The role of virus-infected mononuclear leukocytes (MNLs) in the pathogenesis of neonatal herpetic chorioretinitis in newborn rabbits was investigated. As early as 2 days after inoculating the animals' skins with type 2 herpes simplex virus (HSV-2), infectious MNLs in the infected animals' peripheral blood were found. The virus was associated, for the most part, with MNLs that belonged to phagocytic and adherent cell fractions. Observations by electron microscopy indicated that HSV-2 was actively replicating in the MNLs. It was also found that as few as 80 virus-infected MNLs injected via the right common carotoid artery were capable of inducing the chorioretinal lesions in 50% of the eyes, but that as many as $10^5$ Plf of free virus were required to produce the same lesions in the same percentage of eyes. This result clearly indicated that virus-infected MNLs were far more efficient in producing chorioretinitis than free virus, and may thus play a crucial role in the pathogenesis of herpetic chorioretinitis in newborn rabbits. When $^{111}$In-labeled virus-infected or uninfected MNLs were injected into normal rabbits via the right common carotid artery, the virus-infected MNLs localized more readily in the eye than the uninfected MNLs. The virus-infected MNLs also attached to the cultured vascular endothelial cells significantly more often than the uninfected MNLs. These results suggested that virus-infected MNLs might be easily trapped in the circulation of the eye and, in this way, produce the ocular lesions.


Since Batignani first described newborn herpetic keratoconjunctivitis, the incidence of neonatal herpetic infection has steadily increased. The disease has, in fact, become a serious perinatal complication. This is no doubt due to the widespread increase in genital herpes among young adults, and to the fact that the virus is usually acquired by newborns in the birth canal during delivery. Once the baby's immature defense system has allowed viral infection to take place, ocular and systemic disease can follow.

Among the ocular manifestations, chorioretinitis is next in frequency to external eye infection. Although about 50% of infants recover entirely, severe damage to the ocular fundus can blind many others. Interestingly, all of the isolates from chorioretinal lesions have been type 2 herpes simplex virus (HSV-2). This fact has been partly explained in experimental animals by the different susceptibilities of the skin to the two types of HSV.

In an earlier study, we established an animal model in which chorioretinitis developed in newborn rabbits shortly after their skin inoculations with HSV-2. We also showed that infectious HSV-2 could be isolated from mononuclear leukocytes (MNLs) of the peripheral blood and less frequently from the plasma of infected animals. Although it seemed likely in these studies that the virus was transferred to the eye from the blood, the exact mechanism by which the chorioretinitis occurs has remained unclear. In the present study, we have investigated the role of virus-infected MNLs in the pathogenesis of chorioretinitis, and have attempted to define the pathogenic mechanisms by which HSV-2-infected MNLs produced the chorioretinal lesions.

Materials and Methods

Animals

The experimental animals were litters of New Zealand white rabbits 2–7 days old. Pregnant rabbits were usually purchased from a local vendor at midterm, or the newborns were acquired from our vivarium. Studies using these animals were performed in conformance with the ARVO Resolution on the Use of Animals in Research.
Virus

The Curtis strain of type 2 herpes simplex virus (HSV-2) was propagated in Vero-cell monolayers that were maintained with Eagle's minimum essential medium (MEM: Gibco, Grand Island, NY), supplemented by antibiotics and 2% heat-inactivated fetal calf serum (HIFCS) (Microbiological Associates, Walkersville, MD). The titer of the stocked virus was $1.5 \times 10^6$ PFU/ml.

Inoculation of the Skin of Newborn Rabbits With HSV-2

As described earlier, newborn rabbits were inoculated subcutaneously with Curtis strain of HSV-2. In brief, the diluted virus suspension ($10^4$ PFU/0.01 ml) was inoculated subcutaneously at each of four sites on the skin of the backs of newborn rabbits with a tuberculin syringe and 26-gauge hypodermic needle.

Separation of Mononuclear Leukocytes From Blood

As previously described, cardiac blood drawn from skin-infected newborn rabbits was heparinized, and MNLs were isolated by means of gradient centrifugation on the lymphocyte separation medium (LSM; Bionetics, Kensington, MD). The plasma layer was also collected for the detection of free infectious HSV. Red blood cells were removed from the MNL fraction by the hypotonic treatment described by Fallon et al. Although heparin has been known to inactivate HSV, the concentration of heparin used in this experiment (5U of heparin/ml of blood) had no effect on the infectivity of HSV-2.

