Experimental Ocular Onchocerciasis in Cynomolgus Monkeys

IV. Chorioretinitis Elicited by Onchocerca volvulus Microfilariae

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Onchocerciasis is a major cause of blindness worldwide, and much of the blindness is caused by onchocercal chorioretinitis. In an experimental animal model for ocular onchocerciasis, intravitreal injections of 10,000 live Onchocerca volvulus microfilariae isolated from infected humans into the eyes of cynomolgus monkeys (Macaca fascicularis) resulted in patchy, progressive loss of retinal pigment with pigment clumping. Areas of pigment loss were less extensive in animals that had been sensitized with microfilariae. Intravitreal injections of dead O. volvulus microfilariae resulted in mild vitritis with relatively less clinical change noted in the retina and choroid. Histopathologic examination revealed thinning and loss of outer retinal layers with pigment migration into the retina, and inflammation was more pronounced in eyes that received live microfilariae. Clinical changes appeared in eyes receiving live microfilariae before the development of significant antibody or cell-mediated immune responses. O. volvulus microfilariae appear to be more suitable than O. lienalis microfilariae in producing lesions which resemble human onchocerciasis in the primate model. Invest Ophthalmol Vis Sci 32:1499–1507, 1991

Onchocerciasis, a filarial infection caused by Onchocerca volvulus, affects more than 18 million people worldwide¹ and is a leading cause of blindness. A characteristic chorioretinitis and chorioternal scarring account for nearly one half of this blindness. The pathogenesis of the fundus lesion is unclear in part due to the scarcity of affected human eyes for pathologic studies and the lack of detailed longitudinal ophthalmologic study of affected patients.

An experimental animal model using intravitreal injections of O. lienalis microfilariae in cynomolgus monkeys recently facilitated the study of the immunopathology and pathogenesis of onchocercal chorioretinitis.²⁻⁴ Chorioretinal changes, including retinal pigment epithelial hypertrophy, hyperplasia, and loss of pigment, and choroiditis involving primarily eosino-philms were produced by intravitreal injections of up to 500 live O. lienalis microfilariae. However, O. lienalis, a related species to O. volvulus, is the species which infects cattle. Ocular inflammation elicited by O. lienalis microfilariae in the experimental primate model was much greater than that seen in human ocular onchocerciasis. We tried to determine whether O. volvulus microfilariae isolated from infected humans would elicit onchocercal chorioretinitis in the eyes of cynomolgus monkeys and whether this would more closely resemble human ocular onchocerciasis both clinically and histopathologically. Both live and dead O. volvulus microfilariae were used in intravitreal injections to determine whether direct invasion of the retina and choroid was necessary to cause chorioretinal changes resembling human ocular onchocerciasis. Humoral and cell-mediated immune responses to onchocercal antigens were studied to help elucidate the role of immune mechanisms in the pathogenesis of onchocercal chorioretinitis.

Materials and Methods

Skin biopsy specimens were obtained from humans with onchocerciasis who were undergoing therapeutic nodulectomy on the Liberian Agricultural Company rubber plantation, Grand Bassa County, Liberia. All individuals were characterized by onchocercal nodules (>5) and high densities of microfilariae in the
Skin (>70 microfilariae/mg skin). Specimens containing microfilariae were cryopreserved in 6% dimethyl sulfoxide in RPMI-1640 media (Gibco, Grand Island, NY) and stored in liquid nitrogen. To prepare microfilariae for injection, the specimens were thawed at 37°C, washed with fresh RPMI-1640 medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of chloramphenicol, and incubated at room temperature in microtiter wells. After 1 hr, live microfilariae that had emerged from the specimens were washed in RPMI-1640, filtered in a sterile column containing a fine cellulose sponge, centrifuged at 400 × g, and resuspended in distilled water to remove residual erythrocytes by osmotic shock. They were then washed with RPMI-1640, centrifuged twice, and resuspended to Dulbecco’s phosphate-buffered saline (PBS). To prepare dead microfilariae for intravitreal injection, live microfilariae in PBS were submerged suddenly in liquid nitrogen, then immediately thawed. All microfilariae were observed to be dead after this freeze–thaw treatment.

