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Herpetic Keratitis in Athymic (Nude) Mice

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The inflammatory response to herpes simplex virus infection of the cornea was studied in athymic nude (nu/nu) and heterozygote (nu/+) BALB/c mice. Although athymic mice were highly susceptible to HSV infection and died 13 to 17 days after corneal inoculation, they failed to develop necrotizing keratitis of the cornea. Heterozygote mice survived the initial viral infection, but many of these mice developed necrotizing keratitis and permanent corneal scarring. Light and electron microscopy showed numerous inflammatory cells (polymorphonuclear leukocytes and lymphocytes) in the corneas of heterozygote mice, but not in the athymic mice. These studies suggest that the immune system plays a dual role in herpes simplex virus infection of the cornea: protection against dissemination of the virus and immunopathogenesis of necrotizing keratitis in the cornea.

Evidence from several sources indicates that cell-mediated immunopathogenesis involving T-lymphocytes may be primarily responsible for the tissue necrosis and opacity associated with chronic herpetic stromal keratitis in the cornea (4-6, 8). The initial inflammatory response to herpes simplex virus (HSV) infection of the rabbit cornea is characterized by intense stromal edema and epithelial keratitis in the majority of the infected animals (7). As the initial inflammation subsides, virus can no longer be recovered from the infected corneas (8, 13). However, many of these animals subsequently develop necrotizing keratitis which results in permanent stromal opacity and scarring. Lymphocytes accumulate in the inflamed corneas and are frequently found in intimate contact with stromal keratocytes, suggesting a T-lymphocyte attack on a target cell (6, 8).

Immunoelectron microscopic studies of productively infected corneal cells in vitro (4) showed that HSV antigens were distributed over the entire surface of infected cells, often concentrated over focal areas of plasma membrane thickening. Similar studies of tissue sections from a virus-infected rabbit cornea with early necrotizing keratitis demonstrated HSV antigens in association with the cell surface of stromal keratocytes (5). These surface antigens would allow the stromal keratocytes to be recognized as foreign by the host immune system, just as HLA antigens of an incompatible tissue graft are recognized as foreign (11). Thus, the chronic stages of herpetic stromal keratitis could be analogous to a host-versus-graft type of immune reaction (4, 6, 8).

The congenitally athymic nude mouse is deficient in T-lymphocytes and thymus-dependent immunological functions and readily accepts xenografts from a variety of sources (12). Therefore, a study of herpetic keratitis in athymic nude mice should enable us to evaluate the contribution of T-lymphocytes and thymic-dependent functions in the immunopathogenesis of this common ocular infection. If our hypothesis is correct, athymic mice should not develop necrotizing keratitis, whereas normal mice should.

MATERIALS AND METHODS

Mice. Athymic nude (nu/nu) mice and phenotypically normal heterozygote (nu/+) pathogen-free BALB/c mice were obtained from Grand Island Biological Co. These mice were maintained in a germfree isolette during the study. In some experiments athymic nude mice were also obtained from the National Institutes of Health, and normal BALB/c or Swiss-Webster mice were used as controls.

Virus. The RE strain of HSV type 1 (HSV-1) passaged in human embryonic kidney cells (Microbiological Associates) in Eagle medium with 2% fetal bovine serum was used. A culture of infected cells demonstrating 90% cytopathic effect was frozen and thawed to obtain a cell lysate containing infectious virus at 10^7 plaque-forming units (PFU) per ml as determined by titration on tube cultures of human embryonic kidney cells.

Corneal inoculation. The corneas of anesthetized (methoxyfluorane or pentobarbital) mice were heavily scratched with a 27-gauge needle. A cotton swab saturated with a 1:10 or 1:100 dilution of the stock virus preparation was rubbed on the scratched corneas to initiate a virus infection.