Detection of Free Infectious HSV in Blood

One-tenth of a milliliter each of serial 10-fold dilutions of the plasma was placed on duplicate wells of Vero-cell monolayers. After 1 hr of incubation, the wells of Vero-cells were overlaid with MEM containing 1% methyl cellulose and 10% HIFCS. After incubating the plate at 37°C for 4 days, plaques were counted by fixing the cells with formalin and crystal violet solution.

Infectious Center Assay of Mononuclear Leukocytes

The isolated MNLs were suspended in 0.2 ml of RPMI 1640 medium and the cells were counted. Then 0.1 ml of this cell suspension was placed gently on duplicate wells of Vero-cell monolayers. After 3 hr of incubation to allow the cells to settle down, the wells of Vero-monolayers were carefully overlaid with MEM containing 1% methyl cellulose and 10% HIFCS. After incubating the plate at 37°C for 4 days, we counted the number of infectious centers by fixing the cells with buffered neutral formalin and staining with 1% crystal violet solution.

Electron Microscopy of MNLs Isolated From Skin-Infected Newborn Rabbits

The peripheral MNLs were isolated from the skin-infected newborn rabbits 3 days after virus inoculation and were immediately fixed at 4°C with 1.5% glutaraldehyde in 0.1 N cacodylate buffer (pH 7.4). The cells were then postfixed with 1% OsO4, treated with 0.5% uranyl acetate, and further processed for examination as previously described.

Separation of Nonphagocytic Cells by Yeast Treatment

As described by Ohashi et al, pooled, heparinized cardiac blood of newborn rabbits was mixed with a 1:10 volume of yeast-cell suspension in PBS (1 X 10^9 particles/ml) and incubated for 45 min at 37°C with occasional shaking. After the mixture was centrifuged on LSM at 800 g for 30 min, the lighter cells (non-phagocytic cells) that sedimented at the interface were pooled, washed twice with RPMI medium, and suspended in 1 ml of RPMI medium supplemented with 10% HIFCS and 2 mM glutamine.

Separation of Glass-Beads-Adhering Cells

According to the method of Shortman et al, peripheral MNLs were partially purified from pooled, heparinized cardiac blood by LSM gradient centrifugation. Finally they were suspended in bicarbonate-free MEM (BC(-)MEM) (Gibco, Long Island, NY), supplemented with 50% fresh rabbit serum (FRS), and were applied to the glass-beads column at 37°C, and the flow rate was set for the cells to stay in the column for 10 min. The column was then washed with BC(-)MEM supplemented with 25% FRS. The cells flowing out of the column were pooled as glass-beads-nonadherent (GBNA) cells.

The medium was then switched to 0.02% EDTA in phosphate-buffered saline solution (pH 7.4), supplemented with 2% FRS. After two column-volumes of washing, the column was sealed and rolled to agitate the beads gently. The cells released from the beads were extensively washed out with EDTA solution and pooled as glass-beads-adherent (GBA) cells. Smears of each fraction were stained with esterase, and GBA cells contained more than 95% esterase-positive cells.

In Vitro Infection of Mononuclear Cells With HSV

Isolated MNLs, suspended in 1 ml of RPMI medium supplemented with 10% HIFCS and 2 mM glutamine, were incubated with the Curtis strain of HSV-2 (MOI
for 18 hr at 37°C in 5% CO₂. At MOI of 10, the highest proportion of MNLs (6%) were infected with HSV. The infected MNLs were washed five times with 10 ml of RPMI to get rid of as many cell-free virus particles as possible. The washed cells were resuspended in 1 ml of RPMI medium.

Injection of HSV-Infected MNLs Into Common Carotid Artery

The newborn rabbits were anesthetized systemically with an intramuscular injection of ketamine (25 mg/kg)-xylazine (10 mg/kg) cocktail. Following the median incision of the skin of the neck, the right common carotid artery was exposed by careful separation of salivary glands and muscles, and we slowly injected the artery with 0.1 ml of an HSV-infected MNL suspension (10⁶ cells). We then compressed the artery with a sterile cotton applicator for a minute to stop the bleeding and irrigated the skin wound with antibiotics. By performing the infectious center assay with an HSV-infected MNL suspension each time, we checked the size of the inoculum received by the newborn rabbits. Usually, 3–6% of the MNLs were infected after overnight incubation with HSV at MOI of 10.

