Initial Herpes Simplex Virus Type 1 Infection Prevents Ganglionic Superinfection by Other Strains

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Received 15 July 1981/Accepted 13 November 1981

The ganglia of rabbits infected with a relatively benign strain of herpesvirus (E-43) and challenged with either of two virulent neurotrophic strains (MP or McKrae) were found to be colonized only by the initial benign infecting strain. Primary infection with the E-43 strain resulted in milder disease when the animals were infected with MP or McKrae strains and also prevented colonization of the ganglion by these strains. Neutralization with anti-glycoprotein C, plaque morphology, cytopathic effects, reconstruction experiments, and restriction endonuclease analysis indicated that the virus recovered from the ganglion was the initial infecting E-43 strain; no traces of the challenging MP and McKrae strains were found. The challenging McKrae strain was shed for several weeks in a few animals, but the virus isolated from the trigeminal ganglia of these animals was the primary infecting E-43 strain. These results suggest that initial infection with a relatively benign strain of herpesvirus may prevent superinfection of the ganglion (but not necessarily the end organ) by highly virulent herpes simplex virus strains and could have significant implications in the consideration of immunization against this disease in humans.

It has been estimated that up to 90% of the population has antibodies to herpes simplex, whereas only about 25% display clinical disease. Among those with clinical disease, the recurrence rate and the severity of the disease are highly variable.

Herpes simplex is the most common cause of corneal infection. Some people with no apparent history of corneal infection shed herpesvirus in the tears, whereas others manifest disease of widely varying severity. Although host factors may determine the degree of severity to some extent, it seems likely that differences among virus strains may be equally important in determining not only the manifestations of clinical herpetic disease, but also the rate of clinical recurrences.

It is well known that isolates of herpes simplex virus type 1 (HSV-1) vary widely in their virulence. Some strains are neurotrophic and produce encephalitis, some produce little or no corneal disease, and others produce blinding corneal disease in animals. Wander et al. (10) documented these strain differences and their effect on the cornea and showed that such differences are relatively independent of virus inoculum size. Other studies (Y. M. Centifanto-Fitzgerald, T. Yamaguchi, H. E. Kaufman, M. Tognon, and B. Roizman, J. Exp. Med., in press) suggest that the severity of infection and even the morphology of the epithelial ulcer is specifically genetically determined and that recombimants of different parent strains produce intermediate types of corneal disease, with epithelial disease and stromal disease segregating separately.

Using nucleic acid mapping, other workers (2, 11) examined the ganglia from cadavers and found evidence that, although multiple ganglia of a single individual may be infected, all ganglia appear to be infected by the same virus strain. This suggests that each individual is colonized by a single virus strain and that the cervical ganglia may be resistant to colonization by more than one strain of HSV-1. Other studies in mice have shown that previous exposure of animals to HSV-1 provides some protection not only in reducing the severity of infection but in reducing the tendency toward ganglionic colonization (9). The mechanism for such protection is unclear.

Price and co-workers (7) reported that substantial protection was achieved when animals were immunized and challenged with HSV-1, but no protection was seen when HSV-2 was used as the immunizing and challenging agent. In contrast, McKendall (3) reported that immunization with HSV-1 was effective against acute and latent infection by HSV-2.

It seems likely that immune mechanisms could mediate a situation in which, after the initial infecting strain colonizes the ganglion, subsequent infection with other HSV-1 strains...
might be less effective in producing disease, and, in addition, the second strain might be prevented from colonizing the sensory ganglia.

The purpose of this study was to determine whether the primary infecting strain appeared to be the only permanent resident of the ganglia and whether it would prevent superinfection by the second strain, even though the second strain might become well established at the site of infection for a significant period of time, and whether infection with a relatively mild strain of herpesvirus would (i) ameliorate infection by a second strain; (ii) reduce shedding and recurrences by the second strain, even if it was a highly virulent neurotrophic strain known to produce recurrences; and (iii) lower the death rate produced by such a virulent superinfecting strain.

MATERIALS AND METHODS

Cells and viruses. HEp-2 and RK-13 cells (Microbiological Associates, Rockville, Md.) were grown in basal minimal medium supplemented with 10% fetal calf serum, 1% glutamine, sodium bicarbonate, and antibiotics. RK-13 cells grown in the same medium were used for neutralization experiments and phenotypic studies. Vero cells were used to grow the virus used in DNA studies.

