Vaccinia-infected rabbit cornea: a transmission electron microscopic study.

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In a previous paper describing the morphologic appearance of vaccinial lesions in infected rabbit corneas, it was noted that the cells within the deep layers of the epithelium were the first cells to manifest the infection and subsequently degenerate. The purpose of the present study was to investigate the virus and epithelial cell interaction and to describe the propagation of the corneal disease.

Animals. The experimental animals were young, healthy, white New Zealand male rabbits weighing approximately four pounds.

Virus. Vaccinia virus was isolated from glycerinated vaccinia lymph (Connaught Laboratories, Toronto, Ontario, Canada) and subcultured in primary rabbit kidney cells. The technique of Oh and Yoneda was utilized. In this study the supernatant of the tissue-culture fluid from the eighth passage was used which produced a virus titer of $10^9$ plaque-forming units per milliliter in chick embryos.

Methods of inoculation. Punctate lesions of the...
rabbit corneas were made by gently stippling the corneas with a nylon bristle. One eye of each rabbit was used as a control and the other eye was infected by applying three drops of the vaccinia suspension directly onto the cornea. 

Specimens for electron microscopy. The rabbits were killed at nine hours, 24 hours, three days, five days, and 10 days after inoculation. The eyes were enucleated and fixed in fresh, cold, 3 per cent glutaraldehyde and buffered to pH 7.3 with 0.2M sodium cacodylate. The specimens were postfixed in 1 per cent osmium tetroxide, embedded in Araldite, and stained en bloc with 2 per cent uranyl acetate. Thin sections of these corneas were examined by transmission electron microscopy using Siemens and RCA electron microscopes.

Occurrence of viral lesions of the cornea. The corneas of the control eyes healed within 12 hours, at which time there was no fluorescein staining. In virus-inoculated eyes, clinically apparent lesions were not visible until 24 hours, by which time tiny grey areas were noted which gradually enlarged. Within 48 hours punctate epithelial ulcers were present which stained with fluorescein. The epithelial lesions progressively enlarged for 3 to 5 days and then spontaneously improved so that by 10 days most of these epithelial lesions had healed. Those animals which eventually developed stromal disciform lesions had clinically apparent stromal disease by Day 6, but the animals were killed before the stromal disease had resolved. One animal was not killed until 3 weeks following inoculation by which time the stroma had become extensively vascularized.

Distribution of viral particles and viral-infected cells. In the corneas of animals killed at nine hours and 24 hours no virus was found by electron microscopy despite prolonged searching. The corneas of animals killed at Day 3 and Day 5 had abundant ulcerated lesions in which vaccinia virus could be demonstrated. The characteristic location of the early corneal involvement was deep in the corneal epithelium where both free viral particles and viral-infected cells were found (Figs. 1 and 2). The mature viral particles, virions, were readily recognized by their disc-shaped core of electron-dense material (nucleoid) surrounded by a thick layer of viroplasm with an outer coat of two very thin membranes (Fig. 2, inset). Mature vaccinia virus particles measured approximately 250 to 300 microns in length and 200 to 250 in width. In the deeper layers of the epithelium of the Day 3 specimens more cells were infected and more free virus particles were present than in the superficial layers.
Fig. 3. A vaccinia viral factory in the cytoplasm of the corneal epithelial cell. This viral factory consists of a matrix of fine granular material and numerous circular profiles containing material of varying electron density. In some particles this material appears to have condensed. This viral factory corresponds to the Guarnieri body (× 39,000).

Cell and virus interactions. The entry of vaccinia virus into cells via phagocytic vesicles is a constant finding which has been previously reported.\(^3\)\(^-\)\(^7\) In the infected corneas in this study mature virions appeared to enter the cells by phagocytosis in a vesicle (Fig. 2). The walls of the vesicles peel away, “uncoat,” a process described in vaccinia-infected tissue-culture cells.\(^4\)\(^-\)\(^7\) This may be the mechanism by which the virus contents finally invade the cytoplasm of the cell.

Within cells near the edge of the ulcer, distinct areas (“viral factories”) can be recognized in the cytoplasm composed of a very fine-grained matrix and sharply defined circular membranes which enclose matrix material (Figs. 1 and 3). These “factories” correspond to the Guarnieri bodies seen by light microscopy. The circular membranes have been identified as immature virus particles.\(^3\)\(^-\)\(^6\) The material within these immature particles usually appears more electron dense than the viral factory matrix, but particles with even denser material surrounded by a mantle of translucency are also present (Fig. 3). Since relatively few transitional forms were found between round immature virus and the complex mature forms, it can be assumed that this transition takes place relatively rapidly.

