Viral Antigens in the Immune Ring of Herpes Simplex Stromal Keratitis

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• **Corneal tissue obtained during superficial keratectomy from a patient with herpesvirus disciform keratitis was studied by immunoelectron microscopy. Clinically, this cornea had a dense central infiltrate with a circumferential opaque ring histologically resembling the immune ring described by Wessely. Histologically, along the line of altered keratocytes and ground substance, an infiltration of inflammatory cells was found. Herpesvirus particles were seen by electron microscopy in the corneal stroma, but these virus particles had abnormal, noninfective forms such as empty capsids and incomplete virions. By immunoelectron microscopy with a peroxidase-labeled antiherpesvirus antibody reagent, herpesvirus antigens were localized in the corneal keratocytes and in the corneal stroma. The major localization of the virus antigens was in association with the herpes virions and surrounding vacuoles in the keratocyte nucleus and in the corneal stroma in the area of degenerating keratocytes. These findings support the view of a hypersensitivity mechanism in the pathogenesis of herpes simplex virus disciform keratitis.**

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The pathogenesis of stromal herpetic keratitis is still unresolved. Experimental animal models of herpes simplex virus (HSV) keratitis have been considered to have an immunologic basis.[1-3] The persistence of HSV antigens in the corneal stroma and access of specifically sensitized lymphocytes from the limbus to the cornea could be responsible for the chronic recurrent corneal inflammation in herpes keratitis by chronically activating an immune reaction.[1-5]

Herpes stromal keratitis (HSK) without ulceration can appear to be stromal edema with stromal haze. Stromal infiltrates can have a characteristic partial or complete ring pattern. These rings are identical to the appearance of the Wessely ring produced in the rabbit corneas by intrastromal injection of purified HSV antigen in animals previously systemically sensitized to HSV.[2] By specific fluorescent antibody staining procedures of corneal tissues from these animals, HSV antigen, host antiviral antibody, and serum complement have been found to be deposited at the site of the local accumulation of inflammatory polymorphonuclear (PMN) cells.[2]

We report herein the results of electron microscopy and immunoelectron microscopy studies on a cornea specimen from a patient with HSK with an immune corneal ring.

REPORT OF A CASE

A 6-year-old girl was first seen in the emergency room with a three-week history of a keratoconjunctivitis of the left eye that was being treated topically with a mixture of dexamethasone sodium phosphate and neomycin sulfate (Neodecadron) and chloramphenicol. On examination, her right eye was normal with uncorrected visual acuity of 6/6 (20/20). Her left eye had a pinhole visual acuity of 6/15 (20/50), a diffuse punctate keratitis, a central stromal haze, and no signs of anterior chamber inflammation. A diagnosis of HSK was made, and the patient's left eye was treated topically with idoxuridine every two hours and 0.1% dexamethasone every six hours. Over a period of four weeks there was gradual improvement, medications were tapered, and there was a residual small central stromal scar with an uncorrected visual acuity of 6/9 (20/30). However, several attempts to taper completely off steroids over the succeeding three months always resulted in recurrent active stromal disease. The eye remained "quiet" on a single topical dose of 0.1% dexamethasone every third day. Four months after the initial treatment, the patient was unavailable for follow-up for one year, after which she returned to the emergency room with a dendritic lesion, increased stromal haze, and pinhole visual acuity of 6/15 (20/50). She was treated with idoxuridine ointment five times daily and 5% homatropine methylbromide twice daily. With clearing of the epithelial defect, 0.1% dexamethasone was started topically three times each day. With a quieting of the active disease, the steroid was tapered to a single dose every other day. However, one month later keratouveitis developed and her visual acuity was 6/60 (20/200). Dexamethasone was started every four hours along with idoxuridine ointment five times each day. With this treatment there was gradual improvement of the keratouveitis, but four weeks later a central epithelial defect was noted, with increased stromal edema and vascu-

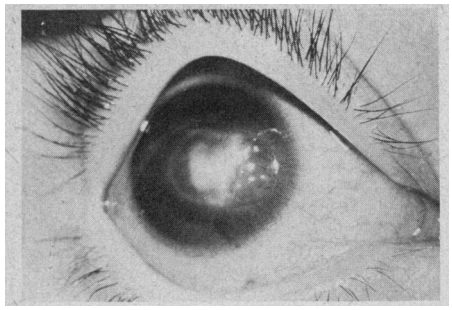

Fig 1.—A 6-year-old girl has recurrent herpes stromal keratitis with opaque corneal ring.

