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Characteristics of an Adenovirus Type 19 Conjunctivitis Isolate and Evidence for a Subgroup Associated with Epidemic Conjunctivitis

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Although adenovirus type 19 (Ad19) was first described in 1955, this virus was not associated with disease until its isolation from outbreaks of conjunctivitis in 1973. A strain of Ad19 isolated from a case of conjunctivitis in Seattle in 1974 was compared with the reference strain (3911). Plaque number and size were enhanced by 30 mM $MgCl_2$. Low pH and chloroform treatment had no effect on either strain's activity, but the two strains were sensitive to pH 8. Growth curves were characteristic of adenoviruses, but differences were seen in the amount of virus released. The ratios of particles to plaque-forming units (approximately 10,000:1) were similar for both. Both virus preparations contained high concentrations of group-specific complement-fixing antigen. Cross-reactions were seen by hemagglutination inhibition and immunoelectron microscopy between antisera to Ad8, Ad9, and Ad10 versus both strains of Ad19, but were not seen by neutralization. We would like to suggest, based on exclusive conjunctivitis association and cross-reactions, that the four cross-reacting serotypes, Ad8, Ad9, Ad10, and Ad19, represent a subgroup of adenoviruses specifically associated with conjunctivitis.

Adenovirus type 19 (Ad19) was first isolated by Bell et al. in 1955 from a child with trachoma in Saudi Arabia (2, 3). No reports of its association with outbreaks of disease appeared in the literature until 1973, although retrospective serological evidence (11) suggested a high prevalence (85%) of Ad19 infections in individuals in Mali and Zaire. In early 1973, Ad19 was isolated from an outbreak of conjunctivitis in the eastern United States (20); later that year and during 1974, its isolation from conjunctivitis outbreaks was reported in Canada (5), Belgium (11), Scotland (1), London (8), and elsewhere in the United States (4, 17). During summer and fall of 1974, Ad19 was isolated in our laboratory from conjunctival specimens from patients with conjunctivitis having a distinct hemorrhagic component resembling acute hemorrhagic conjunctivitis recently shown to be associated with enterovirus type 70 (31). Although adenoviruses were frequently isolated during a surveillance study for respiratory agents in families (the Seattle Virus Watch, 1965 to 1969), Ad19 was not encountered (7, 14). As a result of its sudden appearance in association with conjunctivitis, it was of interest to investigate a representative strain of these Ad19 isolates to determine whether its properties varied from those of the 1955 reference strain of Ad19 and whether either strain was serologically related to the other adenovirus strains associated with conjunctivitis.

MATERIALS AND METHODS

Cell culture. A human fetal tonsil diploid cell line, FT, and two human heteroploid lines, KB and HeLa-M, were used for Ad19 isolation, propagation, and plaque assay, respectively. Growth medium for FT and KB cells was autoclavable Eagle minimal essential medium (MEM; Flow Laboratories Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (MEM-10). The HeLa-M cells were grown in suspension culture using MEM-10 with an increased concentration of L-glutamine (6.4 mM) and lacking divalent cations. MEM-1 was used as maintenance medium for HeLa-M and KB cultures, while Leibowitz-15 medium (Flow Laboratories) supplemented with 2% fetal bovine serum was used as maintenance medium for FT cells. All media contained 100 U of penicillin and 100 μg of streptomycin per ml.

Virus propagation. One of the Ad19 strains (CJ009), isolated in our laboratory from conjunctival swabs from a patient with conjunctivitis, was chosen as a representative strain for study. After isolation on FT cells, it was passed twice in FT, 6 times in HeLa, and 10 times in KB cells. KB cell monolayers in 32-ounce (0.946-liter) bottles were inoculated with passage 10 KB fluid. After 3 days of incubation at 37°C, the cells and supernatant were homogenized in a VirTis 45 homogenizer (The VirTis Co., Inc., Gardiner, N.Y.), mixed with Genesolv D (2:1), agitated for 5 min,

and centrifuged at 2,000 rpm for 10 min. The supernatant was removed, dispensed in 1-ml portions, and frozen at −70°C for use in later experiments. Ad19 strain 3911 was obtained from the Research Resources Branch, National Institue of Allergy and Infectious Diseases, Bethesda, Md., as KB cell culture fluid. Strain 3911 was propagated in FT cells to raise the titer, followed by two passages in KB cells. A virus pool was produced by infecting KB monolayers in 32-ounce bottles. Cells and fluid were harvested, homogenized, and treated with Genesolv as described above. Samples (1 ml) of the final virus pool were frozen at −70°C.