In Vitro Adherence Assay of MNLs

This assay was performed according to a slight modification of the method of Hashimoto et al.¹⁸ Endothelial-cell monolayers were prepared in 24-well plates (16 mm in diameter) from secondary rabbit endothelial cells. In-labeled infected and uninfected MNLs were prepared in the same way as already described in the preceding section. We added to triplicate wells of endothelial cell monolayers 0.3 ml of a labeled, infected or uninfected MNL suspension (2 × 10⁴ cells/well) in Dulbecco’s modified Eagle’s medium supplemented with 10% FRS, and incubation of the plate was continued for 2 hr at 37°C. Nonadherent MNLs were then removed by several cycles of gentle washings with warm HBSS. Finally, 1 ml of 0.1 N NaOH was added to the wells to lyse the adherent cells, and the radioactivity recovered in the NaOH solution was counted with a gamma counter. The degree of adherence was expressed in percent by dividing the mean radioactivity in triplicate sample wells with the radioactivity initially added to the wells.

Results

HSV-2 Infected Mononuclear Leukocytes and Free HSV-2 in the Blood of Newborn Rabbits Infected Subcutaneously With HSV-2

As previously reported, HSV-2 could be recovered as early as 2 days after the infection from only MNLs in three of seven rabbits, from both MNLs and plasma in one of seven rabbits, and from only plasma in none of seven rabbits. On postinfection day 4, however, the virus could be detected from only MNLs in three of eight rabbits, from both MNLs and plasma in five of eight rabbits, and from only plasma in none of eight rabbits. There were, on the average, 100 times more HSV-infected MNLs than free infectious HSV-2 particles in the blood.

Electron Microscopy of HSV-2 Infected Mononuclear Leukocytes

To determine whether virus particles were replicating in MNLs, cells isolated from the blood of infected newborn rabbits were examined by transmission electron microscopy. Two samples were examined: the total MNL cell population, and glass-adherent cells. Virus-infected cells were found in both specimens. Although we could not identify by their ultrastructure all of the MNLs in which virus was detected, some of the glass-adherent cells contained a considerable number of lysozymes and thus appeared to be mononuclear phagocytes (Fig. 1). Nucleocapsid of viruses in all stages of development were found in the nuclei. Mature enveloped virus particles were most commonly localized.
Identification of HSV-Infected Mononuclear Leukocytes Isolated From Skin-Infected Newborn Rabbits

To identify which subpopulation of MNLs were carrying HSV, we fractionated the MNLs from skin-infected newborn rabbits and performed the infectious-center assay with each purified cell fraction. The cell fraction devoid of phagocytic cells produced only seven infectious centers, compared with the unfractionated whole MNLs that produced 110 infectious centers. Similarly, glass-adherent cell fractions of MNLs produced 3250 infectious centers and nonadherent cell fractions produced only 750 infectious centers. These results suggest that phagocytic and adherent MNLs, i.e., monocytes, appear to be major carriers of the virus.

Induction of Chorioretinitis by the Intracarotid Injection of Normal Newborn Rabbits With HSV-Infected Mononuclear Leukocytes

To examine whether HSV-infected MNLs were able to produce chorioretinal lesions, we transferred $10^3$ HSV-infected MNLs into normal newborn rabbits by way of the right common carotid artery, and the eyes of these animals were examined histologically 3 days later. As a rule, we used MNLs infected with HSV-2 in vesicles in the cytoplasm. But where the integrity of the nuclear envelope was impaired, mature encapsulated viruses in vesicles were seen sometimes within the general area of the nucleus (Fig. 1). Electron microscopy clearly showed that the HSV was actively multiplying in the MNLs and suggested that the virus-containing cells are highly infectious. In fact, approximately $10^2$ PFU of HSV could be recovered from a HSV-infected MNL following 3 freeze-thaw cycles of the cells.
in vitro, since a large number of HSV-infected MNLs could be obtained regularly by this method; however, only between 3–6% of the MNLs we used were infected with HSV-2 at MOI = 10. Like the MNLs isolated from HSV-infected animals, these in vitro-infected MNLs largely belonged to phagocytic or glass-adherent cell fractions (Table 1).

The commonest finding in these experimentally infected newborns was a varying degree of choroiditis. Typical inclusion bodies of the Cowdry type A could be seen in the choroidal lesions. Single or multiple retinal folds, with or without underlying choroiditis, were another relatively frequent finding. HSV-2 antigens could be detected in these lesions by immunofluorescent test with HSV-2 antibody. But retinal folds were also seen occasionally around the optic nerve or at the ora serrata of the normal newborn rabbit as they are in human newborn infants (Lange's folds), and for this reason the retinal folds at these sites were not considered as HSV-induced lesions in our infected animals. Neither the optic nerve nor the cornea was involved in the pathological processes. The intracarotid injection of normal newborn rabbits with uninfected MNLs or Latex beads (Cataphote Corp., Jackson, MS) as large as 25 μm produced no ocular lesions.