Three virus strains were used. The E-43 strain has recently been isolated and was passed once in RK-13 cells. The McKrae strain is a clinical isolate that has been used in our laboratory for many years. It produces recurrent disease. This strain is the prototype used in our studies of recurrent disease and viral chemotherapy. It colonizes the trigeminal ganglia and is thymidine kinase positive. The MP strain (obtained from Bernard Roizman, University of Chicago), produces stromal disease and 100% mortality in our rabbits (8).

Plaque morphology and cytopathic effects were studied in RK-13 cells. RK-13 monolayer cultures grown in 4-ounce (120-ml) plastic bottles were exposed to 0.2 ml of virus inoculum. Adsorption of the virus proceeded for 1 h at 37°C with gentle rocking of the cultures. After the adsorption period, 4 ml of complete growth medium was added to those cultures used for the determination of cytopathic effect, and the same medium containing 1% methylcellulose was added to those cultures used for plaque morphology studies. For evaluation of cytopathic effect, the cultures were photographed under phase microscopy; for evaluation of plaque morphology, the cultures were stained with crystal violet or Giemsa stain.

Animal studies. New Zealand white rabbits weighing 1 to 3 kg each were used in the following consecutive experiments. The corneal epithelium was scarified with a trephine, and the lid was pulled tightly, forming a cul-de-sac into which 100 µl of the virus suspension (10^5 PFU) was dropped. The lid was closed and rubbered over the cornea twice. The eyes were examined with the slit lamp every third day for a period of up to 30 days. To determine the severity of the ocular disease produced by the E-43 strain and the rate of colonization of the ganglia, 14 rabbits were inoculated with the E-43 strain, the eyes were examined as described above, and the ganglia were removed and processed for virus recovery.

In one set of experiments, 34 rabbits were infected with the E-43 strain and 5 weeks later were reinoculated with the virulent McKrae strain. Control animals were infected only with the McKrae strain. Severity of ocular disease was graded by slit lamp examination. At 16 days after infection with the second strain, the ganglia from four experimental animals and two controls were removed and processed for virus recovery.

In addition, five animals were infected with the E-43 strain and 15 days later were challenged with the MP strain. The percent mortality was recorded, and the ganglia were removed and processed for virus recovery 6 days after challenge with the MP strain.

A group of 149 rabbits was used to assess the effect of initial infection on the recurrence rate and virus shedding after challenge. The corneal epithelium in each eye was lightly traumatized, and both corneas of each animal were infected with herpesvirus. On day 0, 104 eyes (52 rabbits) were infected with the McKrae strain, and 146 eyes (73 rabbits) were infected with the E-43 strain. On day 34, 80 eyes (40 rabbits) that had previously been infected with the E-43 strain were challenged with the McKrae strain. On days 35 and 37, 50 of these challenged eyes were cultured. Between days 35 and 92, all eyes were examined three times a week by slit lamp biomicroscopy, and cultures were taken of the precorneal tear film with a moistened, cotton-tipped swab. After day 92, the remaining rabbits were killed, and the trigeminal ganglia were removed and processed for virus recovery.

Recovery of virus from ganglia. The procedure followed has been described by Nesburn et al. (4). Surgically removed ganglia were washed extensively with phosphate-buffered saline, digested with collagenase (160 U/mg) for 30 min at 37°C, and treated with trypsin EDTA for an additional 30 min. Calf serum was added to stop the action of the trypsin. Aliquots of these mixtures were seeded into RK-13 cell cultures. The cultures were maintained for 2 weeks with several changes of medium. All positive cultures were subcultured into RK-13 cell cultures (25 cm), dispensed into small vials, and stored at -70°C.

Neutralization experiments. HSV grown in either HEp-2 or RK-13 cells was used in neutralization experiments. A 0.1-ml sample of each virus (300 PFU) was mixed with 0.1 ml of the antiserum and incubated at 36°C for 30 min. At the end of this period, a sample (50 lambda) of the mixture was inoculated into RK-13 cell cultures and allowed to absorb for 15 min before the addition of medium. For the controls, the same amount (PFU) of each virus was combined with medium and treated in the same manner. All cultures were incubated at 36°C for 30 h and stained.