Plaque assay. Tissue culture plates (60-mm diameter; Corning Glass Works, Corning, N.Y.) were seeded with 1.5×10^6 to 2.0×10^6 HeLa cells per plate suspended in 5 ml of MEM-10. After 4 h of incubation at 37°C in 5% CO_2, the medium was aspirated from the plates, and they were inoculated with 0.2 ml of virus dilution or control inoculum. The plates were held at room temperature for 1 h for adsorption, overlaid with 5 ml of overlay medium consisting of 0.5% agarose (Bio-Rad Laboratories, Richmond, Calif.) and MEM-1 with 30 mM $MgCl_2$, and incubated at 37°C in a 5% CO_2 atmosphere. After 3 days of incubation, 2 ml of fresh overlay medium without Mg^{2+} was added to the plates. The plates were reincubated until day 6, when they were fixed with 10% Formalin, the overlay was discarded, and the monolayer was stained with 1% crystal violet. Plaques were counted, and the results were expressed as plaque-forming units (PFU) per milliliter unless otherwise noted. For plaque reduction serum neutralization titers, the virus was diluted to 1,000 PFU/ml and mixed with an equal volume of the appropriate antiserum dilutions. After a 1-h incubation, serum-virus mixtures were plated (0.2 ml/plate) for plaque assay as above. The diluent for preparing serum and virus dilutions and the inoculum for control plates consisted of Hanks balanced salt solution, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 8 mM $NaHCO_3$.

Evaluation of the effect of temperature and $MgCl_2$. Four plaque assays were set up simultaneously, overlaid with overlay media with or without 30 mM $MgCl_2$, and incubated at either 33 or 37°C in 5% CO_2 environments. Plaque diameter was measured with calipers and expressed as the average of 20 plaques from four plates. Cell controls were carried out for each set of growth conditions using diluent as the inoculum.

pH sensitivity. Virus was diluted 1:10 in the appropriate buffer and incubated at 37°C for 1 h. From this reaction mixture, 0.2 ml was removed and diluted 1:10 in cold diluent. The treated virus dilutions were then inoculated into HeLa plates for plaque assay. Buffer controls, with diluent substituted for virus at each pH unit, were plated onto monolayers to determine buffer effect on cells. Buffers used were glycine-hydrochloride (6) at pH 2.0 and 3.0, citric acid-sodium citrate (10) at pH 4.0, 5.0, and 6.0, phosphate buffer (6) at pH 7.0 and 8.0, and glycine-NaCl-NaOH (6) at pH 9.0 and 10.0.

Growth curve. Tubes containing 2×10^5 KB cells per tube were inoculated with 0.2 ml of virus suspension at a multiplicity of infection of 0.1 and incubated at 37°C. At specified intervals, two tubes were removed from the incubator, and the supernatant from the tubes was removed, pooled, and frozen at −70°C. Fresh MEM-1 (1 ml) was then added to each tube, and the tubes were also frozen at −70°C to be assayed for cell-associated virus. After thawing, the cells were scraped from the glass into the media, and the contents of the two tubes were pooled and subjected to three cycles of freeze-thawing. The cell-associated and supernatant virus suspensions were assayed for infectious virus at each time interval.

Electron microscopy. Virus particles were quantitated by a modification of Watson's loop-drop technique (33). A 0.1-ml quantity of virus was mixed with 0.1 ml of polystyrene latex particles (0.481-μm diameter) obtained from Polysciences, Inc., Warrington, Pa., and 0.1 ml of 0.2% phosphotungstic acid, pH 7.0. A 1-μl drop of this mixture was transferred to the surface of a Parlodion-coated 400-mesh grid and dried. Both virus and latex particles were counted, 15 squares from four grids per virus, and the total number of virus particles was determined by comparison with a known concentration of latex particles. Agglutination studies were carried out by the technique of Luton (23) by mixing 0.1 ml of the appropriate antiserum diluted 1:20 with an equal volume of virus, allowing 1 h at room temperature for the reaction to occur, and then transferring a drop to the surface of a Formvar-coated 200-mesh grid (Ernest F. Fullam, Inc., Schenectady, N.Y.). The grid was dried for 1 min and then stained with 2% phosphotungstic acid, pH 7.0. All grids were prepared in duplicate and examined on a JEOL JEM-100S transmission electron microscope. Adeno-associated virus was not seen in any of the preparations examined.

