Morphology of experimental vaccinial superficial punctate keratitis—a scanning and transmission electron microscopic study

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Rabbit corneas infected with vaccinia virus were studied by scanning electron microscopy. The earliest detectable lesion consisted of tiny punctate epithelial pits lined by the intact superficial layers of the epithelium. These lesions were presumed produced by the dissolution of the wing cells with inward collapse of the overlying superficial layer. Examination of more advanced and severe lesions suggested that the further progression consisted of erosion through the superficial layer followed by erosion internally through the basal layer to expose the stroma. With coalescence of adjacent lesions, the punctate appearance of these lesions was lost.

Key words: vaccinia, virus, rabbits, cornea, epithelium, punctate, lesions, scanning electron microscopy, transmission electron microscopy, wing cells, viral factories.

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In this paper an investigation of the morphology and evolution of experimentally induced punctate viral epithelial lesions in the rabbit cornea utilizing scanning electron, transmission electron, and light microscopy is reported. The vaccinia virus was selected as the infective agent in this study as it produces a superficial punctate keratitis in rabbits which is morphologically similar to that produced by other viral, bacterial, and chemical agents in man, is readily identified by transmission electron microscopy, and produces a punctate lesion which can be contrasted with the linear dendritic lesions studied previously.1
Materials and methods

Twelve New Zealand white rabbits were infected with the Connaught strain of vaccinia virus (10^6 infective particles per milliliter). The animals were infected in two different ways.

**Group I.** In five animals the corneal epithelial surface was abraded gently with a rounded sterile platinum spatula in a cross-hatched pattern (10 horizontal and 10 vertical strokes). Two drops of the suspension of virus particles were then dropped onto the corneal surface, the lids were momentarily closed, and the rabbit was returned to its cage. The abrasion healed within a few hours, and within 24 hours randomly scattered central and peripheral punctate areas of fluorescein pooling became visible with the slit lamp. By 36 hours, variations in size of the "stained" areas were observable. Conjunctival hyperemia and a mucopurulent exudate were noted in all animals within 24 hours.

**Group II.** A second series of five rabbits was infected with a 0.05 ml. vaccinia virus subconjunctival inoculum placed between the insertion of the extraocular muscles and the limbus, resulting in the ballooning of the conjunctiva to the limbus. A localized necrotizing conjunctival reaction developed at the injection site in all animals within 24 hours, and by 72 hours, in three of the five rabbits, punctate areas of fluorescein pooling began to appear on the peripheral cornea on the inoculated side.

One eye of each of the remaining two rabbits was infected by a direct inoculation onto the abraded cornea; the other was infected by a subconjunctival injection of the virus. The lesions of the directly inoculated eye evolved as did those of Group I, and the subconjunctival inoculated eye evolved as in Group II.

The animals were put to death at varying times (24 hours to 10 days) following the initial appearance of the corneal lesions. The corneas of animals destined for scanning and transmission electron microscopy were enucleated and placed immediately in fresh, cold 3 per cent glutaraldehyde, and buffered to a pH of 7.3 with 0.2M sodium cacodylate. Following fixation for 12 hours, the specimens for scanning were washed in distilled water for 10 seconds. Four (two from each group) specimens were allowed to dry in air at room temperature. The remaining six specimens were dehydrated in 50 per cent (DMSO) for 1.5 minutes, frozen in liquid nitrogen, and then freeze-dried in a vacuum (Pearse Speed-Evac) evaporator at -70° C. and 0.001 mm. Hg (torr) for 24 hours. After having been dried, the specimens were mounted on a slide with double-stick tape, coated with a thin conducting layer of platinum-palladium alloy, deposited at normal incidence in a vacuum evaporator. The tissues were then placed on the stage of the scanning electron microscope (a modified Japan Electron Optics Laboratory JSM-11 instrument) with the slide at a 45° angle with the scanning beam. The instrument was operated in the secondary electron mode with an accelerating potential of 25 kv and specimen current of 2 x 10^-11 amps.

Photographs of the display cathode ray tube were made with Type 42 Polaroid roll film.

Following 12 hours of glutaraldehyde fixation, 4 by 2 mm. portions of the cornea for transmission electron microscopy were postfixed in osmium and stained en bloc with uranyl acetate and lead citrate.

The corneas of animals destined for light microscopic examination were fixed in 10 per cent formaldehyde and then embedded in paraffin. The corneas were sectioned at 6μ and stained with hematoxylin and eosin.

Results

Regardless of the mode of infection, the lesions seemed to evolve along similar lines. Only three of the five animals infected subconjunctivally developed corneal lesions, and even those with lesions developed fewer than those animals whose corneas were inoculated directly. Moreover, the corneal ulcers in the subconjunctivally inoculated animals did not appear for two or three days, in contrast to the direct corneal inoculated animals which developed lesions within 24 hours.