Reconstruction experiments. Experimental mixtures of the E-43 strain and the MP strain were prepared to determine whether small amounts of one strain (MP as the challenging strain) could be detected in the presence of larger amounts of the other strain.

To this end, three concentrations of E-43 virus (PFU) were mixed with 70 PFU of the MP strain and neutralized with antiserum to glycoprotein C. Theoretically, antiserum to glycoprotein C should neutralize the E-43 strain and allow the MP to survive, because the MP strain lacks glycoprotein C. For the controls,
the same concentrations of E-43 with and without antiserum to glycoprotein C and the same concentrations of MP with and without antiserum to glycoprotein C were used. All flasks were of similar volumes and were subjected to similar temperatures. The mixtures were inoculated into RK-13 monolayer cultures, incubated at 37°C for 36 h, and stained with crystal violet, after which the plates were counted.

Also, RK-13 cell monolayer cultures were infected with known amounts of the E-43 and MP strains to determine whether the MP strain maintains its characteristic syncytium formation in the presence of another virus and whether both viruses can be recovered in the same culture monolayer.

Endonuclease analysis. Confluent monolayer cultures of Vero cells were infected with each virus strain at an input multiplicity of 1 PFU per cell and were allowed to adsorb at 35°C for 1 h. Phosphate-free Eagle minimal essential medium supplemented with 1% calf serum and containing a total of 250 μCi of [32P]orthophosphoric acid (New England Nuclear Corp., Boston, Mass.) was added, and the cultures were incubated for 24 h.

The cells were shaken off the glass and centrifuged for 10 min at 900 rpm in an International centrifuge. The pellet was gently suspended in 0.5 ml of 10 mM EDTA. A 10-μl amount of a 20% sodium dodecyl sulfate solution and 25 μl of pronase (20 mg/ml) were added, and the samples were incubated at 37°C for 24 h.

After this incubation, virus DNA was extracted with one volume of 80% phenol (pH 7.0 with 50 mM Tris base, 0.2 M NaCl) for 5 min on a rocker table. One-third volume of chloroform was added, and the sample was reextracted with one volume of phenol-chloroform (2:1) and dialyzed for 36 h against Tris-EDTA buffer (10 mM Tris-1 mM EDTA [1]).

Restriction endonuclease analyses with HpaI and BamHI were performed under the conditions specified by the manufacturers (Bio Labs, Boston, Mass.). The digested DNA was subjected to electrophoresis in 0.6% horizontal agarose gel slabs at 2 V/cm, using bromophenol blue as a marker. The gels were dried and exposed to Kodak X-ray film for various lengths of time.

RESULTS

Animal studies. Infection of the rabbit cornea with the E-43 strain produced generally mild disease characterized by punctate staining. Overt disease was seen in only a small percentage of the eyes (12%); however, from 6 to 24 days postinfection, virus was recovered from the trigeminal ganglia of 70 to 75% of the animals, a rate consistent with the recovery rate of more virulent herpesvirus strains.

Animals that had been infected initially with the E-43 strain were challenged with either the MP or the McKrae strain. Those challenged with the McKrae strain developed herpetic disease that was less severe and of shorter duration than the disease seen in previously uninfected controls. By day 6 after infection, most of the experimental eyes were clear; the control eyes showed severe disease up to day 11.

In the rabbits challenged with the MP strain, a marked protection from death was observed. In the control group infected with the MP strain only, 100% mortality occurred. In the experimental group infected with the E-43 strain and challenged with the MP strain, 80% survived. The ganglia from four animals from the experimental groups and two animals from the control group were removed and processed as described in Materials and Methods. The recovered virus was frozen and stored at −70°C.

Identification of recovered virus. (i) Experimental group infected with E-43 and challenged with MP. Several dilutions of the recovered virus were inoculated into an RK-13 cell culture to observe cytopathic effects and plaque morphology under methylcellulose overlay. The MP strain does not produce glycoprotein C because of a genetic deletion and forms syncytia in tissue culture regardless of the host, whereas the E-43 strain produces glycoprotein C and forms tight clumps of rounded cells. The cultures infected with virus recovered from the ganglia did not show syncytium formation, and the cytopathic effects were similar to those of the E-43 strain even at very low dilutions, indicating that the MP strain was absent from these cultures.