Four colony-raised cynomolgus monkeys (Macaca fascicularis) were used in the protocol as shown in Table 1. In a separate dose-determining experiment with four other colony-raised cynomolgus monkeys (unpublished), we found the optimal intravitreal dose to be 10,000 live O. volvulus microfilariae. Because the intraocular reaction to intravitreal injection of dead microfilariae was not known, doses of 1000 and 10,000 were used. Two of the four cynomolgus monkeys were first sensitized with subcutaneous injection of 30,000 live microfilariae followed by another subcutaneous injection of 30,000 microfilariae 2 weeks later. Each inoculum was distributed on four abdominal sites and given under ketamine anesthesia. Ten days after sensitization, both sensitized and unsensitized monkeys received an intravitreal injection of either live or dead microfilariae as in Table 1. Control intravitreal injections of PBS alone did not elicit any choriorretinal changes2,3 and therefore were not used in the protocol. A 1-ml tuberculin syringe with a 30-gauge needle was used to inject the 0.1-ml volume containing the microfilariae. Anterior chamber paracentesis was done after the intravitreal injection to relieve intraocular pressure.

Slit-lamp examination, anterior segment photography, direct and indirect ophthalmoscopy, fundus photography, and fluorescein angiography were done at baseline and at 1, 3, 10, 18, 35, 41, and 53 days after intravitreal injection. Inflammation of the anterior chamber was graded according to the scheme of Schlaegel,4 and inflammation of the vitreous space was graded according to the scheme of Kimura et al.5 Blood samples drawn on day 53 were lost due to an equipment failure. On day 53 the animals were killed under pentobarbital anesthesia, and the eyes were preserved in buffered glutaraldehyde and processed for histopathology. Serial sections in the plane of the pupil, optic nerve head, and macula of each eye were prepared.

Enzyme-linked immunosorbent assays for immunoglobulin (Ig) G antibody were done in triplicate using Immulon 2 microplates (Dynatech, Alexandria, VA) coated with 1 μg of O. volvulus adult worm extract per well. Plates were incubated with 10% normal goat serum in PBS for 1 hr at room temperature, with monkey sera at 1:100 dilution for 2 hr at 37°C, and with peroxidase-conjugated goat anti-human IgG (Tago, Burlingame, CA) for 2 hr at 37°C. The substrate was developed as previously described.2 O. volvulus adult worms were obtained by collagenase digestion of onchocercal nodules,7 and a crude adult worm extract was prepared by homogenization of the worms in 0.01 M PBS, pH 7.4, and centrifugation of the homogenate at 16,000 × g for 2 hr.

Cell-mediated immune responses were studied using peripheral blood mononuclear leukocytes (PBML) as previously described.8 These cells were isolated by Ficoll-Hypaque density-gradient centrifugation (Sigma, St. Louis, MO) and resuspended at 1.5 X 106/ml in minimum essential medium supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (all Gibco). Concanavalin A (Sigma, St. Louis, MO) was used as a mitogen at a concentration of 400 μg/ml (100 μg/well) in complete medium. Proliferation assays were done in round-bottom microtiter wells (Linbro, McLean, VA) with 1.5 X 105 PBML and 100 μg of antigen or mitogen in a final volume of 200 μl. Control wells contained 100 μl complete medium in place of antigen or mitogen. Triplicate cultures were incubated in 37°C in 5% CO2 for 3 days, at which time 1 μCi of [3H]-thymidine was added to each well. Cultures were harvested 18 hr later, and the radioactivity was counted in a liquid scintillation counter (Unilux III, Tm Analytic, Elk Grove Village, IL). Stimulation indices (SI) were calculated as follows: SI = mean cpm test well/mean cpm control well.

### Table 1. Experimental protocol

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sensitized</th>
<th>Eye</th>
<th>Intravitreal mf</th>
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</thead>
<tbody>
<tr>
<td>239</td>
<td>Yes</td>
<td>OD</td>
<td>10,000 live</td>
</tr>
<tr>
<td>266</td>
<td>Yes</td>
<td>OD</td>
<td>1000 dead</td>
</tr>
<tr>
<td>331</td>
<td>No</td>
<td>OD</td>
<td>10,000 dead</td>
</tr>
<tr>
<td>369</td>
<td>No</td>
<td>OD</td>
<td>10000 dead</td>
</tr>
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</table>

