METHODS FOR RAPID DETECTION OF HUMAN OCULAR VIRAL INFECTIONS

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Recent methods for detection of viruses in clinical specimens include immunofluorescence, immunoperoxidase, immune adherence hemagglutination, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), and immunoelectron microscopy. Some are useful for the detection of traces of viral antigens but are more complicated and time-consuming than others. Simple techniques of immunofluorescence and negative stain electron microscopy are used for the rapid detection of viruses in human adenoviral, herpetic, rubella, molluscum contagiosum, and vaccinial infections.

MATERIALS AND METHODS

Adenovirus Cases

IN 1977, 50 conjunctival specimens obtained from patients with epidemic keratoconjunctivitis (EKC) were examined by direct immunofluorescence. The specimens were conjunctival swabs or scrapings from patients with acute follicular keratoconjunctivitis. Adenovirus group-antigen specific, fluorescein conjugated rabbit antiserum was used. Titration of this conjugate, dilated in 10% mouse brain suspension in PBS pH 7.5, gave an optimum dilution for use of 1:20. At this dilution, antigen-positive cells stained brilliantly (4+), while antigen-negative cells were only faintly stained but were readily discernible (1+ or less). Conjunctival scrapings were prepared on clean microscope slides and air dried as rapidly as was feasible—preferably on the return-air intake grill of a laminar-flow biologic safety cabinet. Following thorough air drying, the slides were

acetone fixed at room temperature for ten minutes. The slides with the conjugate diluted in brain suspension were incubated for 30 minutes at 37C in a moist chamber, followed by a ten-minute wash in PBS pH 7.4 with intermittent agitation.

The slides were examined at a magnification of ×200, using standard exciter/barrier filter combinations for fluorescein with 200 W Hg-arc illumination.

Scrapings were scored as positive if any cells of normal morphology were observed with typical granular cytoplasmic fluorescence. Scrapings were read as negative if no such cells were found and as inconclusive if only atypical fluorescence and fewer than 50 cells were noted.

CONTROLS.—Fluorescein-labeled preserum from the animals used to produce the antibodies became commercially available only after the study was completed. Before the study was conducted on clinical specimens, the working titer of the conjugates was established at varying dilutions by staining uninfected cells, homologous-infected cells, and heterologous-infected cells. At a working dilution that produced bright-to-brilliant fluorescence with the homologous agents, there was negligible background fluorescence with either uninfected or heterologous-infected (herpes simplex virus 1 [HSV-1], herpes simplex virus 2, and herpes zoster with adenovirus) cells. The conjugates were stored at -80C after reconstitution, and only small amounts of working dilutions were prepared to avoid repeated freezing and thawing.

The clinical specimens were examined with absorbed-conjugate con-trols that were retested approximately monthly with uninfected, homologous-infected, and heterologous-infected cell cultures. During the study, conjugated normal rabbit serum was used as a control conjugate at the same dilution as the virus-specific conjugates. The intention was to determine whether accurate results could be obtained by immunofluorescence without multiple controls.

CELL CULTURE ISOLATION.—Specimens of conjunctival scrapings for viral isolation were collected in 2-ml quantities of Hank's balanced salt solution, with 0.5% gelatin added previously, dispensed and sterilized in 1-gm vials. The specimens were refrigerated until delivered to the virology laboratory, usually within 24 hours. They were treated by adding antibiotics (amphotericin B and PSNB, a standard mixture of penicillin, streptomycin, neomycin, and bacitracin), then inoculated in 0.2 ml volumes into two tubes each of human embryonic lung or kidney fibroblast cells and primary Rhesus monkey kidney cells. Primary human embryonic kidney cells were used when available, as were HeLa 299 cells. Cell cultures were held for a minimum of one month; any cultures that showed nonspecific degeneration earlier were passed to fresh cells of the same type. No further "blind" passages were made. If typical adenovirus cytopathic effects were observed, the cells were held until the effects were estimated to involve well over half of the cell sheet, then harvested for titration and neutralization by the intersecting serum pool scheme. Final serotyping was confirmed using monotypic antisera at a dilution providing approximately 20 antibody units against 100 to 300 TCID$_{50}$.

Cytologic Diagnosis of Herpes Simplex by Direct Immunofluorescence

Adequate smears and scrapings from patients with dendritic corneal lesions were fixed in acetone for ten minutes at room temperature, air dried, inoculated with a 1:20 dilution of conjugate in brain suspension for 30 minutes at 37C, and washed for ten minutes in PBS pH 7.2. With fluorescence microscopy of the slides it was usually possible to differentiate type 1 and type 2 herpes simplex viruses if type-specific conjugates were used.