larization of the cornea nasally. Topical applications of steroids were discontinued and 0.5% chloramphenicol was given topically four times each day. On this regimen the cornea thinned considerably, with a dense central infiltrate and a circumferential immune ring, and there was increased anterior chamber inflammation (Fig 1). The patient was admitted to the hospital, the central infiltrate was cultured, and 1.0% gentamicin sulfate, 0.5% chloramphenicol, and idoxuridine were administered topically every two hours and 1.0% atropine sulfate three times each day. *Staphylococcus epidermidis* grew from the culture of the lids and central infiltrate. After ten days of continuous treatment, including antibiotics, there was only minimal improvement. A superficial keratectomy was performed and conjunctival flap was placed over the cornea. The intense corneal inflammation subsided following this procedure. Two years later the eye remained quiet, with a visual acuity of 3/60 (10/200) secondary to the corneal scar and conjunctival flap. A penetrating keratoplasty is planned.

METHODS

Immediately after keratectomy, the corneal specimen was fixed in 4% buffered glutaraldehyde in 0.1M sodium cacodylate buffer with 0.05% calcium chloride at pH 7.4 for six hours, then placed in buffer until further processing as previously described.[6,7] Briefly, 200-μ blocks are stained with peroxidase-labeled Fab fragment of HSV antibody for 48 hours, washed, and fixed again with paraformaldehyde followed by another wash. The enzymatic activity of peroxidase is disclosed by the incubation of the tissue in the mixture of benzidine and hydrogen peroxide. Under action of the horseradish peroxidase, benzidine is oxidized to an electron-dense product and can be localized by electron microscopy. After washing, the tissues are fixed with 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Five-tenths-micron sections are cut for light microscopy and stained with May-Grünwald-Giemsa. Thin sections for electron microscopy are stained with lead citrate.

RESULTS

Examination of 0.5-μ sections of the corneal specimen disclosed severe inflammation. The cornea was acutely edematous with focal necrosis of the stromal lamellae, swelling and degeneration of the keratocytes, and a dense cellular infiltration (Fig 2). The epithelium was often irregular and denuded in areas. Inflammatory infiltrates were predominately PMN cells, lymphocytes, and only occasionally plasma cells (Fig 3).

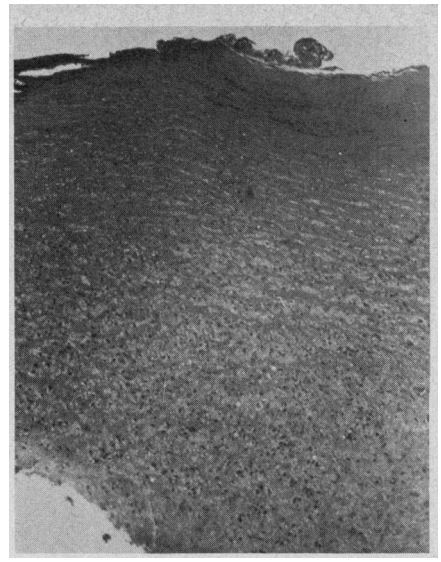

Fig 2.—Corneal ring shows cellular infiltration and swollen fibers (Giemsa, original magnification ×220).

Fig 3.—Polymorphonuclear cells and lymphocytic infiltration of corneal ring (Giemsa, original magnification ×460).

By electron microscopy, corneal stromal necrosis was prominent in the vicinity of degenerating keratocytes. In the areas where the stromal necrosis was prominent, inflammatory cells were not seen. The reason for this was not obvious, but may be that inflammatory factors prevented inflammatory cells from remaining in the area or that they had undergone lysis. The collagen fibers were fragmented, shredded, and disorganized (Fig 4, top). Severe keratocyte degeneration, as evidenced by loss of cytoplasm and nuclear fragmentation, was common (Fig 4, top). Corneal edema was prominent. Structurally complete enveloped herpesvirus particles rarely were found. In more than 268 sections examined in the electron microscope, only two intact complete HSV particles were seen. The usual finding was empty capsids and virions with incomplete forms in severely degenerating keratocytes (Fig 4, bottom). In neighboring areas where normal-appearing keratocytes were found the collagen lamellae were uniform, small, and regularly arranged.

Examination of the corneal tissue by immunoperoxidase electron microscopy demonstrated the presence of an abundance of herpesvirus antigen in the damaged keratocytes (Fig 5, top). Viral antigens were detected on the surface of the virions (Fig 5, top), in the keratocyte nucleus (Fig 5, top), on the nuclear membrane of the keratocyte (Fig 5, bottom), and in the keratocytes undergoing various stages of degeneration (Fig 6, top). Free viral antigens were found also between lamellae of the corneal stroma (Fig 6, bottom). No completely enveloped particles characteristic of infectious virions were seen in the sections stained with the immunoperoxidase antibody reagent. Polymorphonuclear or other inflammatory cells were very rarely found adjacent to the keratocytes, which stained for HSV antigens (Fig 7). Juxtaposition of the inflammatory cells and keratocytes was noted in only one of the fields examined (Fig 8, top). No evidence of herpesvirus antigen or herpesvirus particles was detected in the inflammatory cells (Fig 8, bottom).