Biomicroscopy. The eyes were examined with slit-lamp biomicroscopy at frequent intervals for evidence of viral infection and corneal pathology. The corneas were graded for opacity resulting from edema and cellular infiltration as follows: 0, clear; 1+, slight corneal opacity; 2+, moderate opacity; 3+, severe opacity

with discernible iris; 4+, total opacity with iris not visible. The mean score was determined for each group of mice at each examination.

Histology and electron microscopy. Whole eyes were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The corneas were removed, postfixed in 1% osmium, dehydrated in acetone and embedded in Epon. Thick sections (1 μm) were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and then examined in a Zeiss EM-9 electron microscope.

RESULTS

Biomicroscopic observations. Inoculation of mouse corneas with a heavy inoculum (10^6 PFU/ml) of HSV overwhelmed the immune system, and most of the animals (both athymic and normal) died within 5 to 10 days. In these experiments there was no difference in corneal opacity between athymic and normal mice (Fig. 1).

With lower doses of HSV (5×10^5 PFU/ml), some of the normal mice recovered from the viral infection, but the athymic mice still survived for only 13 to 17 days. During this period the corneas were observed for pathological changes. In the first experiment (Fig. 2), the corneas of the virus-infected mice showed epithelial keratitis and stromal edema within 3 days after inoculation. In normal mice corneal opacity continued to increase, changing in appearance from the hazy opalescence of stromal edema to a white, scar-like opacity indicative of necrotizing stromal keratitis (Fig. 3). Many of the virus-infected mice, both athymic and normal, died after week 1. However, the corneas of the surviving athymic mice slowly cleared as stromal edema resolved (Fig. 2).

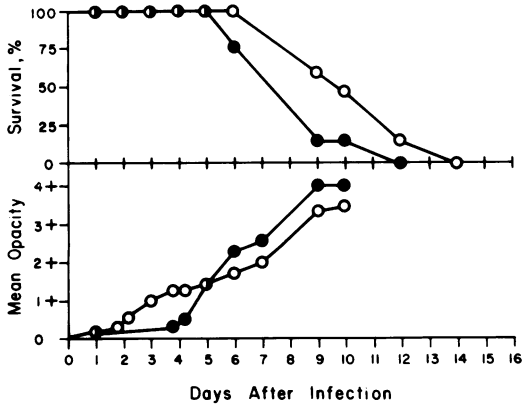

FIG. 1. *Survival and mean corneal opacity of athymic nude (○) and heterozygote (nu/+) BALB/c mice (●) after corneal infection with a heavy inoculum of HSV (10^6 PFU/ml). Each group contained 12 mice at the time of inoculation.*

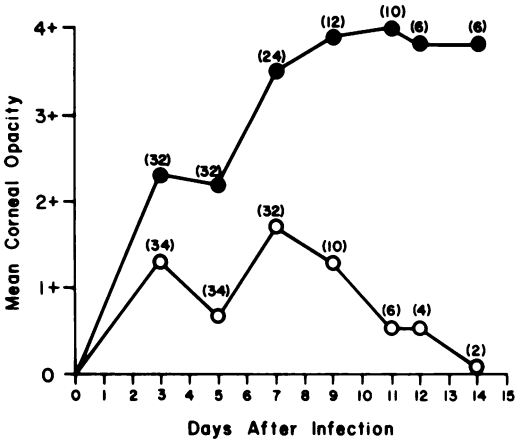

FIG. 2. *Mean corneal opacity of athymic nude (○) and normal (●) mice after infection of the cornea with a moderate inoculum of HSV. The numbers in parentheses represent the number of eyes examined.*