Production of Chorioretinal Lesions by HSV-Infected Mononuclear Cells and by Free HSV

The efficacy of MNL-associated HSV-2 as a means of producing chorioretinitis was compared with the efficacy of free HSV-2. Various doses of HSV-2-infected MNLs or free HSV-2 were injected into the common carotid artery of normal newborn rabbits. The right eyes of these rabbits were examined 3 days later for chorioretinal lesions. As few as 80 infected MNLs were able to cause chorioretinal lesions in 50% of the eyes, while as many as 1,000 Pfu of free HSV-2 were needed to produce such lesions with the same frequency. When 1,000 HSV-infected MNLs were inoculated in the same way, chorioretinitis developed in all of the eyes examined. These results clearly indicated that HSV-infected MNLs were far more efficient in producing the lesions than free HSV.

Retention of HSV-Infected Mononuclear Leukocytes in the Eye

The reason that HSV-infected MNLs produce chorioretinal lesions more effectively than free HSV is unknown. It has been shown, however, that the viral infection of a cell can modify the cell's surface. It is possible, therefore, that the surfaces of HSV-infected MNLs may change so as to favor the production of chorioretinitis. To examine such a possibility, both 10³ HSV-infected MNLs or uninfected MNLs labeled with

| Table 1. In vitro infection of fractionated mononuclear leukocytes of normal newborn rabbits by HSV-2 |
|---------------------------------------------------------------|---------------|----------------|----------------|
| Yeast Phagocytosis                                           | Glass Beads Column |
| Nonphagocytic MNLs                                            | Total MNLs     |
| Nonadherent MNLs                                              | Adherent MNLs  |
| Exp. 1                                                        | 0.9            | 2.3            | 0.7            |
|                                                                 |                | 2.9            | 21.1           |
| Exp. 2                                                        | 0.1            | 5.0            | 2.9            |
|                                                                 |                | 20.0           | 20.0           |

111In were injected into the normal newborn rabbits via the right common carotid artery. Eyes were then enucleated and rinsed with MEM, and the radioactivity retained in both the right and left eyes was measured with a gamma counter. The results are presented in Table 2.

When the total pool of MNLs were used in the experiment, significantly more virus-infected MNLs (120 cells) were present in average in the right eye than uninfected MNLs (35 cells) (P < 0.05). When the glass-adherent fraction of the MNLs were used instead, a larger number of infected MNLs were again retained in the right eye than uninfected MNLs. HSV-infected MNLs were retained in the eyes about six times or

| Table 2. Number of mononuclear leukocytes retained in the right eye of normal newborn rabbits after intracarotid injection of 111In-labeled, HSV-2-infected or uninfected mononuclear leukocytes |
|---------------------------------------------------------------|---------------|
| Estimated Number† of MNLs Retained in Each Eye                | Ratio of UV/(2) |
| MNLs* Injected                                               | Age of Rabbits | Infected MNLs (1) | Uninfected MNLs (2) | |
| Whole                                                        | 7 days        | 102             | 41              | 3.4 |
| MNLs                                                        | 143           | 119             | 117             | |
| Glass-adherent MNLs                                          | 271           | 20              | 20              | |
| Mean‡                                                       | 1287 ± 685    | 216 ± 337       | 5.9             | |
| Mean‡                                                       | 7 days        | 95              | 8               | 9.2 |
| Mean‡                                                       | 74 ± 50       | 8 ± 2.5         | 6               | |

* MNLs = Mononuclear leukocytes
† Estimated number of MNLs retained in each eye was obtained by dividing cpm of the eye with cpm per cell injected.
‡ Ratio of mean number of infected MNLs/mean number of uninfected MNLs.
§ Mean ± 1 SD.
dothelial cells was far greater than the adherence of the virus-infected MNLs to the uninfected MNLs.

To examine this possibility, we compared the adherence of HSV-infected mononuclear leukocytes to cultured rabbit aortic endothelial cells, first of "In-labeled HSV-infected MNLs, and second of "In-labeled uninfected MNLs. As shown in Table 3, the adherence of the virus-infected MNLs to the endothelial cells was far greater than the adherence of uninfected MNLs.

