaou-stained (Fig. 3) and fluorescein-stained (Fig. 4) preparations from the throat scrapings. Infected Papanicolaou-stained cells were characterized by enlargement of the nucleus, lighter staining of nuclear material, and shrinking of the cytoplasm until it formed a narrow ring around the nucleus. Contrast adenovirus-infected epithelial cells (Fig. 3) with scrapings from the buccal mucosa of a volunteer who was uninfected by adenovirus (Fig. 5).

Adenovirus antigens appeared in the nuclei of epithelial cells in the form of 10 to 25 granular clusters (Fig. 6. 7). In other cells the entire nucleus was filled with antigen (Fig. 8). Finally, cells were seen in which the staining was confined chiefly to the cytoplasm with little or no nuclear staining (Fig. 9). As the antigenic material in the nucleus increased and eventually entered the cytoplasm, the cell nuclei increased in size and the cytoplasm became less prominent. Cells at all stages of infection were noted in the same individual; however, cells with cytoplasmic viral antigens were found several times more often than cells with dense nuclear staining.

**DISCUSSION**

Fluorescent-antibody methods were capable of detecting adenovirus antigens in epithelial cell scrapings obtained from infected Marine Corps trainees. In trainees in whom studies were complete, the fluorescent-antibody technique demonstrated virus at approximately the same frequency as standard virus isolation techniques (Table 1); however, a major disadvantage that will require further work with various dilutions of fluorescein-tagged antisera is that conjugates were not completely specific for a given adenovirus type. Furthermore, there is also the possibility that fluorescein-tagged adenovirus antiserum might stain cells obtained from trainees naturally infected with other viral agents such as coxsackie A-21, parainfluenza, or influenza strains.

After reagents were prepared, the fluorescent-antibody method had the advantage of

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**Fig. 3.** Small cluster of exfoliated epithelial cells showing adenovirus cytopathogenic effect. Papanicolaou-stained. Magnification, ×430.
Fig. 1. Cluster of adenovirus-infected epithelial cells similar to Fig. 3 except that staining is with fluorescein-conjugated adenovirus type 4 antiserum diluted 1:20. Glycerine, x600.

Fig. 5. Papanicolaou-stained oropharyngeal epithelial cells from an uninfected volunteer. Note the small, dense nucleus, and the large area occupied by the cytoplasm. Magnification, x430.
Fig. 6, 7. Fluorescent nuclear granules of adenovirus type 4-infected human epithelial cells. The increased nuclear-cytoplasmic ratio indicates that the cell in Fig. 6 appears to be at a slightly more advanced stage of infection than that of Fig. 7. Glycerine, x600.

Rapidity, since a diagnosis could be reached approximately 2 hr after a scraping was obtained, whereas the usual methods required 1 to 3 weeks before virus proliferation caused a cytopathogenic effect in tissue culture and before virus neutralization could be carried out. The cost of cover slips, microscope slides, and reagents for fluorescence microscopy was small as compared with tissue culture tubes, media, and neutralization plates required by the standard procedures.

It was of additional interest that Papanicolaou-stained scrapings demonstrated a cytopathogenic effect similar to that seen in laboratory tissue cultures infected with adenovirus strains. It is possible that a rapid presumptive
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Fig. 8. Two adenovirus-infected cells showing intense specific nuclear staining for adenovirus type 4. Glycerine. ×600.

Fig. 9. Clusters of adenovirus-infected cells in which viral antigen appears mainly in the cytoplasm. This type of staining was seen more frequently than nuclear staining. Glycerine. ×600.

diagnosis of adenovirus disease could be obtained in a large proportion of patients by the examination of Papanicolaou-stained, exfoliated, oropharyngeal cells. This likelihood should be evaluated if future epidemics occur. Of particular interest was the cellular inflammatory response which accompanied pharyngeal adenovirus infections. Leukocytes probably play a role in the immune response to viral respiratory disease. These authors and
many others have suggested that surface neoantigens induced by intracellular virus infection may induce a cellular as well as an antibody-mediated immune reaction to virus-infected target cells (K. Hayashi, J. D. Rosenthal, and A. L. Notkins. Abstr. Anz. Meet. Amer. Soc. Microbiol., p. 235, 1972; references 3 and 11). It is possible that the inflammation present in the respiratory passages of patients infected with lytic viruses, such as adenovirus types, may in part be due to the interaction of specific antibody and sensitized immune cells with virus-infected cells. Notkins and co-workers have demonstrated that surface antigens coded for by the genetic material of intracellular virus may release factors which reacting with infected cells as well as with extracellular virus may release factors which promote the accumulation of inflammatory cells in the nasal and pharyngeal passages.

The predominant leukocyte present in the scrapings was the polymorphonuclear leukocyte, and there were only a few monocytes and lymphocytes present. Clyde has described a leukocyte response to experimental Mycoplasma pneumoniae infection in Syrian hamsters (7). Polymorphonuclear leukocytes formed the predominant cell-mediated response beginning 9 days after infection. After 2 weeks lymphocytes and other mononuclear cells entered the bronchial spaces in large numbers. A characteristic phenomenon was the formation of "rosettes" of immunocytes around epithelial cells which were also characteristic of the response when leukocytes were cultured in vitro. Clyde postulated that such rosettes might be present in sputa obtained from patients with M. pneumoniae pneumonia. His studies of M. pneumoniae disease in hamsters, combined with the observation of cellular response to adenovirus disease in vivo, should prompt further evaluation of cellular immunological responses in adenovirus infections. In particular, it would be interesting to see whether the convalescent stages of adenovirus disease are associated with an increased number of mononuclear leukocytes in the pharyngeal scrapings or nasal wash.

This study clearly confirms earlier work which described the distribution of intracellular antigen in adenovirus-infected cells (2, 14). The later stages of adenovirus infection in vitro were associated with large numbers of cells in which antigen took up residence primarily in the cytoplasm. In this study, adenovirus antigens were located most frequently in the cytoplasm of cells which were obtained from the oropharynges of infected trainees.

After inoculation of throat gargle specimens onto tissue for virus isolation, epithelial cells are frequently seen floating in the media. This study indicated that such epithelial cells may be filled with virus. Single adenovirus-infected cells have been estimated to contain up to 20,000 infectious virus particles (14), and it is these "packages" of virus which may provide the bulk of the infectious virus particles obtained in a throat gargle specimen.

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LITERATURE CITED