The virus recovered from two of these animals was studied by neutralization tests with antiserum to glycoprotein C and polyspecific anti-HSV-1 antiserum. The polyspecific antiserum should neutralize all viruses, and the antiserum to glycoprotein C should also neutralize all viruses except those that do not produce glycoprotein C, e.g., the MP strain. All of the virus recovered from the ganglia was neutralized by the antiserum to glycoprotein C, indicating that no MP virus was present (Fig. 1). The two viruses recovered from ganglia G1 and G2 were neutralized by antiserum to the F strain (HSV-1 prototype) and by antiserum to glycoprotein C. Similar results were obtained with the E-43 strain. Results with the MP strain are shown to demonstrate the specificity of the reaction; this strain was neutralized by the antiserum to F strain and not by the antiserum to glycoprotein C.

Further evidence that the challenging virus strain (MP) could not be recovered from the ganglia was obtained with reconstruction techniques. In these experiments, we showed that small amounts of MP virion can be detected in the presence of increasing amounts of the E-43 strain. Cultures infected with an E-43–MP mixture that had been neutralized by antiserum to glycoprotein C produced only syncytial plaques; the plaque count (70 PFU) was similar to that
observed in the cultures infected with the MP strain alone, indicating that all of the MP virus present in the inoculum had been recovered and that there was no interference between the two strains. Cultures infected with only E-43 virus that had been neutralized by antiserum to glycoprotein C produced no plaques; however, excellent growth was exhibited by the cultures containing non-neutralized E-43 (Fig. 2).

To determine whether each virus retains its characteristic plaque morphology in a mixed infection, a cell culture was infected with both the E-43 and MP strains (Fig. 3). Both syncytia (MP strain) and aggregates of rounded cells (E-43 strain) were seen. This implies that, if the virus recovered from the ganglia of doubly infected animals were a mixture of the primary and challenging strains, both kinds of plaques would be produced. However, the virus recovered from the ganglia of our experimental animals produced only plaques characteristic of the primary infecting strain (E-43). Therefore, we concluded that the second, challenging strain (MP) was not recovered.

(ii) Experimental group infected with E-43 and challenged with McKrae. Several dilutions of the virus recovered from the ganglia were inoculated into RK-13 monolayer cultures. The resulting cytopathic effect and plaque morphology were typical of the E-43 strain, i.e., rounded clumps of cells tightly adherent to the rest of the monolayer.

Also, a sample of the recovered virus (100 PFU) was neutralized with antiserum to E-43 and antiserum to McKrae. Neutralization of the recovered virus with antiserum to E-43 was more efficient than neutralization with antiserum to McKrae strain. The McKrae and E-43 strains were neutralized with homologous antisera as controls.

Restriction endonuclease analysis. The foregoing evidence strongly suggested that the initial infecting herpesvirus strain was the only strain recovered from the ganglia of the doubly infected animals and that the superinfecting strains did not colonize the ganglia and could not be recovered therefrom. However, additional identification of the virus recovered from both doubly infected groups (E-43–MP and E-43–McKrae) was obtained with restriction endonuclease analysis of viral DNA, using HpaI and BamHI enzymes (Fig. 4). The virus isolates from the ganglia of both the animals infected with E-43–MP and those infected with E-43–McKrae showed similar numbers of DNA fragments, indicating that the same virus was recovered from both experimental groups (Fig. 4B and E). The DNA profiles of the recovered viruses were consistent with those of the parent strains.
HSV-1 INFECTION PREVENTS SUPERINFECTION

FIG. 2. Recovery of MP plaques from RK-13 cell cultures infected with mixtures of E-43 and MP strains (crystal violet stain). Well 1, syncytial plaques recovered in cultures infected with 70 PFU of the MP strain and 7,000 PFU of the E-43 strain plus antiserum to glycoprotein C; well 2, control culture infected with 7,000 PFU of the E-43 strain; well 3, control culture infected with 7,000 PFU of the E-43 strain plus antiserum to glycoprotein C; well 4, syncytial plaques recovered in cultures infected with 70 PFU of the MP strain and 7,000 PFU of the E-43 strain plus antiserum to glycoprotein C; well 5, control culture infected with 700 PFU of the E-43 strain; well 6, control culture infected with 700 PFU of the E-43 strain, plus antiserum to glycoprotein C; well 7, uninfected cell control; well 8, control culture infected with 70 PFU of the MP strain; and well 9, control culture infected with 70 PFU of the MP strain plus antiserum to glycoprotein C.