Serology. Complement fixation (CF), hemagglutination (HA), and hemagglutination inhibition (HI) tests were carried out by standard techniques (28). Adenovirus antisera were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases (Ad12, Ad18), from the Scientific Resources Branch, Center for Disease Control, Atlanta, Ga. (Ad8, Ad11, Ad14), or from Microbiological Associates, Bethesda, Md. (Ad1, Ad2, Ad3, Ad4, Ad7). Antisera to Ad9, Ad10, Ad16, Ad19, and Ad29 are courtesy of John P. Fox (30). A pool of human adenovirus-positive CF antisera was used to titrate CF antigen. Sprague-Dawley rat erythrocytes were obtained from the University of Washington Vivarium, and rhesus erythrocytes were obtained from the Regional Primate Center, University of Washington, Seattle, Wash.

RESULTS

Plaque assay. Both virus strains were confirmed as Ad19 by neutralization with anti-Ad19 hyperimmune serum. The conjunctivitis isolate, strain CJ009, had a titer of 10^4 PFU/ml and $10^{3.5}$ 50% tissue culture infective doses per ml, and the reference Ad19, strain 3911, showed a titer of 5×10^5 PFU/ml and $10^{4.5}$ 50% tissue culture infective doses per ml. Maximum plaque sizes and numbers occurred at 37°C in the presence

of 30 mM $MgCl_2$ (Table 1). Titers and plaque size were enhanced by the presence of Mg^{2+} at 37°C for both strains. At 33°C, strain 3911 showed significantly enhanced titer ($P < 0.001$) and plaque size in the presence of Mg^{2+}, whereas strain CJ009 did not show significant enhancement.

Physical characteristics. Adenoviruses are characteristically insensitive to the effects of low pH (15). The viruses were stable to pH values below 8. At pH 8, a slight decrease was seen in strain 3911, whereas strain CJ009 decreased from 100 to 40% of the pH 7 control. Strain 3911 showed a corresponding decrease at pH 9, dropping from 90 to 36% of the pH 7 control, and both virus strains showed 13% activity at pH 10. In addition to their resistance to the effects of low pH, neither virus strain was chloroform sensitive (data not shown).

Growth curves. Growth curves were carried out for both strains. Although two cycles were visible with both strains, probably the result of the unavoidably low multiplicity of infection, the timing was characteristic of an adenovirus growth curve as described by Philipson et al. (27). Strain CJ009 differed from strain 3911. For strain CJ009, the first cycle began with a rise in cell-associated virus at 10 h, leveling off at 16 to 18 h. The second cycle began at 28 h and leveled off at 32 h. Although the cell-associated virus rose first, the supernatant virus titer increased rapidly after 12 h and exceeded the cell-associated virus titer by one-half log after 20 h of incubation, reflecting virus release. The timing of the cycles was the same for strain 3911, with maxima at 18 and 32 h, although neither supernatant nor cell-associated virus dropped below 10^2 PFU/ml. Growth, as measured by cell-associated virus, was characteristic of a two-cycle growth curve, but the supernatant virus, although showing shallow peaks at 18 and 32 h, did not vary from a range of 10^3 to 10^4 PFU/ml. It appeared that most of the virus produced remained intracellular or cell associated because the supernatant did not increase significantly at times when cell-associated virus was at a maximum.

Ratio of particles to PFU. The ratio of particles to PFU was determined for both Ad19 strains (Table 2). Although counts of both PFU per milliliter and particles per milliliter were higher for strain 3911 than for strain CJ009, the ratios of particles to PFU were not significantly different with large numbers of particles in each preparation. However, this technique (33) does not measure the amount of partially disrupted particles or virus protein present in the virus stocks.

Serology. The level of group-specific CF antigen present in our virus stocks was determined by block titration (28) using a pooled human CF-positive antiserum (1:128 titer). Strain CJ009 showed a CF antigen titer of 1:128, whereas strain 3911 showed a titer of 1:64 (Table 2), values which were consistent over three successive experiments. The CF antigen-antibody curve resembled a precipitin curve, with a prozone at high antigen-high antibody concentrations (data not shown).