### Table 3. Adherence of HSV-2-infected and -uninfected mononuclear leukocytes to cultured rabbit aortic endothelial cells

<table>
<thead>
<tr>
<th>MNLs* Tested</th>
<th>Exp. No.</th>
<th>Infected MNLs</th>
<th>Uninfected MNLs</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>1</td>
<td>39.7 ± 1.4‡</td>
<td>27.2 ± 1.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32.3 ± 1.2</td>
<td>21.8 ± 2.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>40.8 ± 4.2</td>
<td>29.3 ± 0.6</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Glass-adherent MNLs</td>
<td></td>
<td>57.4 ± 8.6</td>
<td>32.4 ± 0.7</td>
<td>&lt;0.025</td>
</tr>
</tbody>
</table>

* MNLs = mononuclear leukocytes.† by Student's t-test.‡ Mean of percent adherence ± 1 SD.

more as often as uninfected MNLs (P < 0.05). The radioactivity retained in the contralateral eyes (left eyes) was negligible. These data suggest that HSV-infected MNLs tend to be trapped in the eye's circulation after intracarotid injection.

**Adherence of HSV-Infected Mononuclear Leukocytes to Vascular Endothelial Cells**

One possible explanation for the better retention of HSV-infected MNLs in the eye than uninfected MNLs may be the fact that HSV-infected MNLs attach to vascular endothelial cells better than uninfected MNLs. To examine this possibility, we compared the adherence to cultured rabbit vascular endothelial cells, first of "In-labeled HSV-infected MNLs, and second of "In-labeled uninfected MNLs. As shown in Table 3, the adherence of the virus-infected MNLs to the endothelial cells was far greater than the adherence of uninfected MNLs.

**Discussion**

In previous reports, we described a rabbit model for the study of newborn herpetic chorioretinitis. The unique nature of this animal model is that chorioretinitis develops after the primary HSV-2 infection of a distant area of the skin, and that subsequent pathologic sequelae simulate the sequelae seen in clinical cases. Although our previous studies suggested hematogenous transmission of the virus to the eye, the exact mechanism by which chorioretinitis develops remains unclear.

These earlier studies and the one reported here have shown that a large number of virus-infected MNLs often appear in the blood of skin-infected animals shortly after virus inoculation when no free HSV can be detected in the blood. Moreover, a series of transfer experiments in which we used HSV-infected MNLs or free HSV showed that the infected MNLs were far more likely to produce chorioretinitis than free HSV; in fact, they were about 10 times as likely to do so as free HSV. These results clearly indicated that HSV-infected MNLs can play an important role in the production of chorioretinitis in newborn rabbits.

Although we utilized allogeneic virus-infected MNLs to produce ocular lesions, the chorioretinitis that developed in the newborn rabbits was not the result of graft-vs-host reactions. First of all, the injection of uninfected MNLs failed to produce any pathologic changes in the eye. In any event, the newborn rabbits we used in the study were less than a week old and thus too immature immunologically to induce such reactions. The length of our experiment was only 3 days, moreover, and this was not long enough for the development of such immune reactions. We often observed Cowdry type A inclusions and HSV antigens in the ocular lesions. Also, intracarotid injection of latex beads, which occlude capillaries of the retina and choroid, failed to induce the ocular lesions. These facts strongly suggest that the lesions were, in fact, virus-specific.

Why the virus-infected MNLs are far more efficient in producing the lesions has yet to be investigated. The present study did show, however, that significantly more virus-infected MNLs could be detected in the eye than uninfected MNLs after the intracarotid injection. Since the diameter of the retinal capillaries or choriocapillaris can be as small as 6 μm and since HSV-infected MNLs adhere to the vascular endothelial cells far better than uninfected MNLs (as shown in our present study), HSV-infected MNLs may easily be trapped in the capillaries. These entrapped HSV-infected MNLs, which support active multiplication of HSV, may initiate foci of infection at the site of the entrapment and produce retinal and choroidal lesions.

We should also like to know what subpopulation of MNLs can carry the virus. There have been a number of reports concerning the replication of HSV in the lymphoid cells. It has been reported that HSV can replicate in activated T lymphocytes, macrophages, and MNLs of the newborn. The outcome of the present study with the MNL fraction of skin-infected newborn rabbits suggested that the fraction of the MNLs with phagocytic and glass-adherent properties may carry the virus preferentially. Monocytes may thus be a primary target for HSV-2 in the case of newborn infection. Further studies are needed to determine the validity of this assumption.

In humans, herpetic chorioretinitis can develop by either hematogenous or neuronal routes. It is unlikely, however, that the chorioretinal lesions observed in our rabbit model developed via a neuronal route since no virus could be recovered in the brain prior to...
the appearance of chorioretinal lesions,7-9 and no optic neuritis developed in the eyes with chorioretinitis. It is reasonable to conclude, therefore, that chorioretinal lesions start from chorioretinal capillaries and progress to chorioretinitis.

Key words: herpes simplex virus type 2, chorioretinitis, mononuclear leukocytes, endothelial cells, newborn rabbits

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