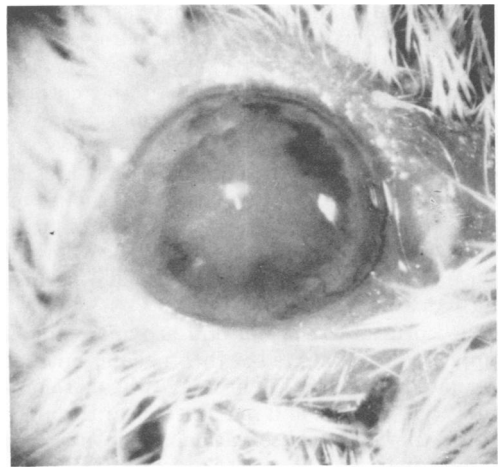

FIG. 3. *Necrotizing keratitis of the cornea 28 days after HSV infection in a normal Swiss-Webster mouse. Note that the cornea is not vascularized.*

The results of a second experiment (Fig. 4) were similar except that the early inflammatory response of both groups of mice was nearly identical during week 1. Thereafter, the corneas of the athymic mice slowly cleared while the normal mice developed opaque corneas as a result of necrotizing keratitis. Figure 5 shows the eye of an athymic mouse with stromal edema on day 8 postinfection, and Fig. 5b shows the same eye on day 13 shortly before death. Although the surrounding tissues were heavily infected, the cornea remained clear.

In an attempt to prolong the survival time of the HSV-infected mice, a 1:100 dilution of stock virus suspension was used to inoculate the corneas. With the lower dose of virus inoculum (10^5

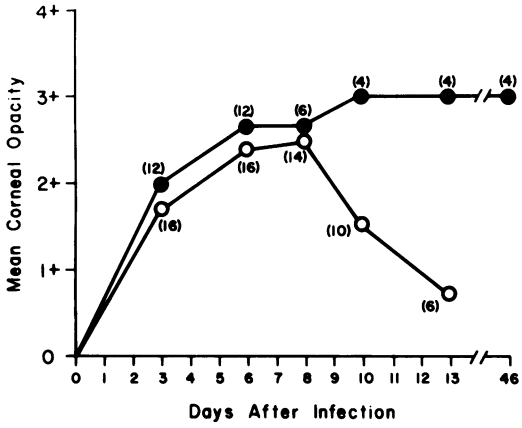

FIG. 4. *Mean corneal opacity of athymic nude* (○) *and normal* (●) *mice after infection of the cornea with a moderate inoculum of HSV. The numbers in parentheses represent the number of eyes examined.*

PFU/ml) the initial inflammatory response was much reduced in normal mice and barely apparent in the athymic mice (Fig. 6). However, the athymic mice still succumbed to the corneal inoculations, while many of the normal mice developed opaque corneas. Figure 7a shows the clear cornea of an athymic mouse, and Fig. 7b shows the opaque cornea of a normal mouse on day 17 postinfection with the lower dose of virus. The athymic mouse soon died. The normal mouse continued to live, but retained a permanently opaque cornea.

The corneas of normal and athymic control animals were scratched with a needle, but not inoculated with HSV. These eyes healed completely within 24 h, and remained clear throughout the period of observation. None of these animals died.

Histology and electron microscopy. Light and electron microscopic observations of the opaque corneas of normal mice with necrotizing keratitis showed that numerous inflammatory cells were present (Fig. 8). Both polymorphonuclear leukocytes (PMN) and mononuclear cells were seen. The mononuclear cells, presumably lymphocytes, were often found in intimate contact with stromal keratocytes (Fig. 9), as described previously, in rabbit and human corneas (6, 8).

Relatively few inflammatory cells (PMN) were found in the corneas of virus-infected athymic mice obtained at the time of death. However, numerous virus-like particles morphologically resembling HSV were seen in corneal nerves (Fig. 10), indicating the probable mechanism by which the virus is transmitted to the central nervous system. Several of the athymic nude mice were observed continuously turning or rolling over before death, suggesting neurological involvement.

