Cytopathology of adenovirus keratitis by replica technique

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Abstract
Corneal replicas were made from the severely affected eyes of 12 patients presenting with typical signs and symptoms of epidemic keratoconjunctivitis. Adenovirus was isolated from the eyes. Histopathological study of the replicas and cytology of diseased cells removed with the replica showed diffuse mild oedema of the epithelium, with scattered moderately swollen and deformed cells. The clinically observed punctate lesions histologically consisted of markedly swollen cells that had become oval or rounded. Fusion of these cells led to small syncytial formations, and the development of pseudopodia-like processes was occasionally observed. Loss of cell contents resulted in the formation of plicae on the surface of many cells. Two types of inclusion bodies were detected in the epithelial cells. The first type, intranuclear vacuolar inclusions, contained homogeneous material. The second type of inclusions were round, dense bodies, that developed in the homogeneous material of intranuclear vacuolar inclusions. The dense bodies contained the replicating and maturing virus particles. After cell degeneration the dense bodies become extracellular. Making a corneal replica had a beneficial effect on the clinical course of adenovirus keratitis.

The in vivo corneal replica technique was developed to study the histopathology of superficial corneal lesions in different types of keratitis without disturbing the exact position of the epithelial cells in relation to each other, as this technique permits us to make an imprint of the corneal surface. In addition, in contrast to the normal cells, diseased superficial cells are easily removed with the replica. Flat mounts of these cells allow study of their cytological details.

Previous reports on this technique from our laboratory have described the histology and histochemistry of normal superficial corneal epithelium, the replicative stages of the herpes simplex virus type 1 (HSV-1) in vivo, and the morphological and histochemical alterations in the HSV-1 infected rabbit epithelium. These studies have not only confirmed the observations on HSV-1 replication in vitro, they have also further elucidated the process of virus release from the cells after viral multiplication. Similar cytopathological alterations were detected when human dendritic and punctate lesions were investigated by means of corneal replicas.

In this paper the cytopathology of adenovirus keratoconjunctivitis (epidemic keratoconjunctivitis) is presented. The results will be discussed in relation to the similarities to or distinguishing features from the histopathology of human dendritic and punctate herpetic keratitis, also studied by the replica technique.

Subjects and methods
Corneal replicas were made from the more severely affected eye of 12 patients who presented with the typical signs and symptoms of epidemic keratoconjunctivitis. The criterion of patient selection was the presence of epithelial punctate corneal lesions (Fig 1). Before the replica was made conjunctival swabs were taken for virus culture, and conjunctival scrapings were made. The scrapings were stained with Giemsa stain and examined by light microscopy. The technique of making a replica has been described in detail elsewhere. In short, the procedure is as follows: (a) instil one drop of a topical anaesthetic three times at five minute intervals; (b) keep the eye open with an ocular speculum; (c) dry the tear film by blowing air for about one minute; (d) paint a thin layer of collodion in amyl acetate (3%-4% solution, w/v)

Figure 1 Punctate lesions on the cornea in epidemic keratoconjunctivitis caused by adenovirus. There is some subepithelial opacification.
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Figure 2 Scattered moderately swollen epithelium cells show morphological alterations. The cells have a tendency to become round. Small round dots are artefacts in the replica. (a) Oblique illumination microscopy. (b) Phase contrast microscopy. (×180)

Figure 3 A focal punctate lesion appears bright in contrast to the surrounding cells. The lesion is formed by markedly swollen cells and syncytiu (arrows). The borders of the lesion are irregular and not circumscribed. Phase contrast microscopy of the replica (×180)

Results
Punctate lesions did not reappear in the healed epithelium in nine patients after the replica was made. In three patients one to three punctate epithelial lesions developed, which disappeared within one week. There was no significant subepithelial opacification at the site of these lesions. In every patient punctate epithelial lesions in the untreated second eye persisted for 10 to 14 days, and marked subepithelial opacities developed at the site of epithelial lesions. These opacities did not disappear for several weeks or several months.

Virus Culture
Adenovirus was isolated from all eyes.

Cytopathology
Scrapings. Giemsa stained conjunctival scrapings showed a large number of lymphocytes and degenerated conjunctival epithelial cells with nuclear vacuolation. Inclusion bodies were not found in the material scraped from the conjunctiva.

Replicas. Phase contrast microscopy and...
acetone, flat mounts of diseased cells were first examined unstained and then again after staining. Most of the cells removed with the replica belonged to the areas of punctate lesions (Fig 5).

Cells from the punctate lesions showed stronger staining of their nuclei and cytoplasm than observed in normal epithelium cells, indicating a rise in their nucleoprotein content. In all replicas alterations in cell membrane were noted in many cells, which showed plicae on the cell surface by oblique illumination microscopy or dark lines by phase contrast microscopy (Fig 6).

With the latter technique part of such a cell that had developed surface plicae appeared lighter than the rest of the cell. In stained slides this part of the cells was faintly coloured. These observations suggest that viral infection induces loss of cell contents, resulting in shrivelling of the cell walls. In fact, in many specimens empty envelopes of cell membranes devoid of any detectable cell contents were observed.

In other cells, especially at the periphery of the syncytia, oblique illumination microscopy showed small surface elevations, like pebbles projecting from the surface (Fig 6). By phase contrast microscopy of unstained cells, and light microscopy of stained slides, these surface elevations were found to be caused by cytoplasmic vacuoles. The nuclei of many cells also contained different sized vacuoles, whereas round dense bodies were present in other cells. Sometimes the dense bodies were located inside the nuclear vacuoles.

