Hartley guinea pigs were injected with microfilariae (MF) of *Onchocerca lienalis* as a model for acute inflammatory responses to MF in human *Onchocerca volvulus* infection. IgG autoantibody reactive with a 3 M KCl extract of guinea pig cornea was detected by ELISA in the serum of guinea pigs injected with *O. lienalis* MF three or more times subconjunctivally, or two or more times subcutaneously. Administration of the microfilaricides diethylcarbamazine citrate and ivermectin did not alter the proportion of animals expressing autoantibody or the mean autoantibody titer. The severity of acute conjunctival inflammatory reactions to MF was similar in animals with and without circulating autoantibody. Although autoantibody responses did not correlate with acute conjunctival inflammatory reactions to dead MF, the ability of MF to induce formation of an antibody reactive with a component of autologous cornea suggests that autoimmune mechanisms might participate in chronic onchocercal lesions in the cornea, e.g., sclerosing keratitis. Invest Ophthalmol Vis Sci 29:827-831, 1988

*Onchocerca volvulus* infection afflicts an estimated 30 to 50 million humans worldwide. In hyperendemic areas, 10% of all persons infected and 50% of adults over 40 may be blind due to corneal or retinal inflammatory lesions caused by *O. volvulus*.1 Corneal disease in onchocerciasis may occur as punctate keratitis or sclerosing keratitis. Punctate keratitis consists of greyish-white opacities composed of acute inflammatory cells and localized edema which form around dying or dead microfilariae (MF).1 These opacities resolve without leaving obvious permanent damage when the microfilarial debris has been degraded by the inflammatory cells. Sclerosing keratitis consists of a fibrovascular pannus which usually begins from the inferior and temporal limbus, and progresses to cover the entire cornea.1 Punctate keratitis is more often found in light infections of shorter (less than 10 years) duration, while sclerosing keratitis is associated with heavy infections of longer (10 years or more) duration.1 Immunopathologic mechanisms mediated by IgE antibody and eosinophils are thought to be of importance in punctate keratitis, while the mechanisms responsible for sclerosing keratitis are largely unknown.2-5

In order to identify the immunopathologic mechanisms responsible for the corneal inflammatory lesions of onchocerciasis, we have developed an animal model using MF of *Onchocerca lienalis*, a parasite of cattle closely related to *O. volvulus*.2-4 Subconjunctival injection of *O. lienalis* MF in guinea pigs resulted in penetration of MF to the central cornea, and formation of opacities resembling the punctate keratitis of human onchocerciasis around individual MF when these MF die.4,5 Enumeration of the punctate corneal lesions and measurement of corneal neovascularization associated with the inflammatory reactions has been used to quantitate the effects of microfilaricidal drugs and of anti-inflammatory agents on the acute corneal reactions to dead MF.5 It has been suggested that autoimmune mechanisms may be involved in the development of ocular lesions in onchocerciasis.1 Experimental studies in rabbits have demonstrated that autoimmunization with a 3 M KCl extract of autologous cornea, followed by intracorneal injection of lymphokines, can induce severe corneal inflammation.6 In the present study serum IgG antibody responses to a 3 M KCl extract of guinea pig cornea were examined in guinea pigs injected with *O. lienalis* MF. Autoantibody was detected by ELISA in sera from guinea pigs given subconjunctival injections of MF at 3-4-week intervals over a period of 14 weeks, or subcutaneous injections of MF at two week intervals over 4 weeks. No obvious correlations were found between autoantibody levels and acute corneal inflammatory reactions to MF, or between serum autoantibody responses and treatment with microfilaricidal drugs. The ability of MF in extraocular sites to induce autoantibody reactive with cornea suggests that autoimmune chronic corneal inflammatory disease could occur in human onchocerciasis, for example in long-standing infections.