Rubella

Lens aspirate from a 2-year-old patient with clinical ocular rubella was examined by immunofluorescence and negative stain electron microscopy.

Indirect immunofluorescence was performed with two human sera, pretested by a standard rubella hemagglutination-inhibition antibody (HAI) test. The positive serum had a titer of 1:640, and the negative serum, a titer of less than 1:10, by HAI. These sera were then used in an indirect immunofluorescence test on rubella-infected and uninfected BHK-cells, a susceptible line used for laboratory propagation of rubella virus. Titration of the positive serum revealed a working titer of 1:40;

working titer is defined as a dilution that gives brilliant (4+) fluorescence of infected BHK-21 cells and minimal (±) fluorescence of noninfected cells. The conjugate used was a 1:25 antihuman IgG rabbit serum. The lens smears were fixed in acetone for ten minutes at room temperature, ringed with fast-drying enamel and overlaid with positive and negative sera, incubated for 30 minutes at 37C, washed in PBS pH 7.4 (also used to dilute the serum), stained with the antihuman IgG for 30 minutes at 30C, washed, mounted in polyvinyl alcohol pH 7.5, and examined with ultraviolet illumination at ×200.

Preparation of Specimens for Negative Stain Transmission Electron Microscopy

The conjunctival and corneal scrapings were fixed in 2.5% buffered glutaraldehyde. The suspension was placed in drops in formvar-coated grids that were then stained for approximately 30 seconds in 3% phosphotungstic acid at pH 6.5. The specimens were examined by electron microscopy.

RESULTS

The patients had conjunctival hyperemia and folliculosis (Fig 1). Early subepithelial corneal infiltrates were present in 60% of the

Fig 1.—Conjunctival Hyperemia and follicles of lower lid in epidemic keratoconjunctivitis one week after infection.

Fig 2.—Intense nuclear and cytoplasmic immunofluorescence of adenovirus in infected epithelial cells with direct immunofluorescence method (×200).

Fig 3.—Specific stippled nuclear fluorescence and cytoplasmic fluorescence in conjunctival epithelial cells infected with adenovirus (×150).

Fig 4.—Herpes simplex keratitis three days after infection showing dendrite stained with fluorescein (×66)

Fig 5.—Typical branched pattern of dendritic form of herpes simplex keratitis four days after infection.

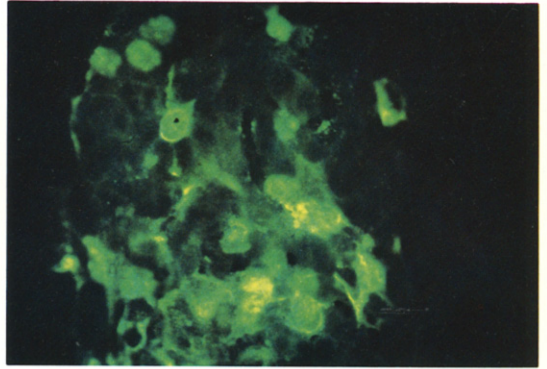Fig 6.—Specific cytoplasmic fluorescence of epithelial cells from herpes simplex dendrite.
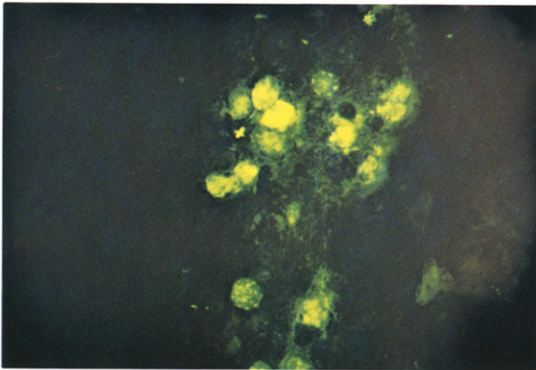
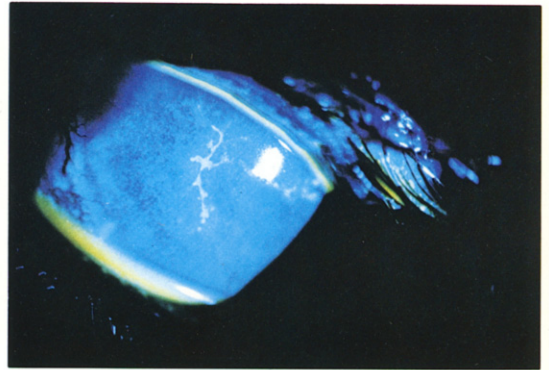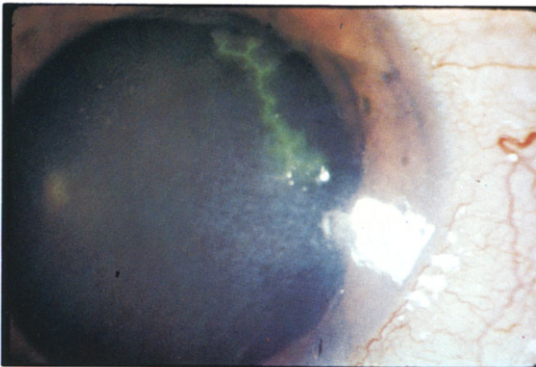FIGURE 1

