

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.

With the advent of adenovirus vaccines and the possibility of the use of chemotherapeutic agents for virus infections (5, 9, 10, 15), the rapid diagnosis of infections causing acute respiratory disease becomes ever more important. Fluorescent antibody methods have been used to detect in the tissues of patients a variety of viral agents including influenza virus (17), *Herpesvirus hominis* (1), and long-incubation hepatitis virus (8), not to mention the widespread application of fluorescent-antibody techniques for the rapid diagnosis of bacterial infections in a clinical setting. Furthermore, fluorescent-antibody techniques have been used to study the synthesis of adenovirus antigens in tissue culture preparations (2, 14).

Recurring epidemics of adenovirus disease in a Marine Corps population have enabled this laboratory to prepare and test fluorescent-antibody reagents for the rapid detection of adenovirus antigens infecting oropharyngeal cells. 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 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, 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.

MATERIALS AND METHODS

The methods for preparation and conjugation of antisera have been described in detail (12).

Tissues. Continuous human epithelial (HEp-2) cells, HeLa-M cells, primary human embryonic kidney (HEK), and human embryonic fibroblast cells (WI-38) were maintained in Eagle minimum essential medium supplemented with 2 to 5% heat-inactivated fetal calf serum.

Virus prototypes. Adenovirus type 3 (strain G.G.), adenovirus type 4 (strain RI-67), and adenovirus type 7a (strain S-1058) were free from mycoplasma contamination and were provided by Sylvia Cunningham, Medical Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Antisera. Adenovirus antigens were prepared in HEp-2 or HEK cells and specific antisera were produced in white rabbits (4). Preimmunization bleedings were obtained by cardiac puncture. On day 1, the rabbit was inoculated subcutaneously with 4.0 ml of undiluted adenovirus antigen emulsified in 4.0 ml of complete Freund's adjuvant (Difco); day 7, 1.0 ml of undiluted antigen was inoculated intravenously and 5.0 ml of antigen intraperitoneally; day 14, 1.5 ml of antigen was inoculated intravenously and 5.0 ml intraperitoneally; day 21, 2.0 ml of antigen was inoculated intravenously and 5.0 ml intraperitoneally; day 28, trial bleeding. Antisera prepared in this way titered 1:512 to 1:2048 against 100 median tissue culture infective dose of type-specific adenovirus by neutralization.

Serology. Rabbit serum-neutralizing adenovirus antibodies were titered in microplates by the method of Rosenbaum, Edwards, and Sullivan (Amer. Pub. Health Ass. 97th Annu. Meeting, Philadelphia,

1969). Dilutions of serum and virus were incubated at room temperature for 2 hr and applied with a suspension of HEp-2 cells to the wells of a microtiter plate (U-shaped wells, Cooke Engineering). Neutralizing antibody titers were read after 3 days of incubation.

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 3.52 M $(\text{NH}_4)_2\text{SO}_4$ (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 sodium chloride was diluted in 0.5 M pH 9.0 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 1 ml of packed cells/3 ml of FITC-conjugated antisera as was done. Preincubation of antigen with unconjugated specific antisera blocked specific staining by fluorescein-tagged antisera.

Fluorescein-tagged antisera were titered 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 Zetopan research microscope using a 3.0-mm BG 12 exciter filter and a GG 9 or GG 13 barrier filter.

Trainees. A total of 37 Marine Corps trainees were studied with oropharyngeal scrapings and a throat gargle for virus isolation. Acute sera were obtained from all trainees; however, because of conflicts in training, convalescent sera were studied from only 10 trainees. 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 1).