DISCUSSION

The role of cell-mediated immunity in the control of HSV infection is well established (2, 3, 10). The thymus-dependent functions of the immune system protect normal mice against dissemination of the virus from a localized site of infection (10). Thus, the finding that congenitally athymic nude (nu/nu) mice are highly

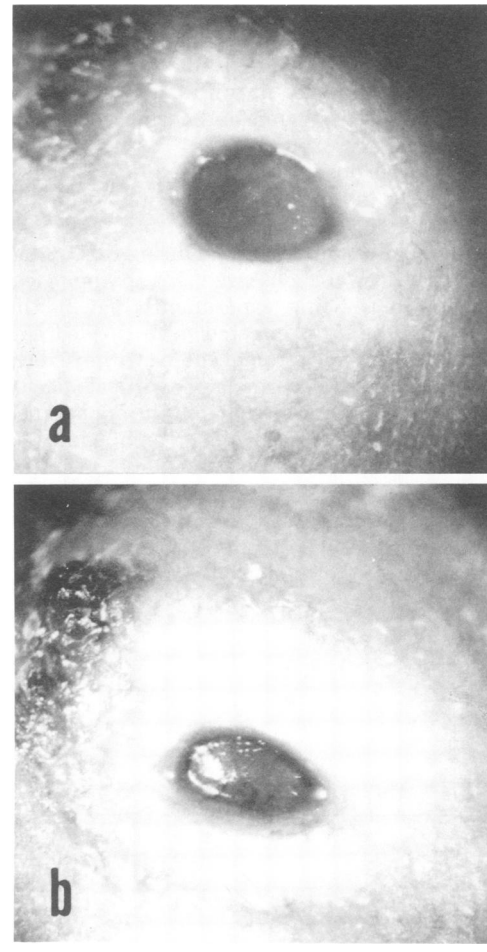

FIG. 5. *The eye of an athymic nude mouse on day 8 postinfection (a); the same eye on day 13 (b) just before death of the animal. Although severe stromal edema was apparent on day 8, necrotizing keratitis did not develop and the cornea was noticeably clearer when the animal died. Note the dissemination of infection to surrounding tissues (upper left).*

susceptible to HSV infection is consistent with these observations.

According to our concept of the immunopathogenesis of herpetic stromal keratitis, the host immune system recognizes HSV-infected corneal cells as foreign because of the presence of virus antigens associated with the surface membrane (4, 5). Sensitized T-lymphocytes then migrate into the inflamed cornea in a manner similar to the host response to an incompatible tissue graft (6, 8). The observations described in this report are consistent with this interpretation. Thus, normal mice may survive a viral infection of the cornea, but many develop opaque corneas. Athymic mice, however, cannot survive the viral infection, but the corneas of these animals are always clear at the time of death. In HSV infection of the cornea, the host immune system plays a dual role: (i) protection against dissemination of the infection to other parts of the body, and (ii) immunological response to infected corneal cells resulting in permanent stromal opacity. It would be of interest to know whether these two effects result from the same immunological mechanism.

The thymus-dependent functions of the mammalian immune system are not fully understood. The finding that T-cell-depleted animals produce antibodies against some antigens but not to others suggests that collaboration between T- and B-lymphocytes is a requirement for antibody synthesis in some instances (1, 9). Therefore, we cannot rule out the participation of antibodies in the induction of necrotizing keratitis, as opposed to cell-mediated cytolysis by T-lymphocytes alone. The absence of inflammatory cells in the corneas of HSV-infected athymic mice is consistent with the role of sensitized lymphocytes in producing chemotactic lymphokines for PMN (14). These cells (PMN) may also be required for the induction of necrotizing keratitis.

Additional studies of the cell-mediated immune responses to HSV infection of the cornea in normal and athymic nude mice should prove to be extremely useful for understanding the mechanism of immunopathogenesis in herpetic keratitis.