FIGURE 2

FIGURE 3
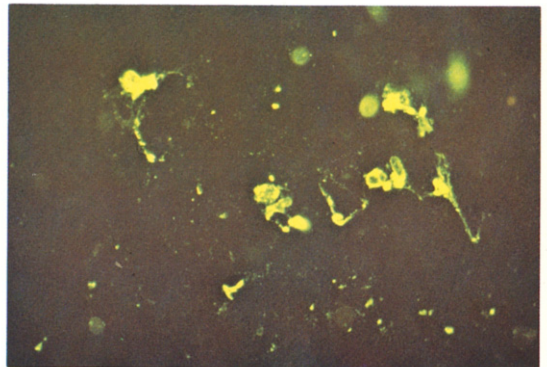

FIGURE 4

FIGURE 5

FIGURE 6

patients, preauricular nodes were present in 80%, and conjunctival pseudomembranes were present in approximately 10%. Of 50 specimens from patients with EKC, positive immunofluorescence for adenovirus was present in 22 cases. Cytoplasmic fluorescence was marked (Fig 2), but stippled nuclear fluorescence was also observed (Fig 3). Cultures and typing showed that 20 of these were a hybrid type 10-19, one was type 3, and one was type 11. The hybrid type 19 showed neutralization with type 10, and a type 19 hemagglutinin was demonstrated by hemagglutination inhibition. This was confirmed by Nathalie J. Schmidt, PhD, Viral and Rickettsial Disease Laboratory, California Department of Health, Berkeley, and by J. Hierholter, PhD, Respiratory Virology Branch Center for Disease Control, Atlanta. The results of viral isolations and immunofluorescence showed good correlation. Typing was confirmed by neutralization with single antiserum from the National Institutes of Health (NIH) catalogue and by the National Communicable Disease Center, Atlanta. Positive results were obtained only in subjects examined within one week of onset of infection. Electron microscopy of negative stained preparations disclosed icosahedral adenoviruses measuring 65 to 70 nm in diameter. Intact capsids were present in some preparations, while in others the capsids were disrupted.

In three persons with herpetic dendritic keratitis (Fig 4 and 5), positive immunofluorescence was present (Fig 6); all were herpes simplex hominis type 1. Herpes simplex virions obtained from scrapings of dendritis lesions measured 100 nm in diameter and displayed mostly intact capsids (Fig 7).

A 2-year-old boy with ocular rubella had unilateral cataract (Fig 8) and microphthalmos. Smears of the cataractous lens revealed positive immunofluorescence with the positive serum in the form of diffuse and granular cytoplasmic fluorescence, and diffuse streaks of amorphous extracellular material.

Molluscum contagiosum lid lesions displayed circumscribed nodules with a central umbilication (Fig 9). Scrapings stained with hematoxylin-eosin revealed large basophilic cytoplasmic inclusions in the granular layer. Negative stain electron microscopy disclosed brick-shaped virions (Fig 10) measuring 250×200 nm with the usual interlacing pattern of surface threads. In one patient with a vaccinia lid lesion (Fig 11), both mulberry (M) and capsule (C) forms were observed (Fig 12 and 13).