TABLE 1. *Effect of temperature and Mg^{2+} on the growth of two strains of Ad19*[a]

Temp (°C)	Mg^{2+}	Strain CJ009		Strain 3911	
		Titer (PFU/ml)	Plaque size[b] (mm)	Titer (PFU/ml)	Plaque size[b] (mm)
37	+	5.50×10^{3}[c]	1.04	7.66×10^{5}[d]	1.72
37	−	2.32×10^{3}[c]	0.65	2.58×10^{5}[d]	0.64
33	+	3.73×10^{4}[e]	0.63	6.76×10^{5}[d]	0.99
33	−	2.20×10^{4}[e]	0.43	1.12×10^{5}[d]	0.55

[a] The results presented are the average of two experiments.
[b] Average diameter of 20 plaques per experiment.
[c] $P < 0.01$.
[d] $P < 0.001$ by Student's t test.
[e] Not significant, $0.1 < P < 0.2$.

TABLE 2. *Properties of two strains of Ad19*

Strain	PFU/ml	Ratio of particles to PFU[a]	CF antigen titer[b]	HA titer[c]
CJ009	10^4	25,000:1	128	64
3911	5×10^5	9,600:1	64	1,024

[a] Particle number was determined by the method of Watson (33). The difference is not significant, $P > 0.05$, by an approximate test of the significance of the difference in the logarithms of the data used to derive the ratio.
[b] Results are expressed as the reciprocal of the highest dilution of virus causing complete CF with a known positive anti-CF serum.
[c] Results are expressed as the reciprocal of the highest dilution of virus causing complete HA of Sprague-Dawley rat erythrocytes.

Adenoviruses can be grouped according to the species of erythrocytes that they agglutinate (29). Ad19 is known to be in HA group II, and both strains showed the characteristic pattern of complete HA with rat erythrocytes (Table 2) and insignificant HA with rhesus erythrocytes (data not shown).

A large number of adenovirus serotypes, 2, 3, 4, 7, 8, 9, 10, 11, 16, 19, and 29, are associated with conjunctivitis (17, 20). Antisera for these types were tested against Ad19 strains CJ009 and 3911 by HI using rat erythrocytes. Only antisera to Ad9, Ad10, and Ad19 showed HI titers for both Ad19 strains, and Ad8 antiserum inhibited strain 3911 only (Table 3). In both the homologous and heterologous reactions, the antisera showed two- to fourfold-higher titers for

strain 3911 than for strain CJ009. Of the types associated with conjunctivitis, Ad8, Ad9, Ad10, Ad19, and Ad29 are all in HA group II. Although Ad8, Ad9, Ad10, and Ad19 appear to be related, anti-Ad29 serum showed no HI titer for either Ad19 strain.

Immunoelectron microscopy was used to further test these adenovirus antisera. The results were consistent with those seen by HI (Table 3), and, in addition, the reaction between Ad8 antiserum and strain CJ009, which was not detected by HI, was detected by this more sensitive technique. None of the other antisera tested, aside from antisera to Ad8, Ad9, Ad10, and Ad19, showed any activity against the Ad19 strains. Figures 1a and b show the homologous reactions (++) for Ad19 strains 3911 and CJ009, respectively. Figures 1c and d show examples of + reactions, strain 3911 versus anti-Ad9 serum, and − reactions, strain 3911 versus anti-Ad12 serum, respectively.

The four antisera showing HI titers for the Ad19 strains were tested for neutralizing activity by plaque reduction neutralization. The hemagglutinating and neutralizing antigens are known to be at different sites on the virus, so further cross-reactivity would indicate multiple relatedness. However, this was not seen by neutralization (Table 3). If 50% reduction were to be considered significant neutralization, only strain 3911 was neutralized by antisera to Ad8 and Ad9 and only at a 1:10 dilution. CJ009 was not neutralized by any of the cross-reacting antisera at any dilution. The differences already noted between the two strains in the homologous system were evident by neutralization also.

TABLE 3. *Titers of four type-specific antisera against Ad19 strains CJ009 and 3911*

Antiserum to:	Strain CJ009			Strain 3911		
	HI[a]	IEM[b]	PRN[c]	HI[a]	IEM[b]	PRN[c]
Ad8	<20	+	<10	40	+	10
Ad9	40	+	<10	80	+	10
Ad10	80	++	<10	320	++	<10
Ad19	160	++	160	640	++	320

[a] HI titers are expressed as the reciprocal of the highest dilution of serum completely inhibiting HA. Results are representative of three experiments.

[b] IEM, Immunoelectron microscopy. All tests were carried out with antiserum at a 1:20 dilution. The predominating types of agglutination were: −, single virus; +, groups of two to three viruses; ++, large clumps of virus.

[c] PRN, Plaque reduction neutralization. Results are expressed as the reciprocal of the highest serum dilution causing greater than 50% reduction in plaque number as compared with virus control and are representative of four experiments.