mf = Microfilariae.
**Table 2. Clinical findings of the retina and choroid**

<table>
<thead>
<tr>
<th>Animal</th>
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<th>Day 1</th>
<th>Day 10</th>
<th>Day 35</th>
<th>Day 53</th>
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<tbody>
<tr>
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<td>Normal</td>
<td>Slight retinal</td>
<td>Moderate pigment</td>
<td>Moderate pigment</td>
<td>Moderate pigment</td>
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<td></td>
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<td>pigment changes</td>
<td>loss temporally</td>
<td>loss temporally</td>
<td>loss temporally</td>
</tr>
<tr>
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<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>OS</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>331 OD</td>
<td>Normal</td>
<td>Normal</td>
<td>Moderate pigment</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>OS</td>
<td>Normal</td>
<td>Normal</td>
<td>Slight pigment</td>
<td>Normal</td>
<td>Died</td>
</tr>
<tr>
<td>369 OD</td>
<td>Normal</td>
<td>Normal</td>
<td>Moderate patchy</td>
<td>Moderate patchy</td>
<td>Normal</td>
</tr>
<tr>
<td>OS</td>
<td>Normal</td>
<td>Normal</td>
<td>pigmentation loss</td>
<td>pigmentation loss</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*O. volvulus* adult worm crude antigen was prepared in complete medium for a final concentration of 50 and 25 µg/well. Pooled whole guinea pig retinas were homogenized in PBS, 0.15 NaCl, 0.01 M phosphate, pH 7.4, and centrifuged at 10,000 x g for 10 min. The pellet was discarded, and the supernatant frozen at −80°C. Crude retinal antigen was diluted in complete medium and used at 100 and 50 µg/well.

The study was conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

**Results**

Clinical findings in the retina and choroid of the animals are summarized in Table 2. Intravitreal injection of 10,000 live microfilariae elicited chorioretinal changes resembling onchocerciasis in both sensitized and unsensitized animals. Intravitreal injection of 1000 or 10,000 dead microfilariae generally elicited much less pronounced chorioretinal changes. Inflammation of the anterior segment was generally more pronounced in eyes that received live microfilariae; these findings are summarized in Figures 1A and 1B. Inflammation of the vitreous space generally appeared earlier in animals that had been sensitized (Figs. 2A–B).

Intravitreal injection of live *O. volvulus* microfilariae in the right eyes of sensitized monkeys (#239 and #266) resulted in moderate anterior chamber cell and flare which appeared by day 1 and persisted for approximately 3–6 weeks after intravitreal injection. Live microfilariae were present in the anterior chamber of animal 239 until day 53 when the animals were killed. Live microfilariae were observed in the anterior chamber of animal 266 until day 45 at which time this animal died from an unrelated cause. (Autopsy revealed leukemic infiltrates in the bone marrow.) Inflammation of the vitreous was present by day 10 in both animals and decreased by day 35. Both animals showed patchy loss of retinal pigment in the temporal periphery of the eyes that received live microfilariae. The patches of retinal pigment loss gradually grew larger in area from day 10 until death (Fig. 3A). Fluorescein angiography revealed patchy loss of...
pigment from the retinal pigment epithelium with choroidal hyperfluorescence (Fig. 3B). There was no late leakage seen on fluorescein angiography from the areas of choroidal hyperfluorescence.

Intravitreal injection of dead microfilariae in the left eyes of sensitized monkeys (#239 and #266) resulted in mild to moderate anterior segment cell and flare; this reached a peak by days 10–18 and then disappeared by day 41 after injection. No microfilariae were observed in the anterior chamber. Inflammation of the vitreous was present by day 10 in the left eye of animal 266 (received 10,000 dead microfilariae). At day 18, cells were observed adjacent to the dead microfilariae in the vitreous. No inflammation of the vitreous was observed in the left eye of animal 239. Slight mottling of the pigment was present in the temporal periphery of the left eye of animal 239 by day 35, and no chorioretinal changes were observed in the left eye of animal 266.

Intravitreal injection of live *O. volvulus* microfilariae in the right eyes of unsensitized monkeys (#331 and #369) resulted in a mild to moderate anterior chamber cell and flare which was most pronounced at days 1 and 18 and gradually decreased. Live microfilariae were noted in the anterior chamber of both animals after injection; these were present in animal 331 until day 53. Mild inflammation of the vitreous was
The histopathologic findings are summarized in Table 3. Foci of chronic inflammatory cells were present in the ciliary body of all eyes except the left eye of animal 331. Foci of chronic inflammation were larger and more numerous in the ciliary body of the eyes that received live microfilariae (Fig. 6) than in eyes that received dead microfilariae. Both eyes of animal 239 had thinning and loss of outer retinal layers and retinal pigment epithelium in the temporal periphery with inflammation in the overlying vitreous. In the right eye of animal 266, microfilariae were present in the vitreous with a light scattering of eosinophils, mononuclear cells, and occasional giant cells (Figs. 7A-B); in the left eye degenerating microfilariae were present with occasional multinucleated giant cells and eosinophils. The areas of slight retinal pigmentation changes noted on clinical examination of the right eye of animal 266 were not confirmed by histopathology.