DISCUSSION

Recent advances in electron microscopy as well as immunologic techniques have facilitated the rapid detection of viruses in clinical specimens. A hapten-sandwich procedure was reported for immunospecific labeling of cell surface antigens with markers visible by scanning electron microscopy (SEM).[1] Antihapten antibody was used to link hapten-modified tobacco mosaic virus and bushy stunt virus. Viral identification by SEM of preparations stained with fluorescein-labeled antibody was described by Springer et al,[2] using hemagglutination of chicken erythrocytes by influenza virus as a model. Transmission electron microscopy has been widely used for the study of viral structure and morphogenesis, although the low concentration of viral particles in some clinical
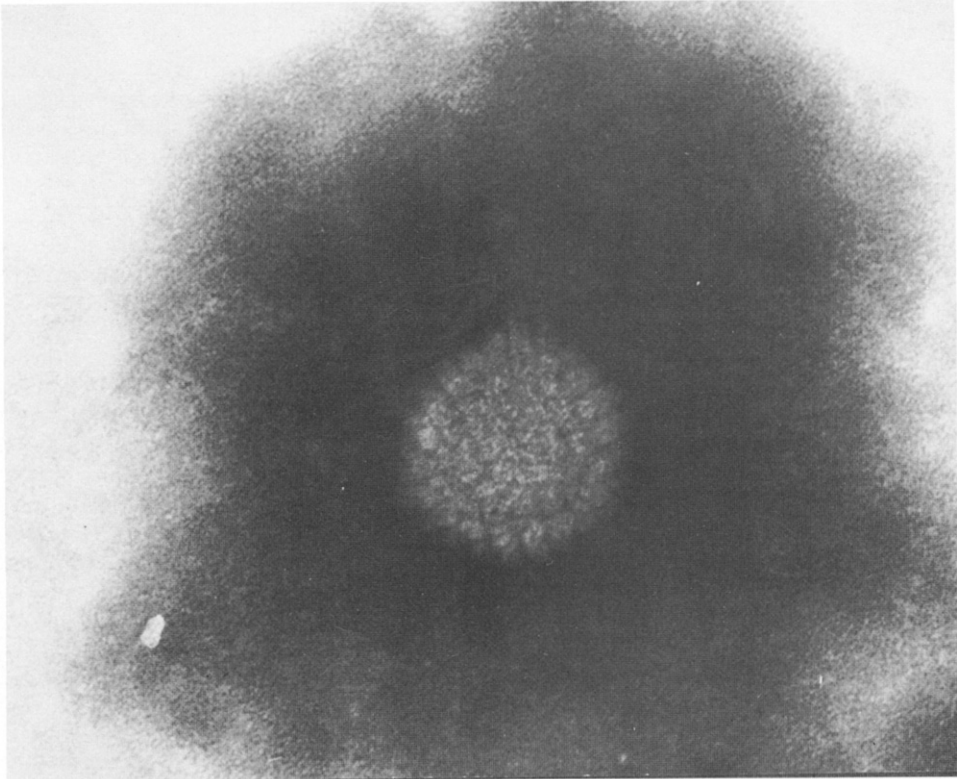
Fig 7.—Herpes simplex. Intact capsid with numerous capsomeres (negative stain, original magnification ×60,000).

Fig 8.—Ocular rubella with unilateral microphthalmos and cataract.

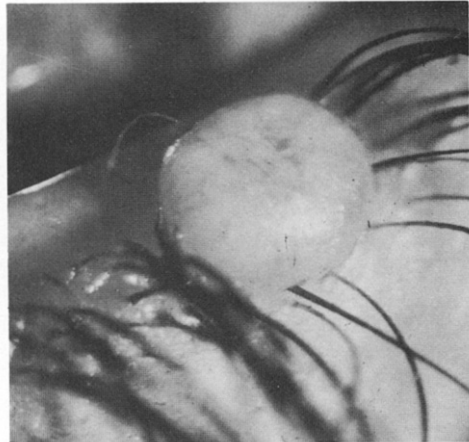Fig 9.—Molluscum contagiosum. Nodular lesion of lower lid.