DISCUSSION

Ad19 has been studied previously by a number of workers interested in its characteristics (16, 18, 24, 25, 35). Recent reports of its association with epidemic conjunctivitis and its isolation in this laboratory stimulated these investigations. Our initial findings suggested that this serotype required diploid fibroblasts for primary isolation, in contrast to other serotypes isolated in this laboratory (14). Additionally, the reference strain 3911, received in our laboratory as infected KB cell culture fluid, required passage in diploid fibroblasts for successful propagation in KB cells.

Growth curves for Ad19 strains CJ009 and 3911 showed that both followed the characteristic curve of adenovirus replication (27), with intracellular (cell-associated) virus production beginning at 10 to 12 h and showing a maximum at 16 to 18 h and with a second cycle maximum at 32 h. Wigand, using a slightly different system, showed a longer lag time before virus production (38, 39). Virus release was visible in the strain CJ009 supernatant growth curve at 20 to 22 h and again at 32 h. However, the strain 3911 supernatant virus showed maxima which, although visible, were much less striking and never exceeded the cell-associated virus titer. The high level of virus still present in the supernatant (10^3 to 10^4 PFU/ml) may mask release.

The differences seen between the growth curves of strains 3911 and CJ009 could be accounted for by differences in the multiplicity of infection, strain 3911 having a higher multiplicity of infection as demonstrated for Ad5 by Wigand and Kumel (37). It is also possible that incomplete particles or free fiber (26) may block virus binding, although in the case of particles we have shown that the ratios of particles to PFU, which are comparable to that described by Green et al. (16), are virtually the same for both Ad19 strains. Because we counted only those particles that appeared intact, it is possible that enough disrupted particles or separate capsomers might be present in the strain 3911 virus stocks to influence its growth cycle, and, because our virus stocks are not band purified, this cannot be ruled out.

It has been reported that Mg^{2+} enhances both adenovirus (40) and rhinovirus (13) growth and plaque size by facilitating virus adsorption and release. We have shown that the presence of 30 mM $MgCl_2$ enhances plaque size and number for both Ad19 strains. Although incubation temperature may influence plaque size, its effect on virus titer was much less striking. The differences in plaque size between the two Ad19 strains probably reflects both passage history and strain differences.

Immunologically, the two strains of Ad19 show some differences. CF antigen titers were