The right eye of animal 331 had extensive loss of outer retinal layers and retinal pigment epithelium in the temporal periphery (Fig. 8) with aggregates of mononuclear cells in the subjacent choroid. In the left eye there were rare chronic inflammatory cells in the vitreous and the retina and choroid were normal. In the right eye of animal 369 there was a focus of chronic inflammatory cells and eosinophils in the choroid and subjacent retinal pigment epithelium; in the left eye there was a light scattering of chronic inflammatory cells and eosinophils in the vitreous, and the retina and choroid were normal. No microfilariae were found in the retina, choroid, or sclera of any of the animals. In general, pathologic changes in the retina and choroid were relatively more pronounced in eyes that received live microfilariae than in those that received dead microfilariae.

Sensitization of systemically immunized monkeys was confirmed by detection of IgG to onchocercal microfilarial antigens in serum by enzyme-linked immunosorbent assay (Fig. 9). Both sensitized animals (#239 and #266) had an IgG titer 10 days after receiving the second subcutaneous injection of microfilariae. Both unsensitized monkeys had an IgG titer

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**Table 3. Summary of histopathologic findings**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Eye</th>
<th>Inflammation, ciliary body</th>
<th>Inflammation, vitreous</th>
<th>Thinning, loss, outer retina</th>
<th>Retinal hemorrhage</th>
<th>Pigment migration</th>
<th>Inflammation, choroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>239</td>
<td>OD</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
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<td>+</td>
<td>+</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>266</td>
<td>OD</td>
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<td>++ (mf)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>+</td>
<td>++ (mf)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td>369</td>
<td>OD</td>
<td>+++</td>
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<td>+++</td>
<td>+</td>
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<tr>
<td></td>
<td>OS</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

mf = Microfilariae present.
Fig. 6. Large focus of chronic inflammatory cells in the ciliary body with light scattering of chronic inflammatory cells in the vitreous at day 46 in sensitized animal (#266) which received intravitreal injection of 10,000 live microfilariae.

Fig. 7. (A) Microfilaria in the posterior vitreous with light scattering of chronic inflammatory cells and eosinophils in right eye of animal (#266) which received live microfilariae. (B) High-power magnification of cells in the vitreous showing mononuclear cells, eosinophils, and a multinucleated giant cell.
within the first 3 weeks after intravitreal injection of microfilariae, indicating they were sensitized by this route.

Cell-mediated immune responses to *O. volvulus* adult worm crude antigen are shown in Figure 10. Sensitized animals produced positive T-cell responses at the time of intravitreal injection, and the magnitude of these responses continued to increase over the next 18 days. Unsensitized animals had no apparent T-cell response at the time of intravitreal injection but became positive 18 days after injection, again demonstrating that they had become sensitized by this route. None of the animals had a significant response to crude retinal antigen at any time, and lymphoproliferative responses to mitogen were normal in all the animals throughout the experiment (data not shown).

**Discussion**

We demonstrated that live *O. volvulus* microfilariae injected into the vitreous of primates can produce chorioretinal changes which resemble human onchocercal chorioretinitis, and these changes more closely resemble the human disease than the primate model that uses *O. lienalis* microfilariae. In this primate model, live microfilariae were observed in the anterior chamber for many weeks, and they were observed in two of the animals until they were killed. Anterior chamber inflammation was present in primate eyes that received intravitreal injections of live or dead microfilariae. Live microfilariae are often present in the anterior chamber in human ocular onchocerciasis, but usually there is no accompanying cell and flare. Some humans have significant iridocyclitis in ocular onchocerciasis, and this appears to be similar to the anterior segment inflammation in the primate model. The introduction of a large number of microfilariae into the primate eye at one time may cause the in-

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**Fig. 8.** Extensive loss of outer retinal layers with migration of pigmented cells into the inner retina in unsensitized animal (#331) which received intravitreal injection of 10,000 live microfilariae.

**Fig. 9.** IgG activity against *O. volvulus* microfilarial antigen. ELISA A492 × 1000 for serum at 1:500 dilution is plotted against time. Horizontal dashed line denotes minimum positive (mean for normal serum plus 3 × SD).

**Fig. 10.** Cell-mediated immune responses against *O. volvulus* adult antigen. Stimulation index (CPM [3H]-thymidine incorporation with antigen/incorporation without antigen) is plotted against time. Dashed line indicates minimum positive value (two-fold stimulation above background).
creased amount of inflammation in the anterior chamber.