Compared with the air-dried specimens, the corneas prepared by freeze-drying techniques demonstrated better preservation of their surface microvilli (seen at higher magnifications), however, the topographic morphology of the lesions at magnifications up to 1,000 times showed no observable difference between the air- and freeze-dried specimens.

**Evaluation of lesions.** Figs. 1 to 3 show different areas of the same cornea prepared by air drying and viewed with the scanning electron microscope. These figures demonstrate lesions in different stages of evolution. This rabbit was infected by direct inoculation onto the abraded corneal surface and put to death four days later. In Fig. 1 (original magnification x1,000) a lesion is seen which is believed to correspond to those
which appear at 24 hours. Localized rounded indentations of the epithelium averaging 50μ are observed. We believe that a lesion of this size covered on its outer surface with pooled fluorescein corresponds to those initially seen with the slit lamp (at x16 magnification). The surface layer of the epithelium is seen to be intact, both between and within the lesions. Lines believed to represent cell junctions can be traced into the indentations from the adjacent uninvolved surface epithelial cells. Fig. 2 (original magnification x100) shows an area of cornea in which more advanced or severe lesions in different stages of evolution are seen. Lesion A is believed to represent a relatively early and small lesion. Presumably this lesion would be the earliest in which true staining with fluorescein could be demonstrated clinically as the surface epithelial cells appear to be interrupted. Lesion B is larger and more advanced than A and the surface layer of cells centrally demonstrate increased degenerative changes. Several spheroidal structures (arrow) are now apparent in this area. These are thought to represent degenerating epithelial cells and/or inflammatory cells. The surrounding epithelium appears to be affected with the intact superficial layers tending to collapse inward. The outer border of the lesion is now well defined. These changes are accentuated in Lesion C (seen in Fig. 3 at a higher magnification) where centrally the superficial layer of cells has disappeared. The undermining of the edge (arrow) of the lesion is readily apparent.

Fig. 1. Early vaccinial lesion consisting of rounded indentation of the epithelium. Lines believed to be intact cell junctions (arrow) can be traced into the indentations. (Scanning electron micrograph, original magnification x1,000.)
Figs. 2 and 3. (Fig. 3) Epithelial lesions in different stages of evolution: (Lesion A) demonstrates an early lesion with beginning degeneration and interruption of the surface epithelium; (Lesion B) more advanced than A with more marked degenerative changes of the surface epithelium (arrow). The intact surrounding epithelium collapses inward; (Lesion C) demonstrates further progression and erosion of the lesion. Undermining of the edge of the epithelium (arrow) with inward collapse of the surrounding epithelium is apparent; (Lesions D and E) the outer border of D is irregular and coalescence with E appears imminent, resulting in a geographic shape. (Scanning electron micrograph, original magnification x100.) (Fig. 3) Higher power view of Lesion C. Undermining with inward collapse of surrounding epithelium is readily apparent. The basal layer of the cells in the center of the lesion appears to be intact. The flattened surface layer of cells become spheroidal as the edge of the eroded epithelium is approached. (Scanning electron micrograph, original magnification x300.)

The basal layer of the epithelium appears intact. The superficial layer of cells as they approach the edge of the lesion lose their normal configuration becoming spheroidal. The undermining with collapse of the surface epithelium is readily apparent. In Lesion D, Fig. 2, the outer border of the lesion is irregular and coalescence with an adjacent lesion (E) seems to be imminent. The phenomenon of coalescence was observed frequently in all of the infected corneas, but no dendritic or branching lesions were seen. Further progression of the vaccinial ulcers leads to erosion through the basal layer to bare stroma (Fig. 4). Fig. 5 demonstrates the light microscopic features of a lesion at the same stage of development as that shown in Figs. 2, C and 3. In addition to the undermining and degenerative changes of the epithelial cells at the edge of the lesion, a marked acute inflammation reaction is present. This is quite intense in the bed of the lesion and in the underlying stroma.

Transmission electron microscopy of the basal epithelium of ulcers corresponding in configuration to those seen in Fig. 5 are shown in Fig. 6. Inflammatory cells are present between intact epithelial cells.
Fig. 4. Further progression of vaccinial ulcer with erosion to bare stroma (S). (Scanning electron micrograph, original magnification x300.)

The basal epithelium is seen to contain viral particles in varying stages of evolution. Mature viral particles appear to be entering a basal cell adjacent to its basement membrane (see inset, Fig 6). The mature vaccinia viral particle is readily recognizable by its dumbbell-shaped core of very dense material. Some of the steps involved in the entry of the vaccinia viral particles into the cells can be recognized. The particle contacts the cell membrane, is enclosed within a phagocytic vesicle, and is transported into the cytoplasm.