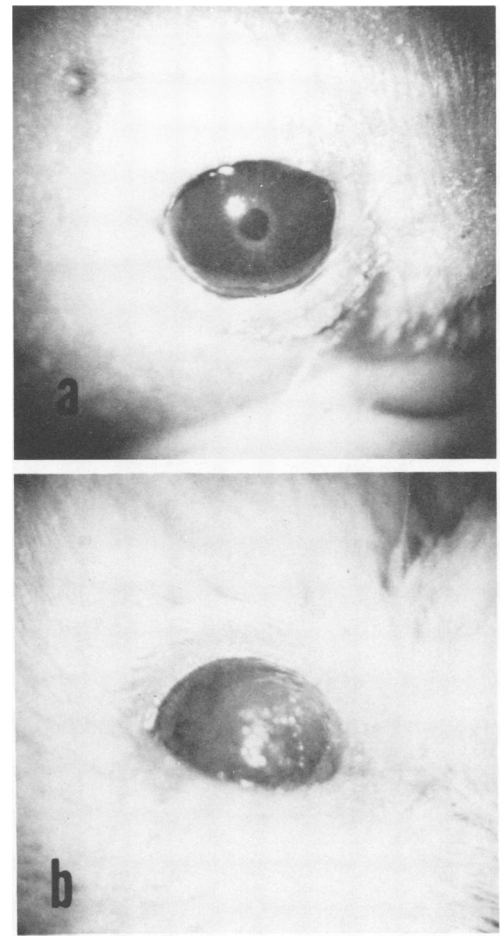

FIG. 7. *The eye of an athymic nude mouse (a) and a normal (nu/+) heterozygote BALB/c mouse (b) 17 days after infection with a light inoculum of HSV (10^5 PFU/ml).*

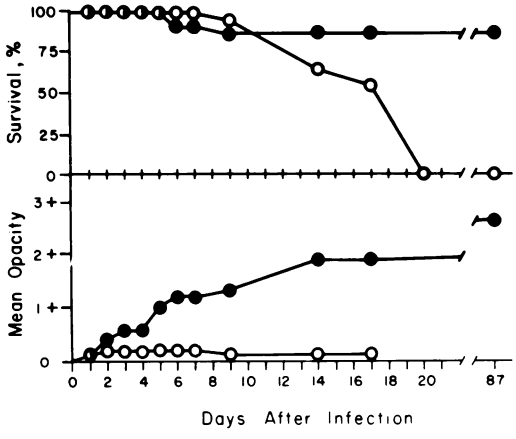

FIG. 6. *Survival and mean corneal opacity of athymic nude (○) and normal (●) heterozygote BALB/c litter mates after infection of the cornea with a light inoculum (10^5 PFU/ml) of HSV. Each group contained 12 mice at the time of inoculation. Five corneas in the remaining 10 normal mice had perforated before the last observation.*