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, 175 trainees were seen at the dispensary for ARD, and adenovirus type 4 was isolated from 25 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 1. Results of staining adenovirus-infected oropharyngeal cells with fluorescein-tagged adenovirus antiserum

Patient	Hospital (H) or outpatient (O)	Virus isolation	Adenovirus complement-fixation		Throat scraping stained with conjugate against:	
			Acute	Convalescent	Adenovirus type 4	Adenovirus type 7
GSK	H	Ad 7	N.T. ^a	N.T.	N.T.	+
CQF	O	Ad 4	<1:2	1:64	+	-
RJS	O	Ad 4	1:8	1:8	-	+
MM	O	Ad 4	N.T.	N.T.	+	-
JML	O	Ad 4	<1:2	1:64	+	-
PRD	H	None	1:8	1:64	+	-
LWW	H	None	1:4	1:32	+	-
RS	H	None	1:4	1:64	-	+
LCP	O	None	<1:2	1:64	-	-
SMM	O	None	1:32	1:32	-	-
DEF	O	None	1:32	1:32	+	-
NG	O	None	1:8	1:8	+	-

^a N.T.: Not tested.

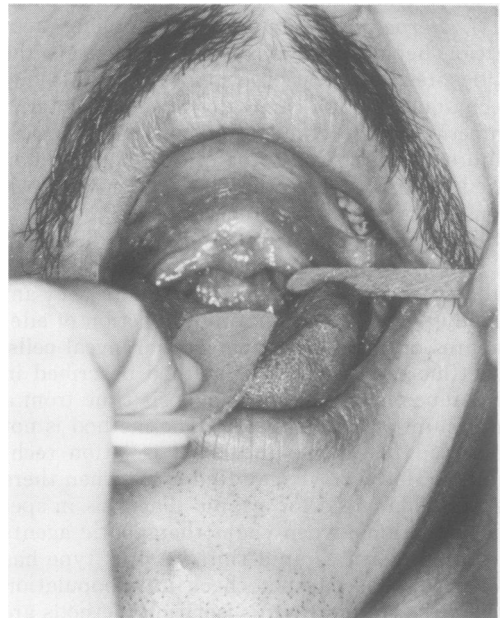


FIG. 1. Method for obtaining epithelial cell scrapings in the field. Erythema was frequently the only physical finding upon examination of the oropharynx of adenovirus-infected trainees.

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 leukocytes 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

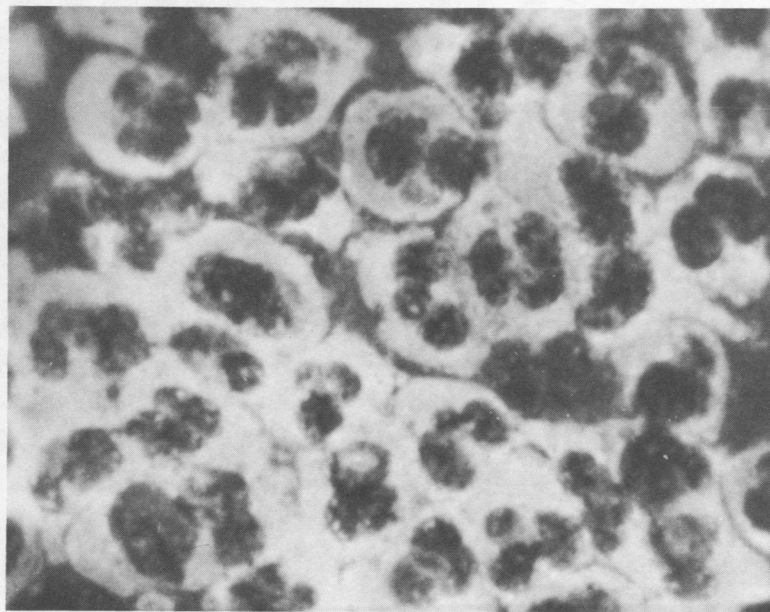


FIG. 2. Autofluorescence of polymorphonuclear leukocytes in preparations of scrapings from the infected oropharynx. This type of green fluorescence can sometimes be confused with the specific fluorescence of stained adenovirus-infected cells. Glycerine, $\times 600$.