COMMENT

A corneal immune ring with purified herpesvirus antigens can be developed in a systemically sensitized rabbit following intracorneal challenge.[2] Corneal rings are seen in patients with stromal herpes keratitis especially of the disciform type. It has been widely hypothesized that these rings are evidence of an immune phenomenon and indicators of corneal hypersensitivity occurring in the course of a herpes infection.[3] This article presents evidence of the localization of HSV antigens and viral fragments in the corneal ring from a

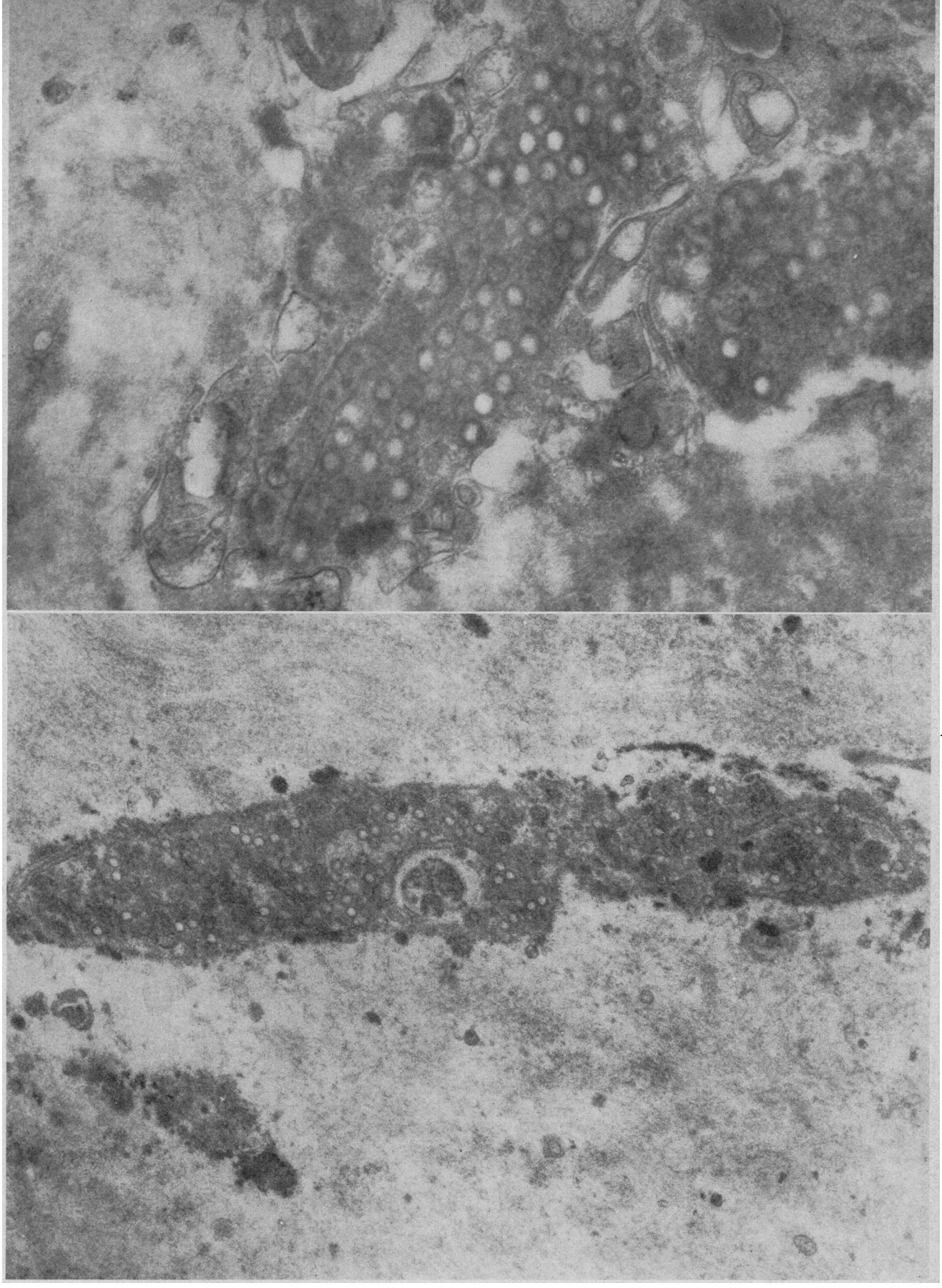

Fig 4.—Top, Corneal stroma. Incomplete virions in nucleus of degener ating keratocyte. Nonperoxidase-stained tissue (original magnification ×53,300). Bottom, Numerous naked particles without dense cores are found. Nonperoxidase-stained tissue. (original magnification ×22,800).