specimens limits application of this tool for viral detection. There have been reports[3,4] that clinical specimens contain adequate numbers of morphologically recognizable viral particles if obtained at an early stage of infection. Horne and Ronchetti[5] reported a negative staining carbon film ultrastructural technique for studying viruses that was
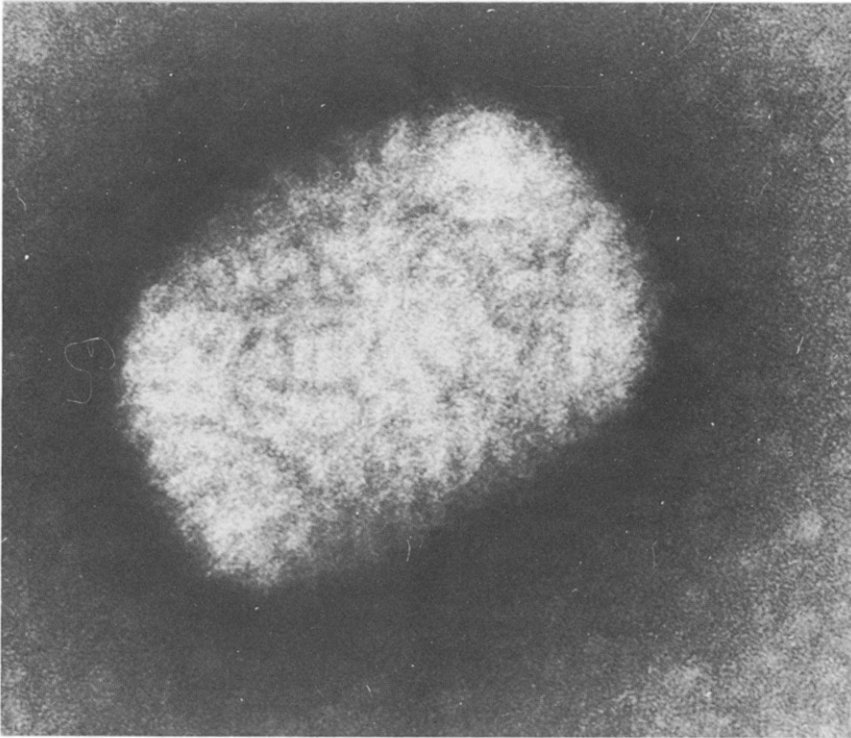
Fig 10.—Molluscum contagiosum. Brick-shaped virion with irregular surface threads (negative stain, original magnification ×74,000).
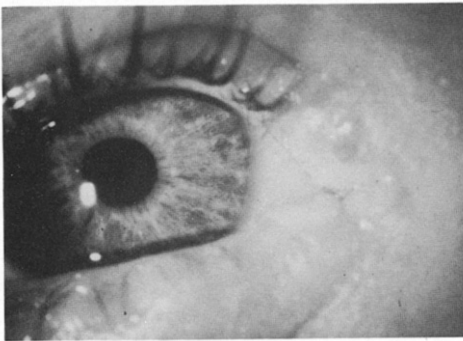
Fig 11.—Vaccinia nodular lid lesions.

used for the preparation of highly concentrated suspensions of mosaic viruses and human adenovirus type 5. The advantage of this technique over conventional negative staining is the formation of two-dimen-

sional and three-dimensional crystalline arrays of viruses. The packing arrangement of the viruses could be varied according to the type of negative stain and pH used during the preparation. Doane et al[6] described a two-hour embedding procedure for intracellular detection of viruses in tissue culture as well as tissue biopsy specimens. The total processing time by this method required two hours compared with an average of 24 to 32 hours by the standard method. However, there was slight decrease of specimen detail with less than optimal staining compared with the standard techniques. Doane[4] reported that identification of viris by immunoelectron microscopy was useful for the study of viral

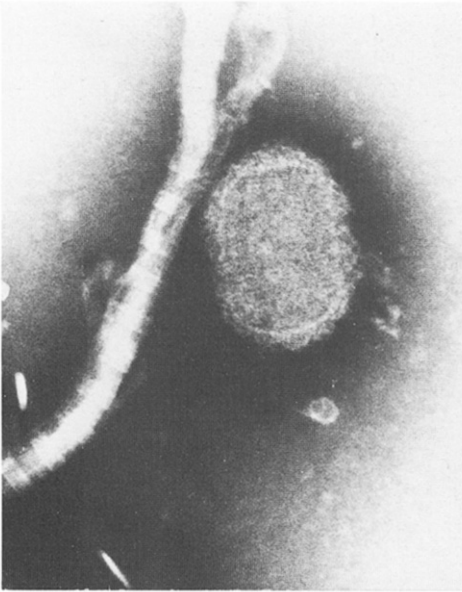Fig 12.—Mulberry (M) virion of vaccinia (negative stain, slightly enlarged from ×21,500).

antigens and antibodies. He was able to identify elusive viruses including rubella, hepatitis-associated antigens, rhinovirus, and coronavirus.

Gardner and McQuillin[7] have described the value of immunofluorescence in the diagnosis of rubella, adenoviral infections, and measles. Immunofluorescence of human viral infections, particularly herpes simplex, has been reported.[8]

The most common causes of viral keratoconjunctivitis are herpes simplex, adenovirus, and chlamydia. The most frequent types of adenoviruses encountered in ocular infections are types 8 and 19; types 3 (pharyngoconjunctival fever), 7, and 10 are sporadically seen. Viral isolations occur during EKC outbreaks, usually in the spring and fall in the United States. Clinically, adenovirus EKC is characterized by the acute onset of a follicular conjunc-tivitis, hyperemia, photophobia, and lacrimation. Diffuse epithelial keratitis followed by subepithelial infiltrates are a frequent finding. All of these features were present in the cases studied. However, the clinical manifestations of the patients with hybrid type 10-19 infection resembled EKC caused by other types of adenoviruses. There was no evidence of systemic involvement.