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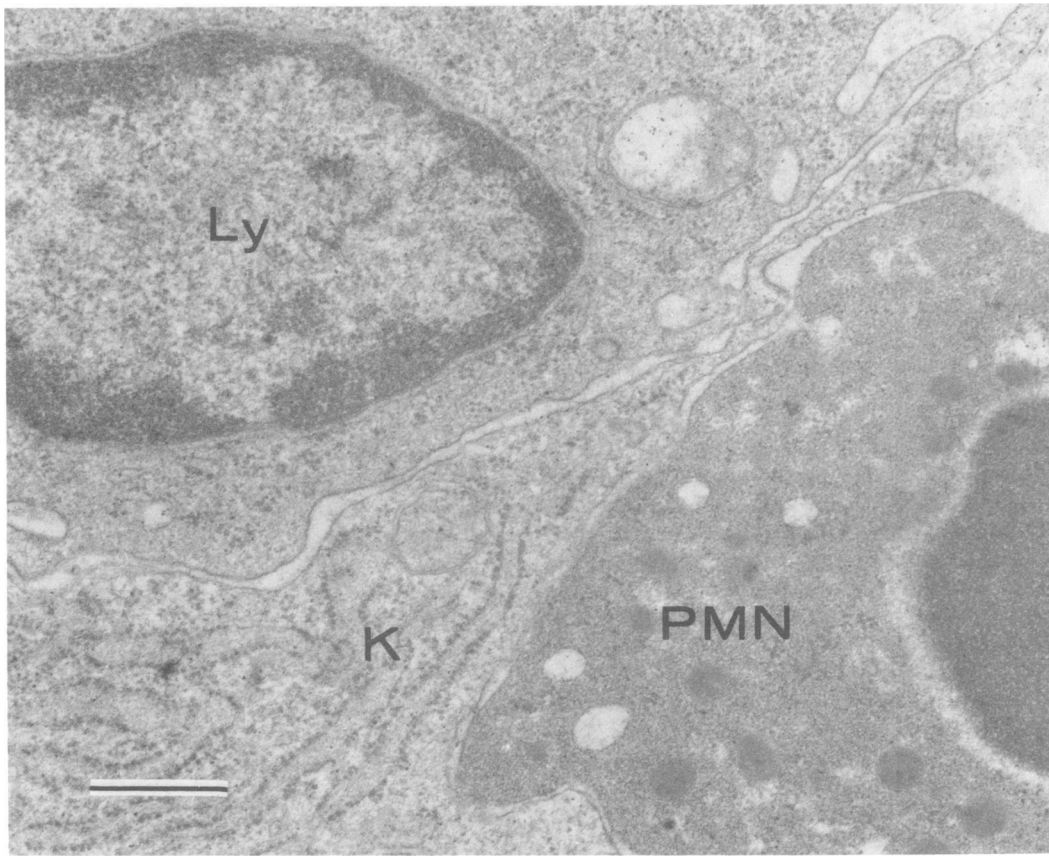

FIG. 8. *Electron micrograph of inflammatory cells in the stroma of a normal BALB/c mouse cornea with necrotizing keratitis at 39 days postinfection. A lymphocyte (Ly) and PMN are seen adjacent to keratocyte (K). Bar, 0.5 μm.*