The adjacent cell demonstrates the pres-
ence of a "virus factory" with newly formed viral particles in various stages of development.

Discussion

It is of interest that both routes of infection (direct inoculation onto the abraded cornea and subconjunctival injection) result in lesions with similar morphology and evolution. Presumably, all layers of the epithelium are exposed to the virus, and it is likely that all layers become infected, but the exact sequence of involvement is not known with certainty.
Fig. 6. Transmission electron microscopic thin section showing entry of mature viral particles into basal part of cell through cell membrane. (Inset) Viral particles (open arrow) at higher magnification. The adjacent cell contains a virus factory (arrow) with viral particles at different stages of development. (Uranyl acetate and lead citrate, original magnification ×14,500. Inset, original magnification ×29,000.)
The study to date suggests that the first cells to manifest the infection and break down are in the wing cell layer. The initial detectable corneal lesions consist of small epithelial pits produced by the breakdown of the deeper cell layers. Whether this is due to a "toxic effect" or due to direct viral replication within cells is unclear. These small pits, which initially may be little larger in diameter than that of a single surface cell, are covered by intact surface epithelial cells. The pits are produced possibly by the dissolution of wing cells with the subsequent inward collapse of the overlying surface epithelium. This feature is probably accentuated by air- and freeze-drying artifacts. With further dissolution of the cells laterally the overlying epithelium becomes undermined, enhancing the tendency to collapse inwards. With extension superficially the surface cellular layers are eroded, and the lesion stains with fluorescein. At this stage, the basal layer is exposed but appears intact. The superficial cells bordering the lesion continue to undergo degenerative changes, becoming somewhat spheroidal in form in contrast to the normal flattened configuration. Finally, the basal epithelium is eroded, exposing the underlying stroma. An intense neutrophilic inflammatory reaction is present in the epithelium and stroma bordering the ulcer. Figs. 1 to 4 represent the different stages of development.

Tissue culture studies have demonstrated that the cytopathic effects of vaccinia include rounding of the infected cells. This change corresponds probably to the spheroidal shapes found in the surface cells at the margin of the ulcers of this in vivo study. A similar rounding up of the epithelial cells was observed by Spencer and Hayes in their scanning electron microscopic investigations of herpes simplex infections of the rabbit cornea. Previous studies on the effects of vaccinia virus in tissue cultures have revealed a characteristic replication cycle of the virus. Following the absorption of a mature viral particle into the cell within a phagocytic vesicle (Fig. 6, inset), the viral outer coats are disrupted simultaneously with the breakdown of vesicle membranes and the release of the deoxyribonucleic acid (DNA)-containing viral cores into the cytoplasmic matrix. Viral material derived from the cores is probably the initial stage in the formation of viral factories (Fig. 6). Later, spherical membranes of immature vaccinia become evident in the factories (Fig. 6). A large portion of these immature particles possess dense nucleoids. The viral material, once it has become segregated within membranes of immature particles, undergoes rearrangement (differentiates) into the dense brick-shaped mature infectious virus (Fig. 6). The mature viral particles are probably released from the cytoplasm when the cell dies and disintegrates.

In this study, viral particles and factories have been demonstrated by transmission electron microscopy in the intact basal layer, but the first noticeable degenerative changes occur in the wing cells. Autoradiographic studies have demonstrated the viral inclusions in all layers of the corneal epithelium and not exclusively the wing cell layer. In normal rabbits, the basal cells become the surface cells in 7 to 11 days. It is conceivable that the basal cells are the initial cells to be infected, but by the time they have undergone microscopically visible degenerative changes, they have become the wing cells. The sequence of events represented by Figs. 1 to 4 occurred within four days following infection. It is possible that vaccinia virus produces increased DNA synthesis analogous to herpes simplex which perhaps may speed up the cell replication cycle.

Within vaccinial-infected tissue cultures it has been suggested that the focus probably starts from a single cell, with a single virus particle, and the infection gradually expands radially. Viral particles can be seen between adjacent cells in both tissue cultures and in our studies,
which suggests that the infection can spread from cell to cell. The implication is that the infection may arise from a point focus, i.e., a single infected cell, and upon the release of viral particles from this focus surrounding cells are infected, and the infection extends centrifugally. The resultant lesion has an enlarging punctate rounded appearance. With coalescence of adjacent lesions, the punctate appearance may be lost with the lesion assuming a geographic configuration. The development of this pattern of extension contrasts markedly with the linear branching dendritic lesions seen in previous scanning electron microscopic studies of herpes simplex keratitis, where it was assumed that guiding factors such as temporary corneal resistance to infection or neural pathway spread played a significant role in the branching phenomenon. It is possible that the rounded, nonbranching lesions of vaccinia develop because all cells at a given locus from the center of the lesion break down at approximately the same time.

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REFERENCES