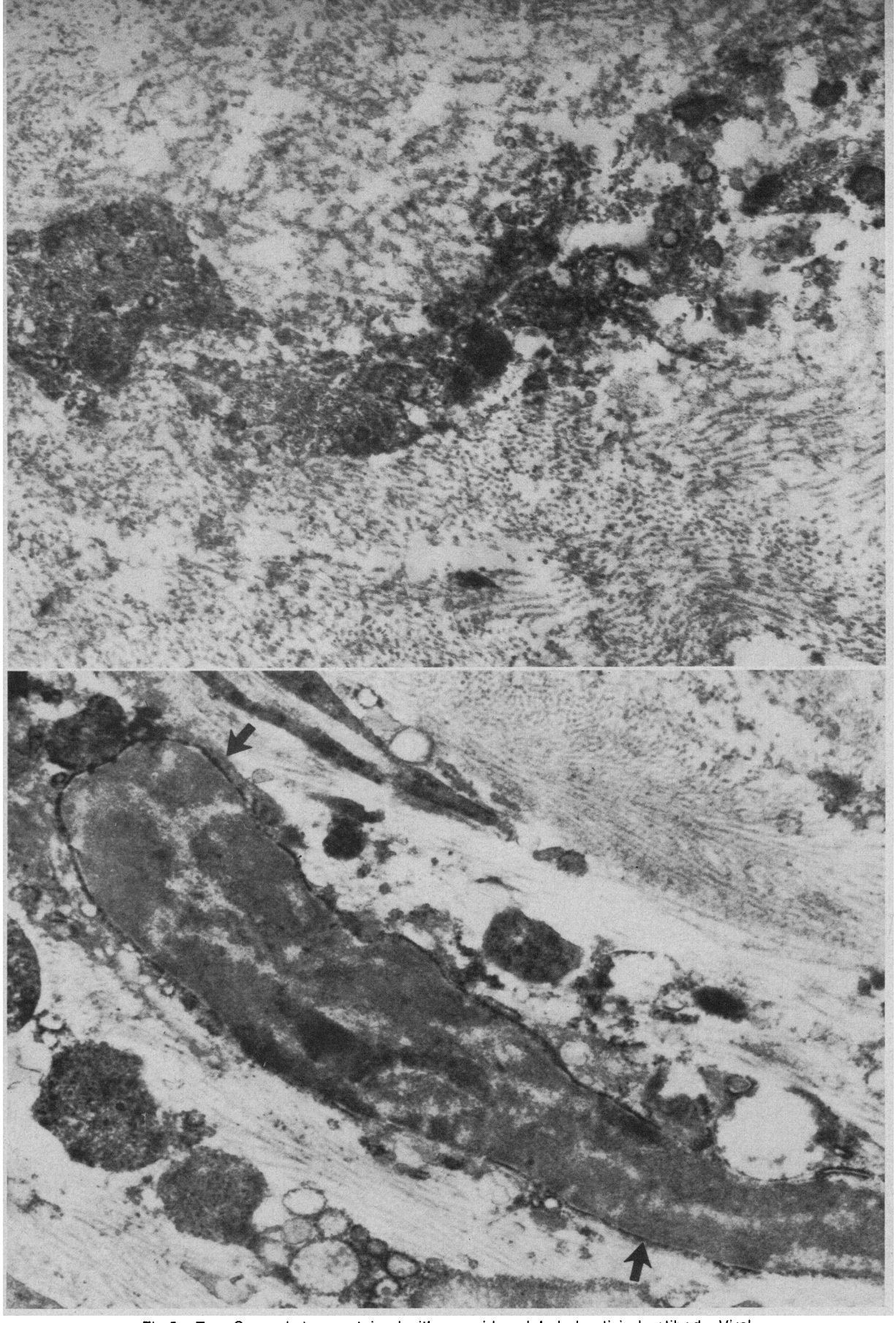

Fig 5.—Top, Corneal stroma stained with peroxidase-labeled antiviral antibody. Viral antigens are localized around capsid in nucleus of disintegrating keratocytes. Collagen fibers are swollen, disarranged, segmented, and shredded (original magnification ×24,000). Bottom, Localization of viral antigens on keratocyte membrane (arrows) (original magnification ×27,000).