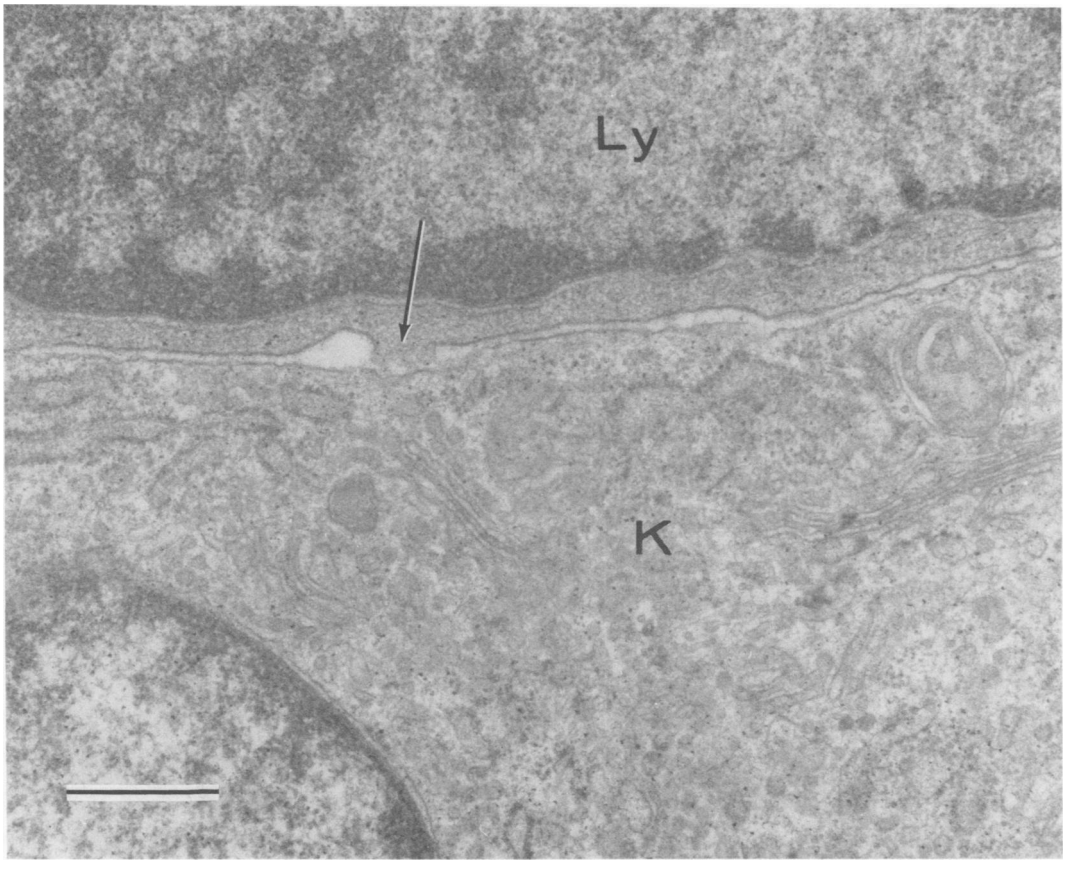

FIG. 9. *Electron micrograph of a lymphocyte (Ly) closely adjacent to a keratocyte (K) in the stroma of a normal mouse cornea with necrotizing keratitis at 39 days postinfection. Note the cytoplasmic projection extending from the lymphocyte toward the keratocyte (arrow). Bar, 0.5 μm.*