Intravitreal injections of *O. volvulus* microfilariae resulted in progressive loss of retinal pigment and pigment clumping, and these two clinical features are also characteristic of human ocular onchocerciasis. The major difference between the posterior segment changes in the primate model and human disease was the more rapid progression of lesions in the animal model. The primate model may be an accelerated version of human onchocerciasis because the numbers of microfilariae are probably much greater than present in human disease. Posterior segment disease in the primate eye evolved over several weeks, whereas human onchocercal posterior segment disease appears to evolve over several years.\(^9\)

*O. volvulus* microfilariae produce a more accurate model of human ocular onchocerciasis than *O. lienalis* in primates because there is less ocular inflammation in both the anterior and posterior segments. In our study, intravitreal injections of 10,000 live *O. volvulus* microfilariae produced less inflammation than intravitreal injections of 500 live *O. lienalis* microfilariae. In previous studies with *O. lienalis*, intravitreal injection was followed by fibrin clot formation and even severe iritis with hypopyon,\(^3,4\) whereas only a moderate anterior chamber cell and flare were noted in our study with *O. volvulus*. Chorioretinal changes in our study were characterized by retinal pigment loss rather than pronounced retinal vasculitis and pigment loss seen with *O. lienalis*. *O. volvulus* appears to be less antigenic in the primate model than *O. lienalis*, the species causing bovine onchocerciasis.

Both live and dead *O. volvulus* microfilariae were injected intravitreally to determine whether direct invasion of the retina and choroid by microfilariae would be necessary to produce chorioretinal lesions. Overall, chorioretinal changes were more pronounced in eyes that received live microfilariae, suggesting the passage of microfilariae through the retina and choroid may play a role in the pathogenesis of onchocercal posterior pole disease. However, one animal (#239) had thinning and loss of the outer retina in both eyes, suggesting that the presence of dead microfilariae alone can contribute to chorioretinal changes. Histopathologic examination of all the eyes did not reveal microfilariae in the retina and choroid, although in previous experiments, within 1 week of intravitreal injection, live microfilariae were found in the retina.\(^3\) In a murine model, onchocercal microfilariae migrated rapidly through ocular tissues within hours to days.\(^10\) In our study, it appears that after 53 days, most of the live microfilariae may have migrated out of the eye or died. This suggests that, to continue the progression of posterior segment disease in the primate model, it may be necessary to repeat intravitreal injections of live microfilariae.

We examined the IgG response to onchocercal antigen and systemic cell-mediated proliferation responses to mitogen and onchocercal microfilarial antigen to correlate these responses with the development of posterior segment lesions. Systemic IgG responses in unsensitized animals occurred within 3 weeks of the intravitreal injection. Cell-mediated immune responses to onchocercal microfilarial antigen were present in all animals by 18 days after intravitreal injection. The cell-mediated responses to Onchocerca were the smallest in animal 266, suggesting that the animal, who died of leukemia, had reduced concentrations of lymphocyte responders. Areas of retinal pigment loss developed within the first day after intravitreal injection of live microfilariae in this experiment and gradually grew more severe. Immunologic sensitization, as demonstrated by circulating IgG antibody and cell-mediated immunity, did not appear to correlate with the early development of chorioretinal lesions. Since pathologic specimens of the eye were not available from the first few weeks of the animal model, the extent of immune-mediated mechanisms in the development of chorioretinal lesions cannot be stated definitely. An apparent lack of correlation between circulating IgG or IgE antibody, local IgG antibody, cell-mediated immunity, and clinical disease has also been previously observed in our earlier studies with *O. lienalis*, suggesting that local inflammatory mechanisms may be of primary importance in the early development of lesions in the posterior segment of the eye.\(^4\) Sensitization may have inhibited later development of patchy areas of retinal pigment loss; the lesions were more extensive in animals that had not been previously sensitized. Our previous studies in the monkey model also showed that unsensitized animals had more extensive disease than sensitized animals.\(^3\) The cell-mediated proliferative response to crude retinal antigen was examined because autoimmunity against the retina has been suggested to play a role in the retinal lesions.\(^11\) Cell-mediated immune responses against the retina were not present in any animal in this study.

Further studies in this model may aid in understanding the disease processes involved and the development of the devastating chorioretinal lesions which are a major cause of blindness in onchocerciasis.

**Key words:** onchocerciasis, Onchocerca, chorioretinitis, animal model, microfilaria

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References


CORRECTION

In the article, “Relations Between Fundus Appearance and Function: Eyes Whose Fellow Eye Has Exudative Age-Related Macular Degeneration,” by Alvin Eisner, Vasiliki D. Stoumbos, Michael L. Klein, and Susan A. Fleming, which appeared in the January 1991 issue of Investigative Ophthalmology and Visual Science (pages 8–20), the third category in the Appendix is labeled “Reticular Drusen Area.” The correct label should read “Drusen Area.” “Reticular” should be the last entry under “Drusen Size.” The publisher regrets any inconvenience this error may have caused.