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

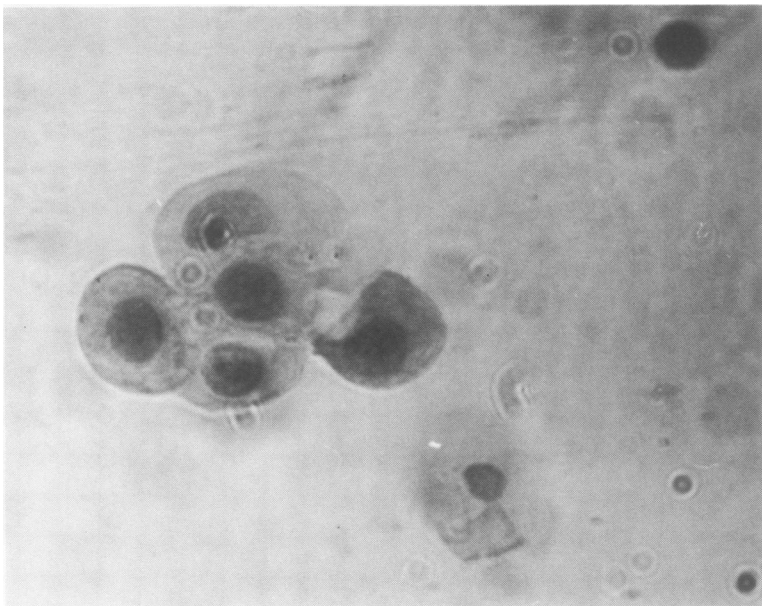


FIG. 3. Small cluster of exfoliated epithelial cells showing adenovirus cytopathogenic effect. Papanicolaou-stained. Magnification, $\times 430$.

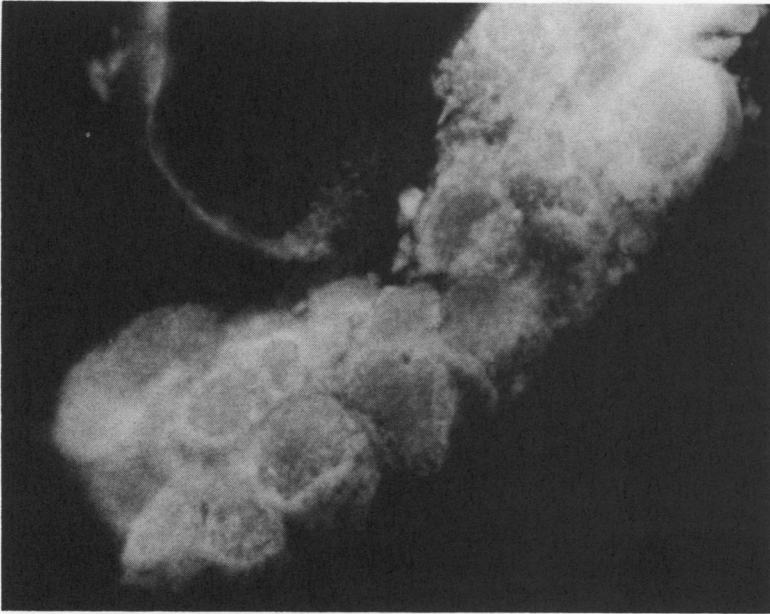


FIG. 4. Cluster of adenovirus-infected epithelial cells similar to Fig. 3 except that staining is with fluorescein-conjugated adenovirus type 4 antiserum diluted 1:20. Glycerine, $\times 600$.