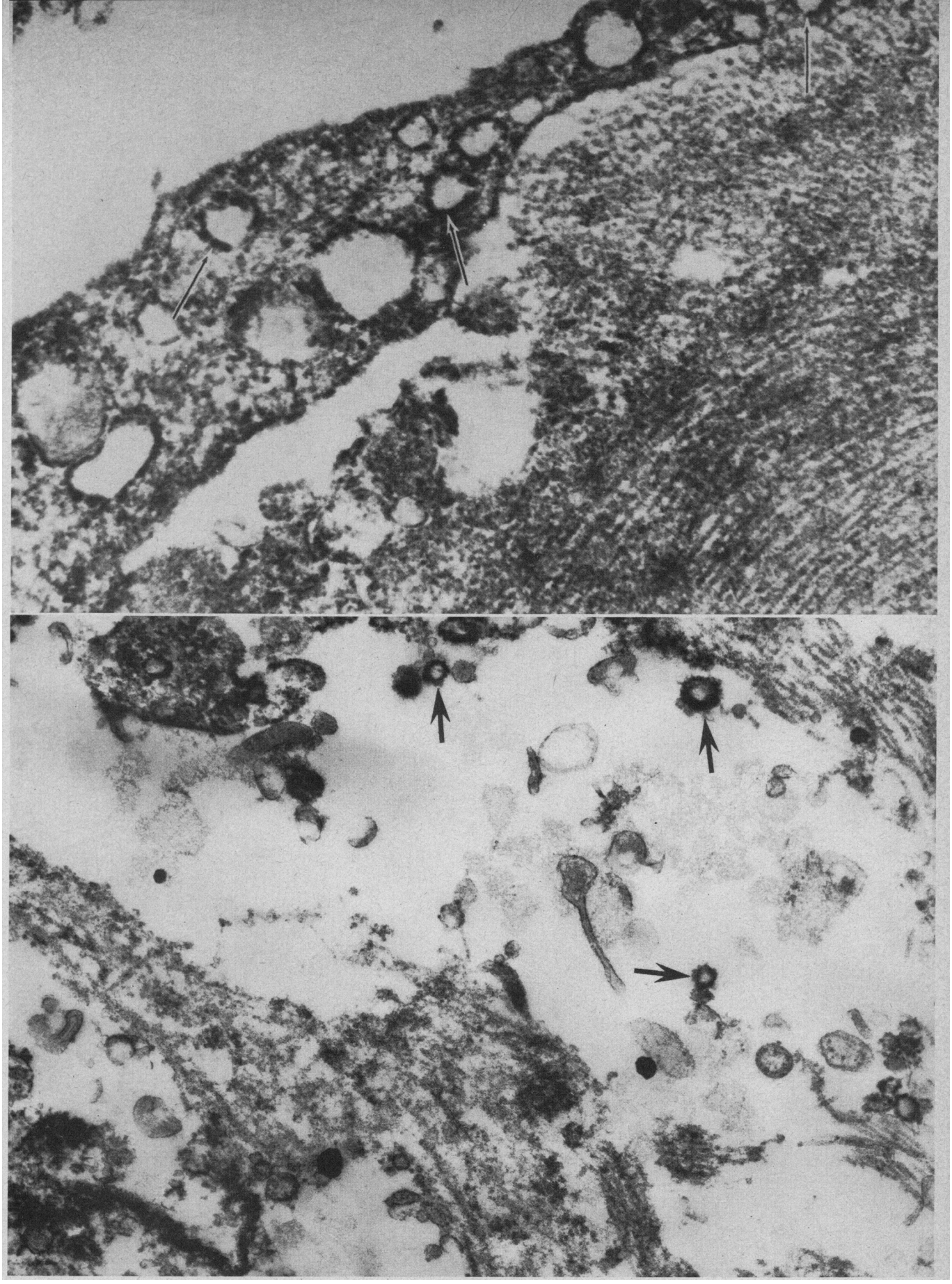

Fig 6.—Top, Corneal stroma stained with peroxidase-labeled antiviral antibody. Viral antigens are localized around vacuoles in keratocyte nucleus (arrows) (original magnification ×40,248). Bottom, Immunoelectron staining for viral antigens localized around viral capsids (arrows) in corneal stroma. Note disintegration of collagen and cellular debris (original magnification ×32,900).