*Materials and Methods.* Microfilariae (MF) of *Onchocerca lienalis* were obtained from the umbilical skin of cattle, cryopreserved, and prepared for injection as previously described.2,4 Groups of 20-30 female Hartley guinea pigs (500-600 g body weight)
were given subconjunctival injections of 0.1 ml of Dulbecco’s Modified Eagle’s medium (DME) containing 5000 Mf. Betamethasone (Schering Pharmaceuticals, Kenilworth, NJ) was given to treatment groups of six to ten animals at a dose rate of 0.1 mg/kg daily beginning 4 days after the subconjunctival injection of Mf. Diethylcarbamazine citrate (DEC, Sigma Chemical Co., St. Louis, MO) was given at 15 mg/kg daily beginning 5 days after the injection of Mf. Betamethasone and DEC were diluted in 0.01 M phosphate-buffered 0.15 N NaCl, pH 7.4, (PBS) and administered by subconjunctival injection. A single dose of 0.2 mg/kg of ivermectin diluted in PBS, was given orally 5 days after injection of Mf. Drug treatments and clinical examinations were conducted in a double-masked fashion. Clinical examinations were performed with a Zeiss slit lamp before the injection of Mf, and 2, 4, 7 and 8 days after injection, as previously described.7

Antigen extracts were prepared from *O. lienalis* Mf and normal guinea pig cornea. *O. lienalis* Mf were disrupted by freeze-thawing and sonication in PBS, centrifuged for 5 min at 8000 g, and supernatants were stored in liquid nitrogen. Guinea pig corneas, with epithelium attached, were stored in 3 M KCl for 2 days at 4°C, centrifuged for 10 min at 8000 g, and dialyzed against PBS. The protein content of the Mf and guinea pig cornea extracts was determined by the Coomassie blue dye-binding assay of Bradford standardized with bovine serum albumin.7

Enzyme-linked immunosorbent assays (ELISA) were performed using Immulon 2 round-bottomed 96-well polystyrene plates (Dynatech, Alexandria, VA) coated with *O. lienalis* Mf antigen (1 µg/well), or guinea pig cornea antigen (5 µg/well), in 0.1 M sodium bicarbonate buffer, pH 9.0, for 18 hr at 4°C. Sera were added at 1:700 dilution in PBS plus 10% FBS for anti-*O. lienalis* antibody assays, or undiluted for autoantibody assays, and incubated for 2 hr at room temperature, or at 37°C. Peroxidase-conjugated goat anti-guinea pig IgG (Cooper Biomedical, King of Prussia, PA) was added at 1:200 dilution and incubated for 1 hr at room temperature, or at 37°C. Substrate development was carried out for 30 min at room temperature using 0.5 mg/ml 5-aminosalicylic acid, pH 6.1. The optical density at 492 nm (OD492) was determined using a Multiskan MC ELISA reader (Flow Laboratories, McLean, VA) with an Apple Ile (Cupertino, CA) computer interface.

Statistical analyses were carried out by one-way analysis of variance, by the chi-squared test, and by the Pearson product-moment correlation.8

**Results.** ELISA for IgG antibody were performed on serum using *O. lienalis* Mf extract or 3 M KCl extract of guinea pig cornea as antigen. Thirty-two normal sera from guinea pigs aged 2 to 5 months gave ELISA optical densities at 492 nm of 0.043 ± 0.040 (SD) against the cornea extract when incubations were conducted at room temperature. When ELISA incubations were conducted at 37°C, the average OD492 for normal sera was 0.297 ± 0.128. Sera were considered positive for autoantibody if the OD492 against corneal extract was equal to or greater than the mean plus 3 SD for normal serum determined under identical conditions.