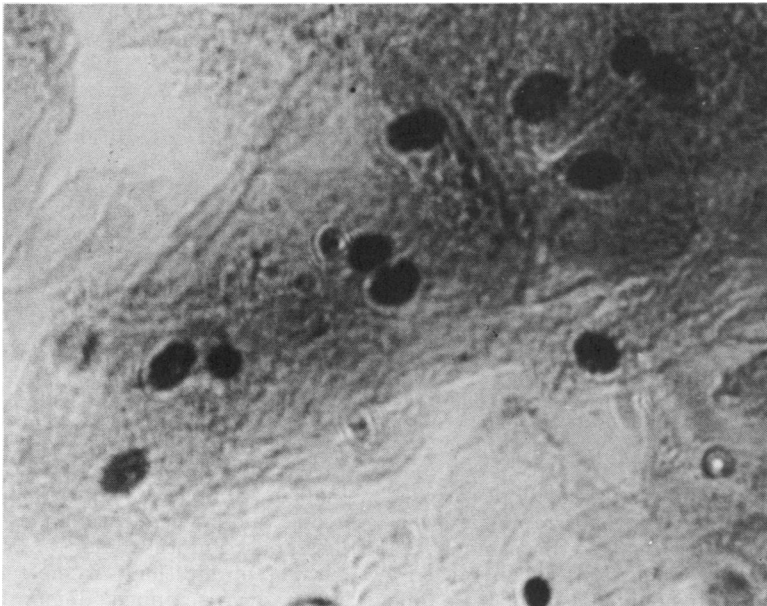


FIG. 5. Papanicolaou-stained oropharyngeal epithelial cells from an uninfected volunteer. Note the small, dense nucleus, and the large area occupied by the cytoplasm. Magnification, $\times 430$.

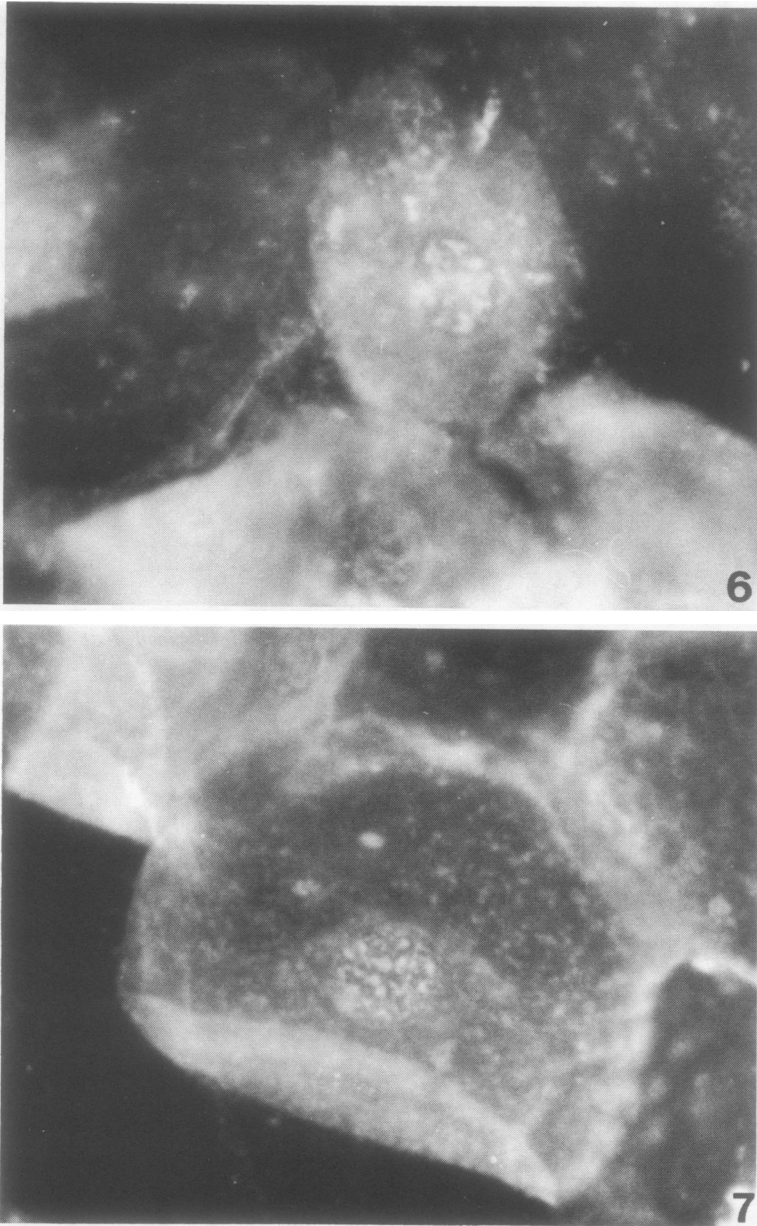


FIG. 6, 7. Fluorescent nuclear granules of adenovirus type 4-infected human epithelial cells. The increased nuclear-cytoplasmic ratio indicates that the cell in Fig. 6 appears to be at a slightly more advanced stage of infection than that of Fig. 7. Glycerine, $\times 600$.