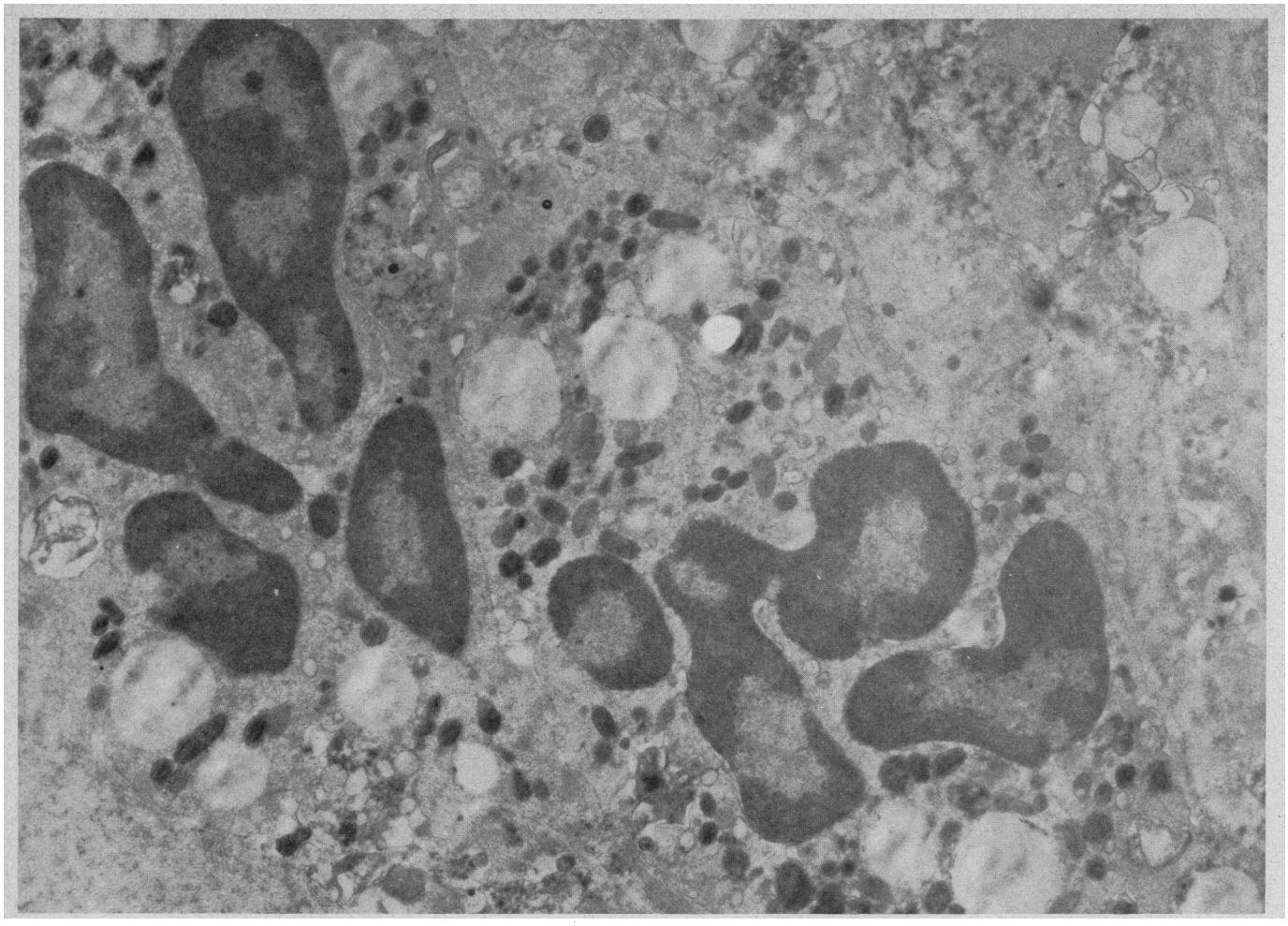

Fig 7.—Inflammatory polymorphonuclear cells in corneal stroma. No virus particles or herpesvirus antigens are detected in vicinity of these cells (original magnification × 13,000).

patient with stromal herpes keratitis. Structurally complete, enveloped virus particles indicative of mature replicating infectious virus rarely were seen in this cornea. Only two were found in 268 sections. We found many viral particles without cores that are considered noninfectious but nonetheless antigenic.[8] Herpesvirus antigens were localized in the damaged corneal keratocytes in the corneal stroma. The peroxidase-labeled antibody stained the viral capsids within the keratocyte as well as HSV antigens located on the nuclear membrane of the keratocyte. Most of the inflammatory cells present were PMN, but herpes virions and HSV antigens were not found in proximity to these cells. The juxtaposition of lymphocytes with infected keratocytes was a rare occurrence in the sections examined. This is in contrast to Metcalf and Kaufman's electron microscopic observations on human corneas obtained from patients with herpetic stromal keratitis.[9] In their report viral particles were found only in two of the eight human corneas studied by electron microscopy. Whether these were complete infectious particles or abnormal noninfectious forms was not stated in their communication. They described lymphocytes in direct contact with degenerating keratocytes, but no viral particles were found and no immunoelectron microscopic studies were performed to detect the presence of viral antigens.