identical to the profile of the control E-43 strain (Fig. 4D). In contrast, the control MP and McKrae strains produced very different profiles (Fig. 4A and C). Several points of difference in the cleavage patterns are noted in Fig. 4 (arrows).

The results of these experiments indicate that the initial infecting strain, and not the superinfecting strain, was the resident virus in the ganglia and that our analytical methods were sufficiently sensitive to make this identification.

Recurrences and virus shedding. The initial infection of rabbit corneas with the E-43 strain caused some mild dendritic keratitis which resolved by day 7, leaving clear corneas. The McKrae strain caused a more severe dendritic keratitis in all of the infected corneas; this disease cleared by day 14. Of the rabbits infected with the McKrae strain, 46 died of systemic viral infection before day 34. In contrast, none of the E-43 group challenged with the McKrae strain died. In the control group, two rabbits infected with the E-43 strain died.

Rabbits infected with the McKrae and the E-43 strains showed spontaneous clinical recurrences consisting of typical small dendrites. Many of the eyes infected with the McKrae strain developed necrotic ulcers and scarring. The E-43–infected corneas remained clear. The frequency of clinical recurrences and virus shedding was similar for all groups, including the animals challenged with the McKrae strain. Spe-
specifically, in the McKrae-infected rabbits, 28 of 30 animals had clinical recurrences, and 6 of 17 eyes showed positive virus isolation in vitro. Of 23 E-43-infected rabbits, 15 had clinical recurrences; 2 of 16 eyes showed positive virus isolation. In the doubly infected group (E-43-McKrae), 22 of 38 animals had clinical recurrences, and positive virus isolation was seen in 6 of 20 eyes. The severity of the recurrent disease was minimal and similar in all groups.

Virus isolated from the precorneal tear film of the E-43-infected, McKrae-challenged rabbits was identified as the McKrae strain in all eyes on days 36 and 37, which were days 1 and 3, respectively, after challenge. Some animals shed McKrae strain virus for as long as 21 days after challenge (Table 1) but shed only the original infecting E-43 strain thereafter. Other animals shed E-43 strain as early as 11 days after challenge with the McKrae strain (Table 1).

The virus isolated from the trigeminal ganglia of these animals was also examined. We recovered virus from 17 of 32 ganglion specimens. In all cases, the virus from either the left or right trigeminal ganglion was identified as the E-43 strain. In general, all of the virus isolated from the trigeminal ganglia was identified as the initial infecting E-43 strain; no McKrae strain virus was found.

**DISCUSSION**

The results of this study seem to have important implications for the understanding of herpetic disease in general. We have shown that when a relatively benign strain, such as E-43, infects and colonizes the ganglia of an animal, subsequent infection with even such virulent strains as MP and McKrae seems to produce milder ocular disease and reduced mortality. In addition, although the superinfecting virus strain does produce a significant local infection, it does not appear to colonize the ganglia.

This finding was documented by several methods in the group of animals infected with the E-43 strain and challenged with the MP strain. The MP strain, which does not make glycoprotein C, is not neutralized by anti-glycoprotein C antibody, whereas the E-43 strain is. Therefore, the MP strain can be detected in a mixture of even small amounts of MP and large amounts of E-43, because the addition of anti-glycoprotein C neutralizes E-43, but does not affect the number or morphology of MP plaques (Fig. 2).

Neutralization with anti-glycoprotein C (Fig. 1), examination of plaque morphology (Fig. 3), and restriction endonuclease digestion of the viral DNA recovered from the ganglia (Fig. 4) indicate that the initial infecting virus strain colonizes the ganglia and that no trace of the superinfecting viruses can be found in the virus recovered from the ganglia of these doubly infected animals.