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

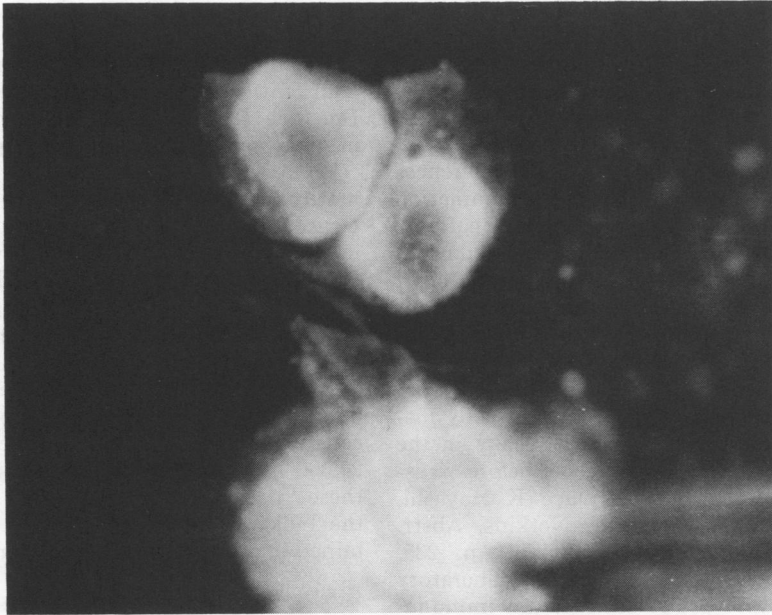


FIG. 8. Two adenovirus-infected cells showing intense specific nuclear staining for adenovirus type 4. Glycerine, $\times 600$.

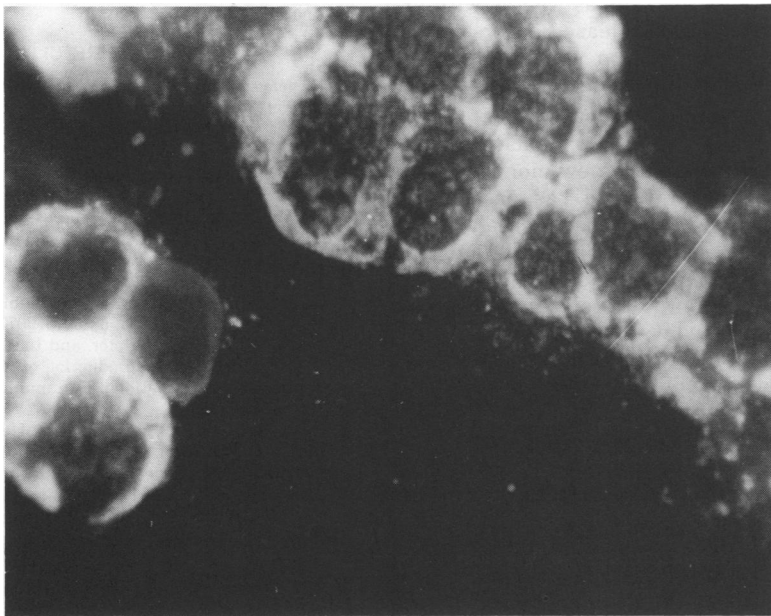


FIG. 9. Clusters of adenovirus-infected cells in which viral antigen appears mainly in the cytoplasm. This type of staining was seen more frequently than nuclear staining. Glycerine, $\times 600$.

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. Ann. 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 neoantigens coded for by the genetic material of intracellular virus induce an antibody-mediated immune response which results in the destruction of infected cells even before virus-specific cell lysis had taken place (K. Hayashi, J. D. Rosenthal, and A. L. Notkins. Abstr. Ann. Meet. Amer. Soc. Microbiol., p. 235, 1972; reference 3). Studies in this laboratory have shown that pre-existing serum immunoglobulin G and A molecules enter the nasal passages during acute adenovirus disease. Specific antibody and complement components 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 a cellular response to adenovirus disease in man, 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 oropharynxes 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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