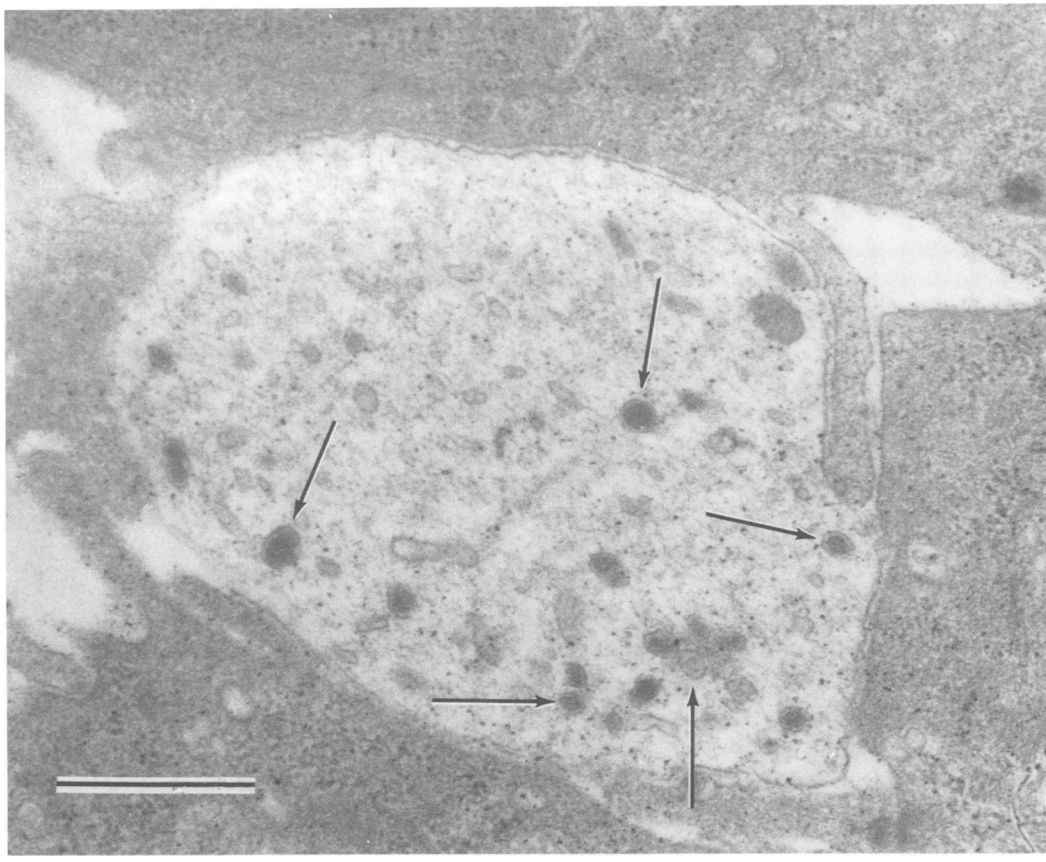

FIG. 10. *Electron micrograph of virus-like particles (arrows) morphologically resembling HSV in a corneal nerve in the epithelium of an athymic nude mouse 10 days postinfection. The diameter of the virus-like particles is 86 to 100 nm. Bar, 0.5 μm.*

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LITERATURE CITED

1. **Burns, W. H., L. E. Billups, and A. L. Notkins.** 1975. Thymus dependence of viral antigens. Nature (London) **256**:654-656.
2. **Ennis, F. A.** 1973. Host defense mechanisms against herpes simplex virus. I. Control of infection in vitro by sensitized spleen cells and antibody. Infect. Immun. **7**: 898-904.
3. **Ennis, F. A.** 1973. Host defense mechanisms against herpes simplex virus. II. Protection conferred by sensitized spleen cells. J. Infect. Dis. **127**:632-638.
4. **Henson, D., R. Helmsen, K. E. Becker, A. J. Strano, M. Sullivan, and D. Harris.** 1974. Ultrastructural localization of herpes simplex virus antigens on rabbit corneal cells using sheep antihuman IgG antihorse ferritin hybrid antibodies. Invest. Ophthalmol. **13**:819-827.
5. **Metcalf, J. F., and R. Helmsen.** 1977. Immunoelectron microscopic localization of herpes simplex virus antigens in rabbit cornea with antihuman IgG-antiferritin hybrid antibodies. Invest. Ophthalmol. Vis. Sci. **16**:779-786.
6. **Metcalf, J. F., and H. E. Kaufman.** 1976. Herpetic stromal keratitis: evidence for cell-mediated immunopathogenesis. Am. J. Ophthalmol. **82**:827-834.
7. **Metcalf, J. F., J. I. McNeill, and H. E. Kaufman.** 1976. Experimental disciform edema and necrotizing keratitis in the rabbit. Invest. Ophthalmol. **15**:979-985.
8. **Metcalf, J. F., and R. W. Reichert.** 1979. Histological and electron microscopic studies of experimental herpetic keratitis in the rabbit. Invest. Ophthalmol. Vis. Sci. **18**:1123-1138.
9. **Mitchell, G. F.** 1977. Observations and speculations on the influence of T cells in the cellular events of induction of antibody formation and tolerance in vivo, p. 230. *In* J. J. Marchalonis (ed.), The lymphocyte. Marcel Dekker, Inc., New York.
10. **Oakes, J. E.** 1975. Role for cell-mediated immunity in the resistance of mice to subcutaneous herpes simplex virus infection. Infect. Immun. **12**:166-172.
11. **Polack, F. M.** 1973. Corneal graft rejection: clinicopathological correlation. *In* Corneal graft failure. Ciba Foundation Symposium 15, Association of Scientific Publishers, Amsterdam.

12. **Reed, N. D., and D. D. Manning.** 1978. Present status of xenotransplantation of nonmalignant tissue to the nude mouse, p. 167-185. *In* J. Fogh and B. C. Giovanella (ed.), The nude mouse in experimental and clinical research. Academic Press Inc., New York.
13. **Schwartz, L. K., M. H. Friedlander, R. Cyr, and J. O. Oh.** 1979. Histopathology of experimental primary herpes simplex keratitis. Invest. Ophthalmol. Vis. Sci. **18**(Suppl.):233.
14. **Ward, P. A., H. G. Remola, and J. R. David.** 1969. Leukotactic factor produced by sensitized lymphocytes. Science **163**:1079-1081.