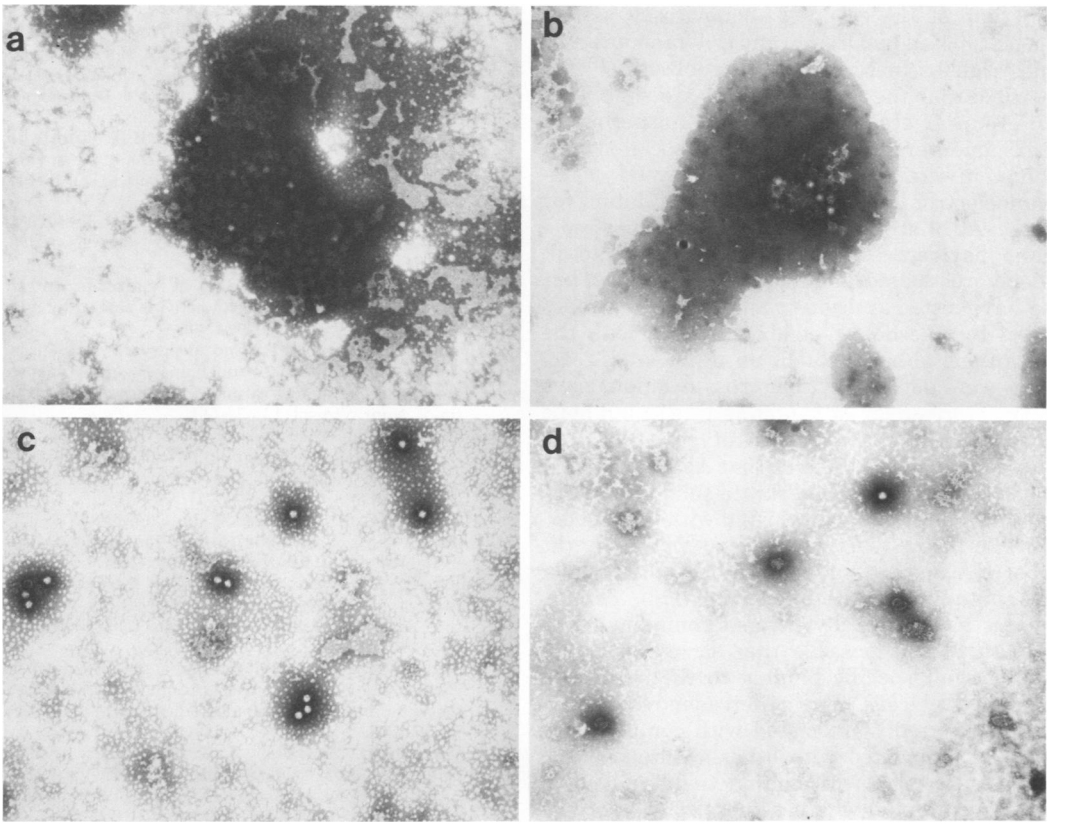

FIG. 1. *Agglutination of Ad19 by type-specific antisera. Undiluted virus was mixed with antisera diluted 1:20, stained with 2% phosphotungstic acid, and examined with a JEOL JEM-100S electron microscope. (a) Antiserum to Ad19 versus strain 3911; (b) antiserum to Ad19 versus strain CJ009. Note the aggregations of virus in both, characteristic of the homologous reaction. (c) Antiserum to Ad9 versus strain 3911; (d) antiserum to Ad12 versus strain 3911. ×15,111.*

high for both strains, with strain CJ009 showing a 2-fold-higher titer than strain 3911; conversely, the HA titer of strain 3911 was 16-fold higher than that of strain CJ009, which may reflect the difference in infectivity titer (Table 2). By HI, the two strains again show a difference in the homologous system, with strain 3911 inhibited by a fourfold-higher dilution of Ad19 antiserum than is strain CJ009. This is probably due to slight strain differences between the reference Ad19 and strain CJ009 which are detected by the reference Ad19 antiserum.

Cross-reactions, which have been reported previously by other workers (19, 20, 22, 34, 36) and in the National Institute of Allergy and Infectious Diseases *Catalog of Research Reagents*, were detected between antisera to Ad8, Ad9, and Ad10 versus both Ad19 strains by HI and confirmed by immunoelectron microscopy. In one case, Ad8 antiserum versus strain CJ009, a cross-reaction was detected by immunoelectron microscopy which was not detected by HI. Evidence that immunoelectron microscopy is a more sensitive technique is also provided by a report of a cross-reaction between Ad7 and Ad11 which was evident only by this system (32). Although some cross-reactivity was seen by neutralization between antisera to Ad8 and Ad9 versus strain 3911, this was only at a 1:10 dilution of antiserum and was not detectable for strain CJ009, thus indicating that cross-reactivity is limited, for the most part, to the hemagglutinating antigen of these viruses.

These cross-reactions would only have been of interest and characteristic of Ad19 were it not for the fact that Ad19 has recently been isolated from several large outbreaks of conjunctivitis with symptoms usually associated with Ad8 (9, 21). The related adenovirus serotypes Ad8, Ad9, Ad10, and Ad19 are rarely isolated, but, when seen, are almost exclusively associated with epidemic conjunctivitis outbreaks, particularly Ad8 and Ad19. We would like to suggest that Ad8, Ad9, Ad10, and Ad19 represent a separate

subgroup of HA group II adenoviruses, as has been proposed by Dreizen and Zolotarskaya (12) and Wadell as described by Hierholzer (18). It is possible that the cross-reactions are simply inter-group II cross-reactions, but antiserum to Ad29, another group II serotype associated with conjunctivitis, showed no activity by HI or immunoelectron microscopy at a 1:20 dilution for either Ad19 strain. In addition, the cross-reactions may represent an antigen common to all adenoviruses associated with conjunctivitis, but we have tested antisera to all of those serotypes listed by Hierholzer et al. (20), and, with the exceptions already noted, no other cross-reactions were detected. Other cross-reactions have been described for Ad8, Ad9, and Ad10, but none is shared between all four serotypes (19). All of these factors suggest to us that Ad8, Ad9, Ad10, and Ad19 represent a separate subgroup of HA group II specifically associated with conjunctivitis. The cross-reacting antigen, apparently the fiber HA antigen which is also responsible for the initial virus-cell interaction (26), demonstrates a serologically defined commonality in structure. It is possible that such a structure may permit specific binding to the surface of conjunctival cells. The other adenovirus serotypes reportedly associated with conjunctivitis are also associated with other syndromes, and their association with conjunctivitis may be a less specific one, whereas these four serotypes are seen to be associated exclusively with epidemic conjunctivitis.

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