Outbreaks of EKC frequently occur in schools, swimming pools, outpatient departments, and hospitals. The epidemics occur from contamination of eye solutions, instruments, and from infected fingers of doctors and nurses.[9,10] Three of the patients in this study were ophthalmologists who had acquired the infection from examining patients with adenoviral infections. Simultaneous nosocomial and community outbreaks of EKC with types 8 and 19 adenovirus were recently described.[10] In a recent epidemic of EKC at a Vietnamese refugee camp in Florida, adenovirus type 8 was recovered in 81% of cases cultured within two weeks of onset of infection.[11]

Dawson et al[12] described adenovirus-like particles in the conjunctiva of one patient and in the corneal epithelium of another by transmission electron microscopy of tissue culture preparations. They found that among 15 patients with EKC caused by adenovirus type 8, 2 had persistent corneal erosions during the acute stage of the disease, 7 had conjunctival scarring, and 9 had inflammatory membranes. An outbreak of adenovirus type 19 in the United States was described by Hierholzer and associates[13] and by Burns and Potter.[14] An epidemic of EKC caused by adenovirus type 19 occurred in London.[15] In this study,
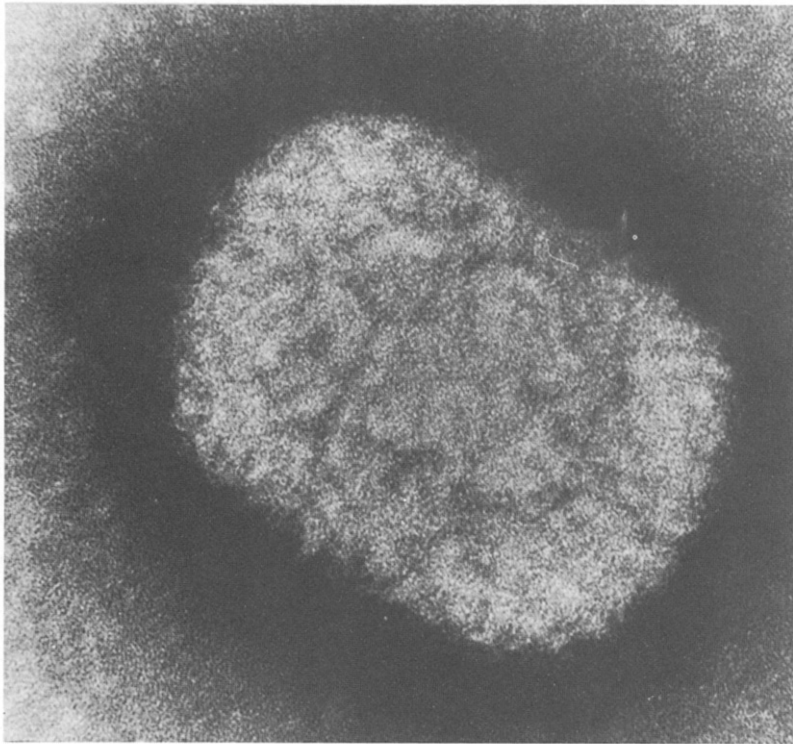
Fig 13.—Capsular (C) form of vaccinia (negative stain, original magnification ×74,000).

adenovirus type 19 was isolated from 21 patients using human embryonic kidney cells. Another report[16] described difficulties encountered in typing adenovirus related to types 10 to 19 in an outbreak of keratoconjunctivitis in Bristol. The same problem was encountered in this study, since the hybrid type 10-19 showed characteristics of type 10 determined by neutralization and of type 19 by hemagglutination inhibition.

In 1969, Ellison and associates[17] compared methods available at that time for the laboratory diagnosis of ocular adenovirus type 3 infections. They used fluorescent antibody staining, examination of conjunctival cytology by the Giemsa and Papanicolaou stains, and standard virus isolations and antibody titers. They believed that virus culture was the most reliable way to identify adenoviral infections, since attempts at more rapid techniques using fluorescence antibody methods were unsuccessful. More recently, however, Vastine and associates[18] used a direct immunofluorescence technique for the diagnosis of acute adenoviral keratoconjunctivitis with positive results in 25 patients with epidemic keratoconjunctivitis. In an earlier report, Schwartz et al[19] described positive immunofluorescence studies in all 39 patients with adenoviral isolation. The fluorescence staining was predominantly present in the cytoplasm, but speckled nuclear fluorescence was also noted.