Every method of detection has its limitations, and there is always the possibility that some virus escaped detection. Within such limits, however, the results of these experiments suggest that an initial infecting strain colonizes the ganglion and the subsequent infection of the ganglion by a superinfecting strain does not occur, even when the second strain is known to

FIG. 3. Photomicrograph (crystal violet stain) of RK-13 cells infected with the MP and E-43 strains. The star-shaped plaque is the result of polykaryocyte formation characteristic of the MP strain; to the right is a plaque showing the clumped, rounded cells typical of the cytopathic effects of the E-43 strain. Magnification, ×65.
be neurotrophic and virulent. This observation is consistent with that of Lonsdale et al. (2) that an individual has only one strain of herpesvirus in all cervical ganglia.

Our hypothesis was again confirmed in the experiments dealing with shedding and recurrences. In animals previously infected with the E-43 strain and challenged with the McKrae strain, this highly neurotrophic superinfecting strain produced only mild disease and did not produce encephalitis and death, although the virus was well established locally and was shed from the eye, in some cases, for 2 to 3 weeks as the primary virus (data not shown). That the secondary infection was a prolonged one with virus shedding is interesting in view of the fact that the secondary infecting strain ultimately appeared to vanish, and shedding of the initial infecting strain resumed after a period of weeks.

The mechanism of protection of the ganglia is unclear but is likely to be immunological. Studies in mice suggest that previous immunization can give from partial to nearly complete protection against the establishment of latency (3). Other reports (5, 6) indicate that not only antibody but also some radiosensitive component may be necessary for this protection. In humans, few consistent differences have been demonstrated between patients who get severe or recurrent herpetic disease and sero-positive controls in the absence of obvious immunosuppression. In fact, a recent analysis of the immune response to herpesvirus infections by Zweerink and Stanton (12) found no difference in circulating antibodies between patients with recurrent herpetic disease and sero-positive patients who have no recurrences. These studies, as well as those reported here, raise the intriguing possibility that, in the absence of gross immunosuppression, differences among infecting viruses may be of even greater importance than differences in host defense in the determination of type and severity of the resulting clinical disease.

Although host immune factors may still determine recurrence rate and the presence of disease and although widespread immunosuppression can make even patients who are not prone to recurrent disease susceptible to this, as to any parasite, it is equally possible that each individual is infected initially with a strain of virus whose genetic makeup largely determines its virulence and disease-producing characteristics. It is clear that superinfection by other HSV-1 strains can occur, but the experiments reported here for this group of viruses suggest the possibility that the superinfecting virus, even though it may shed for a considerable period of time, does not colonize the ganglia. In addition, it appears that, even if prolonged shedding of the superinfecting strain occurs, eventually this strain disappears, and shedding of the initial infecting virus resumes. The concept of an initial colonizing virus strain that is not replaced in the ganglion and that remains as the dominant virus strain in each individual, even though superinfection may occur, needs further substantiation but seems to be supported by this evidence. In addition, the fact that the severity and virulence of herpetic viral disease can be genetically determined by the virus genome may indicate that initial infection basically determines the characteristics of the herpetic disease the individual will have for the rest of his or her life.

FIG. 4. Electrophoretic pattern of viral DNA digested with restriction endonucleases BamHI and HpaI. A, MP strain; B, E-43-MP; C, McKrae; D, E-43; E, E-43-McKrae. Arrows indicate differences among the patterns.
TABLE 1. Identity of virus isolated from rabbits infected with the E-43 strain and challenged with the McKrae strain of herpesvirus

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Type of virus recovered from tear film at following no. of days post-McKrae strain challenge</th>
<th>Type of virus recovered from trigeminal ganglia at &gt;58 days post-McKrae strain challenge</th>
</tr>
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<tbody>
<tr>
<td>1198</td>
<td>McKrae</td>
<td>E-43</td>
</tr>
<tr>
<td>1197</td>
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<td>E-43</td>
</tr>
<tr>
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<tr>
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<tr>
<td>1206</td>
<td>—</td>
<td>E-43</td>
</tr>
</tbody>
</table>

\( ^a \) Cultures negative for virus.

\( ^b \) Virus recovered from right and left ganglia.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service grants EY02389, EY02672, and EY02377 from the National Eye Institute.

Technical assistance was provided by Rosalie Rusinko.

LITERATURE CITED