Dawson et al[10] found herpes virions by electron microscopy in five of 19 corneal specimens from HSK patients undergoing keratoplasty. Two specimens were from one patient who received two grafts consecutively, and virus was found both in the original specimen and in the failed graft. Specific staining for viral antigens was not part of their study. A positive virus culture for HSV was achieved in only one of the 19 corneal specimens they studied. This is consistent with the observations of other workers whose attempts to isolate herpesvirus from the corneal stroma in herpes keratitis have met with failure.

The opaque ring seen in the cornea of our patient is similar to the lines and rings of antigen antibody precipitation that occur in immunodiffusion in agar media. One might envision viral antigens on the surface of keratocytes or liberated from damaged keratocytes diffusing through the stromal tissue spaces. Antibody originating from the circulation and diffusing into the cornea from the limbus or antibody formed from plasma cells at the limbus diffusing through the corneal tissue spaces move toward the viral antigens. A ring of opacification representing a circular deposit of immune precipitates develops when a critical concentration of antigen and antibody is reached. As a concentration gradient develops in the tissue, immune complexes include those in antigen excess that can activate complement. Complement is in part responsible for the accumulation of PMN leukocytes that accumulate at the site of the immune ring.

We have produced an immune ring in the rabbit cornea by the intrastro-

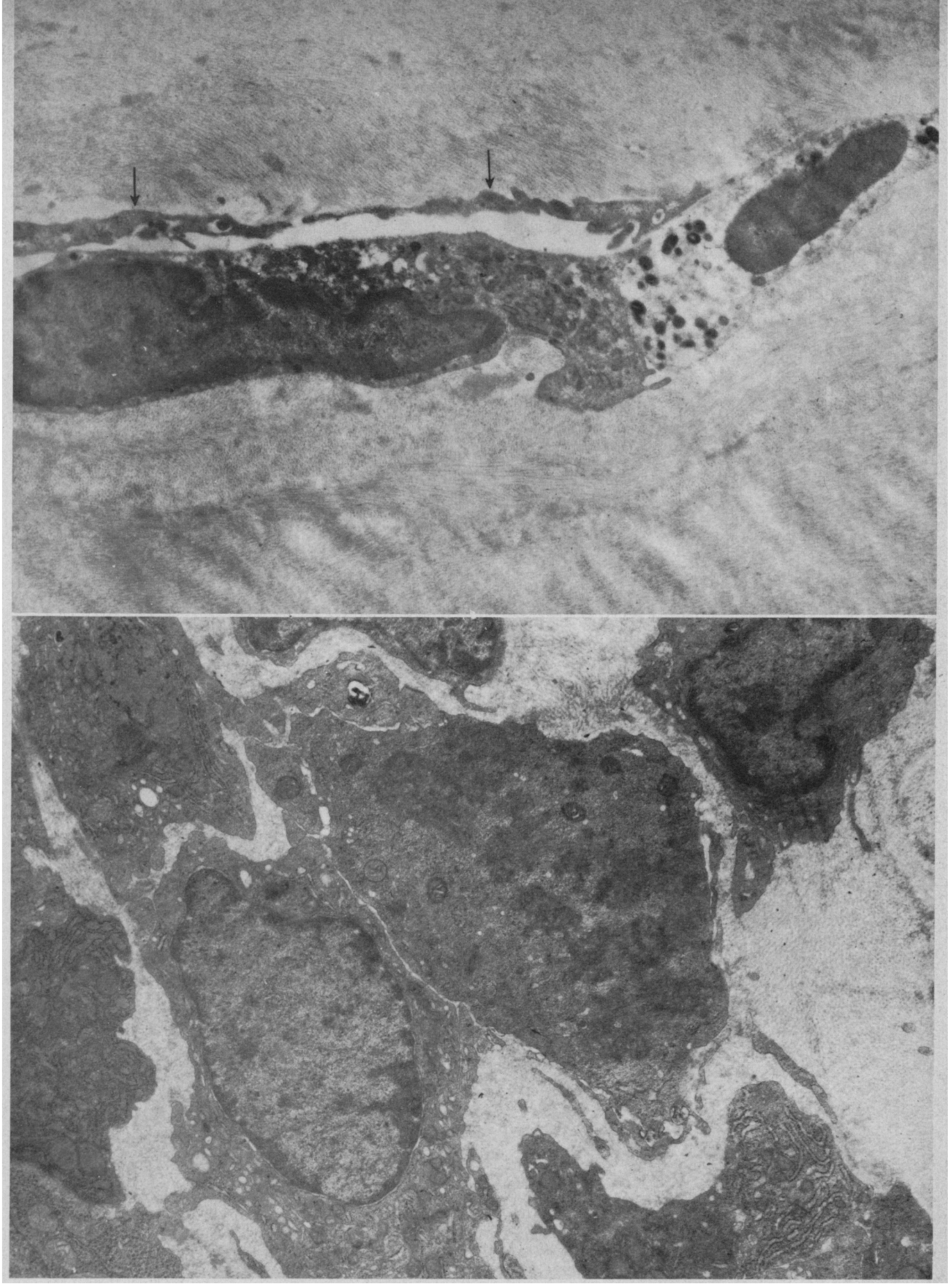

Fig 8.—Top, Inflammatory cells (far right), adjacent to keratocyte (left) and processes of keratocytes (arrows), a rare finding in corneal specimen studied. Note regularity of surrounding collagen fibers. No virions or viral antigens were found (original magnification ×12,560). Bottom, Inflammatory lymphoid cells. Virions or herpesvirus antigens are absent (original magnification ×10,160).

mal infection of purified HSV antigens in animals previously actively sensitized to HSV antigens.[2] This experimentally produced ring represents a local accumulation of inflammatory PMN leukocytes, HSV antigen, host antiviral antibody, and complement, as demonstrated by specific fluorescent antibody labeling. The formation of the ring can be blocked if the animals receive treatment to suppress their PMN response or if the complement component C3 is inactivated with cobra venom factor.[11] This demonstrates that both complement and PMN leukocytes are required for this type of inflammatory response to develop.