In the present study, positive results with immunofluorescence and cultures were obtained in patients examined within the first week of infection. Negative results were found in persons with advanced or chronic disease. Thus, fluorescent antibody staining provides a more rapid and sensitive method for the detection of adenoviruses in infected conjunctival cells than viral culture, which usually requires one to eight weeks for virus identification. The direct immunofluorescent method is more rapid (requiring less than one hour) than the indirect technique, which takes a few hours to perform. Adequate controls are essential for the reliability of immunofluorescence and should be easier with the new commercial preserum conjugates. Frenkel and Piekarski[20] emphasized the problems in the diagnosis of *Toxoplasma* organisms and others by immunofluorescence with appropriate positive and negative controls. The present study showed a good relation of viral isolates to positive immunofluorescence; the latter was confirmed by culture.

Adenovirus virions were also noted in conjunctival scrapings by negative stain electron microscopy. Kajima and associates[21] were unable to demonstrate adenovirus by this method, although the organisms were demonstrated by conventional thin section electron microscopy. This could be due to more superficial smears or scrapings in their cases.

In certain areas, adenoviral infection may be confused with keratoconjunctivitis (AHC) produced by enterovirus type 70.[21] In AHC, the presence of conjunctival hemorrhage, slight folliculosis, and fre-

quent pain in the early stage distinguishes this condition from EKC. In keratoconjunctivitis caused by echovirus 7, associated gastrointestinal symptoms, fever, headache, and lymphadenopathy are present at an early stage, but conjunctival folliculosis was not reported.[22]

The two main antigenic types of herpes virus hominis are type 1, commonly associated with infections of the eye, mouth, skin, and upper body, and type 2, associated with genital infections. Neumann-Haefelin et al[23] reported 154 patients with HSV type 1 and three with type 2 ocular infections.

In the present study, the typical virions of herpes simplex keratitis were readily identified both by immunofluorescence and by negative stain transmission electron microscopy. The patients clinically manifested typical dendritic corneal lesions, which stained with fluorescein. Dawson and Togni[24] observed virus particles in corneal epithelium and stroma of human corneal buttons. The virus may replicate in the corneal stroma without any overt inflammation. Collin and Abelson[25] recently described a case of apparent herpes simplex keratouveitis where the virus was demonstrated by transmission electron microscopy in the cornea removed at the time of a third corneal transplant, indicating that the virus persisted for a considerable period of time. In a case of herpetic iritis, virus was found in the anterior chamber by immunofluorescence and electron microscopy.[26]

In specimens of molluscum, the virions displayed typical brick-shaped structures[27] and were the largest viruses observed. The mol-

luscum lid lesion was a centrally umbilicated lid nodule. Light microscopy showed typical cytoplasmic inclusions in the stratum corneum and granulosum.

SUMMARY

Rapid techniques for the detection of viruses in specimens from patients with epidemic keratoconjunctivitis, herpes simplex keratitis, rubella, vaccinia, and molluscum were used. These included direct and indirect immunofluorescence and negative stain electron microscopy. In 1977, 50 conjunctival specimens from patients with EKC showed positive immunofluorescence in 22 cases. The latter were from subjects with onset of infection of up to one week. Cultures and typing showed that 20 of these were hybrid type 10-19, one was type 3, and one was type 1.

Herpes simplex hominis type 1 was recovered in culture and demonstrated by direct immunofluorescence and negative stain electron microscopy in three patients with herpetic dendritic keratitis. In one patient with ocular rubella, lens aspirate showed positive indirect immunofluorescence. In patients with vaccinial and molluscum lid lesions, virions were demonstrated by negative stain electron microscopy.

REFERENCES

1. Nemanic MK, Carter DP, Pitelka DR, et al: Hapten-sandwich labeling: II. Immunospecific attachment of cell surface markers suitable for scanning electron microscopy. *J Cell Biol* 64:311-321, 1975.

2. Springer EL, Riggs JL, Hackett AJ: Viral identification by scanning electron microscopy of preparations stained with fluorescein-labeled antibody. *J Virol* 14:1623-1626, 1974.

3. Doane FW, Anderson N, Zbitnew A, et al: Application of electron microscopy to the diagnosis of virus infection. *Can Med Assoc J* 14:1043-1049, 1969.

4. Doane FW: Indentification of viruses by immunoelectron microscopy, in Kurstak E, Morisset R (eds): *Viral Immunodiagnosis.* New York, Academic Press, Inc, 1974, pp 237-255.