The intranuclear vacuoles appeared empty by haematoxylin-eosin stain (Fig 7), except when they contained round dense bodies, but toluidine blue and Giemsa staining showed the presence of light blue homogeneous material in them. This material did not show fluorescence by immuno-fluorescence technique. The intranuclear round dense bodies gave a strong positive immuno-

Figure 4  A diffuse epithelial lesion showing rounded, markedly swollen, epithelium cells. Some syncytia are indicated by arrows. Small round dots are artefacts. Oblique illumination microscopy. (× 150)

Figure 5  Flat mounts of diseased cells in punctate lesions after dissolving collodion membrane. (Haematoxylin-eosin, ×45)
homogeneous material, is probably an initial step in virus replication, and the formation of round dense bodies represents the development of adenovirus. The round dense bodies measured from 1 μm to 10 μm. In a few specimens round dense bodies were found in the extracellular spaces.

The swollen, globular cells in the punctate lesions also contained intranuclear vacuolar inclusions. However, round dense bodies were more frequently detected in these cells (Fig 9). Similarly, syncytia in the punctate lesions also contained round dense bodies. The central areas of some syncytmia were necrosed, but the cell debris still contained a few dense round inclusion bodies.

Formation of pseudopodia-like processes from the syncytia was infrequently seen. When present, they were small, usually extending over two to five cell lengths. Leucocytes and ghost cells were not observed in our specimens.

Discussion
Duke-Elder\(^{10}\) summarised the corneal histopathological changes in epidemic keratoconjunctivitis as degenerative alterations in the corneal epithelium and stroma, with infiltration of mononuclear cells at the site of epithelial lesions. Bowman’s membrane is thinned or even disappears. Lund and Stefani\(^{11}\) described the corneal focal lesions in epidemic keratoconjunctivitis as a collection of polymorphs and mononuclear cells.

The predominant mononuclear cell response in the Giemsa stained conjunctival scrapings in our patients is in agreement with the findings reported in the literature.\(^{12}\)

Replica histology of adenovirus keratitis shows mild diffuse oedema of the corneal surface epithelium. The ribonucleoprotein content of the cells is increased, the increase probably induced by the synthesis and accumulation of virus specific proteins. Conversely, some cells show loss of cytoplasm and nuclei, with resulting formation of plicae on the cell surface of shrunken empty envelopes of cell membranes. More affected cells, showing moderate swelling and alterations in their shape and size, are found scattered among the less oedematous cells that still preserve their shape and size. Marked structural cellular changes are observed in the areas of punctate lesions. The cells vary in shape and size, having attained an oval or rounded form. They show a tendency to fuse together, resulting in syncytial formations. Parts of the syncytia undergo necrosis. Only rarely do small pseudopodia-like processes develop from the syncytia.

Although cell swelling, rounding, and syncytia formation also occur in herpes simplex virus keratitis,\(^{1,4,4,4}\) there are distinct cytopathological dissimilarities between the punctate keratitis caused by adenovirus and by herpes simplex virus. The punctate lesions in herpetic keratitis are cytologically more focal in character, and they are always circumscribed by rows of elongated epithelial cells.\(^{4}\) Round the branches of a dendritic figure the elongated cells are arranged in an arcuate pattern.\(^{4,4}\) We have not observed the presence of elongated cells in any other type
Two types of inclusions were detected in this study of adenovirus epithelial disease. The first type of intranuclear vacuolar inclusions containing homogeneous material develop commonly in the moderately altered cells. The homogeneous material in these inclusions is not antigenic in character as judged from the lack of fluorescence by the immunofluorescence technique.

The second type of inclusions are round dense bodies that develop in the homogeneous material of vacuolar inclusions. The dense bodies contain viral antigens, as shown by immunofluorescence technique and have the staining characteristics of DNA. The intranuclear vacuolar inclusions seem to be analogous to the intranuclear A-bodies of the herpes simplex virus replication cycle, wherein the replicating and maturing virus particles appear as A-granules.13 However, the round dense bodies in adenovirus infection are not granular. They measure from 1 μm to 10 μm in size and are abundant in the moderately altered cells and syncytia. In the areas of necrosis they can be observed in the cell debris or even in extracellular positions.

An interesting finding in this study is the lack of any leucocytes in the superficial epithelium, even though abundant lymphocytes and some polymorphs are found in the conjunctival scrapings. Similarly leucocytes have not been observed in the replicas from herpetic dendritic ulcers or punctate keratitis.8,9 Probably the lymphocytes and mononuclear cells penetrate into the deeper epithelial layers, and into the subepithelial stroma, owing to accumulation of viral antigens at these sites.11,13,14

After making the corneal replica, epithelial
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lesions did not form in the regenerated epithelium in most cases, nor in these cases did subepithelial opacities develop. Since amyl acetate, used to dissolve collodion in the replica solution, penetrates into the superficial stroma and kills the cells by fixation, the multiplying virus in the epithelium is probably destroyed by making the replica. Consequently the source of viral antigen which normally incites the mononuclear cell infiltration, resulting in development of subepithelial opacities on the basis of immune action, is eliminated. 13-15

Making a corneal replica in adenovirus eye infection is not only a diagnostic aid: it may also be a therapeutic measure. The therapeutic effect of the replica technique in dendritic ulcers has been reported elsewhere. 16

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