Infected epithelial cells appeared to have a relatively normal appearing fine structure (Fig. 1). In such cells apposition to basement membrane and corneal stroma appeared normal and desmosomal attachments to adjacent cells were present. A large number of mature viral particles were found in the intercellular spaces which suggests, however, that the intercellular attachments of the infected cells have decreased to some extent. Some of the cells with functioning viral factories have ingested further mature viral particles and the initial steps of virus uncoating appear to have occurred (Fig. 1). At the edge of the ulcer many cells contain either mature viral particles and/or functioning viral factories with
Fig. 4. Degenerating epithelial cells in the base of a vaccinial corneal ulcer overlying the stroma (S). The vaccinia-infected epithelial cells are rounded and their plasma membranes are no longer intact (arrows). These cells contain both viral factory material (F) and mature viral particles (M). In the upper center of the photograph there is a polymorphonuclear leukocyte (× 9,000).

Immature forms in every stage of their evolution. Propagation of the lesion. In some areas at the edge of the ulcer numerous mature infectious particles both within and between adjacent cells suggest that the cell attachments may have loosened to some extent. In the bed of an ulcer the infected basal epithelial cells are rounded, appear to be only loosely attached to stroma, and contain both mature and developmental viral forms, some of which are in phagocytic vesicles (Fig. 4). The plasma membrane is disrupted allowing a discharge of virions and cytoplasmic debris into the extracellular space. In addition to the degenerating epithelial cells, polymorphonuclear leukocytes are also present.

In only one instance were a few virus particles found in the superficial layers of the corneal stroma. These particles were not associated with intact keratocytes and may well have entered through a defect produced by the nylon bristle used in inoculating the cornea.

In our previous study, it was noted that the epithelial cells near the corneal ulcer underwent characteristic degenerative changes consisting of a diminution in size and a change to a rounded or spheroidal shape. The rounding of diseased cells at the margins of the ulcers is a cytopathic effect that is a constant feature of tissue-culture studies. It is not entirely clear whether this rounding effect of diseased cells is due to direct viral replication within cells or is merely a nonspecific toxic effect. Possibly these cytopathic effects can occur through the synthesis of virus proteins before viral particle formation. The detachment of intercellular attachments and rounding of cells occurred only in cells with definite viral factories and mature particles. The stromal involvement was limited to finding the occasional virion lying free in the
stroma. We were unable to detect viral particles within keratocytes; however, other investigators using fluorescein antibody and autoradiographic techniques have stated that there is viral replication within the stromal keratocytes.

It has been demonstrated that vaccinia does produce a number of soluble antigens which can be classified broadly into two groups depending upon molecular weight. The high molecular weight antigens (M.W. greater than 200,000) stimulate synthesis of neutralizing antibodies in rabbits. It is possible that disciform keratitis in vaccinia corneal infections may be related to an antibody reaction with vaccinal antigens that have diffused into stroma from the epithelium or that have formed in keratocytes. The efficaciousness of debridement in the therapy of viral epithelial keratitis may result from the removal of antigen-containing epithelial cells as well as the removal of the virus itself.


Key words: rabbit, cornea, transmission electron microscopy, virus, ulcer, virion, factory, Guarnieri body, immune.

REFERENCES


Fundus holography through a wide-angle contact lens.* ARTHUR N. ROSEN.

The wide-angle ophthalmoscope has the potential to become an effective new tool in diagnosing eye diseases. To date, it has been used to record large areas of the fundus with conventional photographic techniques. A unique new way of utilizing the wide-angle contact lens has now been developed. It uses holography to record information about the eye and offers much more information than a single photograph taken with the wide-angle lens alone. The hologram contains information about all parts of the eye visible through the contact lens. Hence, by changing the focus of the viewing device, regions other than the retina (e.g., the vitreous, lens, aqueous, or cornea) can be viewed without recalling the patient for further examination.

The experimental arrangement is shown in Fig. 1. A C. W. Argon laser with an output of 210 mW. in a single longitudinal mode at 514.5 nm. was used as the light source. The laser beam was separated into reference and object waves. The reference wave was spatially filtered and expanded to uniformly fill the holographic film plate. A collimated reference beam was chosen to facilitate projection of the image with the conjugate to the reference beam, if desired. The object wave was delivered to the animal eye through a flexible fiber-optic bundle. This provides a significant advantage over previously reported mirror systems of illumination, since it allows much more mechanical freedom in the system delivering light to the eye, and thus provides greater flexibility in positioning the subject. The exit pupil of the fiber optics is an annulus 8 mm. in diameter and 1 mm. wide, embedded in a specially designed wide-angle contact lens.