In our study, we did not stain for antiviral antibody and complement. We expected to find the viral antigens to be localized in greatest amount near the inflammatory cells as we have noted in the experimental rabbit model. This was not the case, however, and the reason for this difference is not immediately apparent. Within any single field of view with the electron microscope, we did not find keratocytes, viral antigens, or inflammatory cells. At the most, we were able to find several inflammatory cells with adjacent keratocytes but only in one field (Fig 8). There are several possible explanations for this difference. The rabbit model represents an acute inflammatory process. The corneal specimen examined in this study is from an active but chronic corneal infection with elements of both acute and chronic inflammation. The acute inflammatory response with PMN cells, antigen, antibody, and complement are followed by processes of tissue repair. It is not unexpected to find areas of active inflammation adjacent to areas of tissue repair alternating with areas of necrosis so toxic that inflammatory components could no longer remain in the vicinity. We suggest that viral antigens may remain for long periods of time in the keratocyte nucleus or in tissue spaces among degenerating keratocytes and disrupted collagen without stimulating an acute inflammatory response. Viral antigens may be present in areas where active inflammation is present but their receptors are blocked by host antiviral antibody and thus escape detection by the immunoperoxidase reagent. Viral antigen present in excess in the cornea could form soluble antigen-antibody complexes in the areas of inflammatory cells and yet be undetected by the techniques we employed. Further investigations are needed to determine localization of complement in immune corneal rings and to determine the role of the PMN cells in corneal tissue damage or in viral clearance from the corneal stroma.

Our study demonstrates a high incidence of abnormal noninfective forms of herpesvirus in a patient with a stromal herpetic ulcer and prominent corneal immune ring. The finding of incomplete viral particles suggests that the corneal stroma is not a fertile milieu for viral replication. An abortive form of replication takes place as evidenced by the numerous empty particles we found in the degenerating keratocytes. The corneal inflammation that develops appears to be an immunologic response to the presence of both soluble and insoluble herpesvirus antigens produced as a result of this replication.

This patient had both herpesvirus-neutralizing antibody and cellular immunity to HSV antigens demonstrated at the time of keratoplasty. The role of hypersensitivity mechanisms in the pathogenesis of HSV disciform keratitis is further supported by the findings in this study as well as in our previous work that viral antigens can initiate a similar corneal immune ring in a sensitized animal.[2]

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Pedro Fiorello is responsible for the electron microscopy and Patricia Chitjian provided the peroxidase-conjugated reagents.

Nonproprietary Names and Trademarks of Drugs

Gentamicin sulfate–*Garamycin.*
Idoxuridine–*Dendrid, Herplex, Stoxil.*

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