5. Horne RW, Pasquali-Ronchetti I: A negative staining-carbon film technique for studying viruses in the electron microscope: I Preparative procedures for examining icosahedral and filamentous viruses. *J Ultrastruct Res* 47:361-383, 1974.

6. Doane FW, Anderson N, Chao J, et al: Two-hour embedding procedure for intracellular detection of viruses by electron microscopy. *Appl Environ Microbiol* 27:407-410, 1974.

7. Gardner PS, McQuillen J: *Rapid Virus Diagnosis Application of Immunofluorescence Adenoviruses.* Washington, DC, Butterworths, 1974, p 18.

8. Gardner PS, McQuillen J, Black MM, et al: Rapid diagnosis of herpes virus hominis infections by immunofluorescent antibody techniques. *Br Med J* 4:89-92, 1978.

9. Laibson PR, Ortolan G, Dupré-Strachan S: Community and hospital outbreaks of epidemic keratoconjunctivitis. *Arch Ophthalmol* 80:467-473, 1978.

10. Vastine DW, West CE, Yamashiroya H, et al: Simultaneous nosocomial and community outbreak of epidemic keratoconjunctivitis with types 8 and 19 adenovirus. *Trans Am Acad Ophthalmol Otolaryngol* 81:826-840, 1976.

11. Zweighaft RM, Hierholzer JC, Bryan JA: Epidemic keratoconjunctivitis at a Vietnamese refugee camp in Florida. *Am J Epidemiol* 106:399-407, 1977.

12. Dawson CR, Hanna L, Togni B: Adenovirus type 8 infections in the United States. *Arch Ophthalmol* 87:258-268, 1972.

13. Hierholzer JC, Guyer B, O'Day D, et al: Adenovirus type 19 keratoconjunctivitis, correspondence. *N Engl J Med* 290:1436, 1974.

14. Burns RP, Potter MH: Epidemic keratoconjunctivitis due to adenovirus type 19. *Am J Ophthalmol* 81:27-29, 1976.

15. Darougar S, Quinlan MP, Gibson JA, et al: Epidemic keratoconjunctivitis and chronic papillary conjunctivitis in London due to adenovirus type 19. *Br J Ophthalmol* 61:76-85, 1977.

16. Tullo AB, Higgins PG: Epidemic adenovirus keratoconjunctivis. *Lancet* 1 (8061): 442-443, 1978.

17. Ellison ED, Kaufman HE, Little JM: Comparison of methods for the laboratory diagnosis of ocular adenovirus type 3 infection. *Invest Ophthalmol* 8:484-487, 1969.

18. Vastine DW, Schwartz HS, Yamashiroya HM, et al: Cytologic diagnosis of adenoviral epidemic keratoconjunctivitis by direct immunofluorescence. *Invest Ophthalmol* 16:195-200, 1977.

19. Schwartz HS, Vastine DW, Yamashiroya HM, et al: Immunofluorescent detection of adenovirus antigen in epidemic keratoconjunctivitis. *Invest Ophthalmol* 15: 199-207, 1976.

20. Frenkel JK, Piekarski G: The demonstration of *Toxoplasma* and other organisms by immunofluorescence: A pitfall. *J Infect Dis* 138:265-266, 1978.

21. Kajima M, Shiota H, Takashima R: Electron microscopy as a diagnostic procedure for viral infections of the eye. *Twenty-second Concilum Ophthalmol* 2:161-164, 1976.

22. Sadelin K, Tuomioja M, Erkkila H: Echovirus type 7 isolated from conjunctival scrapings. *Scand J Infect Dis* 9:71-73, 1977.

23. Neumann-Haefelin D, Sundmacher R, Wochnick G, et al: Herpes simplex virus types 1 and 2 in ocular disease. *Arch Ophthalmol* 96:64-69, 1978.

24. Dawson CR, Togni B: Herpes simplex eye infections: Clinical manifestations, pathogenesis and management. *Surv Ophthalmol* 21:121-135, 1976.

25. Collin HB, Abelson MB: Herpes simplex virus in human cornea, retrocorneal fibrous membrane, and vitreous. *Arch Ophthalmol* 94:1726-1729, 1976.

26. Kaufman HE, Kanai A, Ellison ED: Herpetic iritis: Demonstration of virus in the anterior chamber by fluorescent antibody techniques and electron microscopy. *Am J Ophthalmol* 71:465-469, 1971.

27. Madeley CR: *Virus Morphology*. Baltimore, Williams & Wilkins Co, 1972, p 36-37.