The time course of expression of IgG autoantibody and antibody to *O. lienalis* in 22 animals injected subconjunctivally with *O. lienalis* Mf is shown in Figure 1A. Injections of Mf were given on days 0, 27, 68 and 84, and DEC, DEC plus betamethasone, or no treatment was given on days 31–35, 71–75, 85–90. The combined data for all treatment groups are shown in Figure 1A. Autoantibody was not detected until 12 days after the third subconjunctival injection of Mf, while anti-*O. lienalis* antibody was present after one injection of Mf. Figure 1B shows the time course of expression of anti-*O. lienalis* IgG antibody and autoantibody following two subcutaneous injections and one intracorneal injection of Mf in 20 guinea pigs. Subcutaneous injections were given on days 0 and 14 and the intracorneal injection was given on day 28. Autoantibody appeared in 10 of 20 animals after one subcutaneous injection and in 18 of 20 after two subcutaneous injections (Fig. 1B). No intracorneal Mf were seen in these animals prior to administration of the intracorneal challenge injection. The peak IgG antibody response against *O. lienalis* was approximately half that observed in animals given subconjunctival injections of Mf. Animals injected subconjunctivally or subcutaneously with DME did not develop detectable autoantibody.

Guinea pigs given subconjunctival injections of Mf had microfilariae in the central cornea within 2 days, and subsequently developed inflammatory reactions as these Mf lost motility and appeared to die. The number of punctate opacities containing nonmotile, degenerating Mf, and the extent of corneal neovascularization, were estimated. The relationship between the numbers of punctate opacities and the presence or absence of autoantibody in the experiment shown in Figure 1A is shown in Table 1. No significant dif-
ference in the number of opacities was observed between animals with or without autoantibody. The Pearson product-moment correlation was used to determine whether any association existed between the amount of autoantibody (ELISA OD$_{492}$) and the number of punctate opacities, the length of corneal new vessels, or the amount of anti-$O$. lienalis antibody (ELISA OD$_{492}$). A weak ($r = 0.39$) but statistically significant ($P < 0.05$) positive correlation was found between the autoantibody and anti-$O$. lienalis antibody titers. No other correlations could be demonstrated.

The effects of treatments with microfilaricides and anti-inflammatory drugs on the autoantibody response were studied in guinea pigs injected subconjunctivally with $O$. lienalis Mf. Figure 2 shows the autoantibody responses of three groups of guinea pigs given subconjunctival injections of Mf on days 0, 27, 68 and 84, and treated with DEC, DEC plus betamethasone, or not treated on days 31–35, 71–75 and 85–90. (The combined data for all treatment groups are shown in Figure 1A.) Figure 2 shows the autoantibody responses of three groups of guinea pigs given subconjunctival injections of Mf on days 0, 27, 68 and 84, and treated with DEC on days 37–39 and 92–96, with ivermectin on days 37 and 92, or not treated. Using the chi-squared test, no statistically significant differences in the proportion of autoantibody-positive animals were found between the groups in either experiment. In both experiments, the effect of drug treatment on the magnitude of the autoantibody response (estimated by using the ELISA OD$_{492}$) was estimated by one-way analysis of variance. No statis-

Table 1. Mean punctate opacities/animal ± SEM for guinea pigs with and without autoantibody on day 89

<table>
<thead>
<tr>
<th>Day</th>
<th>With auto Ab (n = 5)</th>
<th>Without auto Ab (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>31 ± 4.5</td>
<td>31 ± 3.9</td>
</tr>
<tr>
<td>76</td>
<td>17 ± 3.3</td>
<td>25 ± 4.2</td>
</tr>
<tr>
<td>86</td>
<td>27.9 ± 6.3</td>
<td>24 ± 3.6</td>
</tr>
<tr>
<td>88</td>
<td>27 ± 3.0</td>
<td>27 ± 2.5</td>
</tr>
</tbody>
</table>

Mf injections were given on days 0, 27, 68, 84; DEC, DEC plus betamethasone, or no treatment were given on days 31–35, 71–75 and 85–90.

Fig. 1. (A) Anti-$O$. lienalis and anti-guinea pig cornea autoantibody following subconjunctival injection of Mf. IgG, ELISA OD$_{492}$ (X1000) for undiluted serum tested against 3 M KCl extract of guinea pig cornea, for all treatment groups combined. Mean of the ELISA OD$_{492}$ (X1000) for sera from all groups, diluted 1:50 and tested against $O$. lienalis Mf extract. Incubations were carried out at room temperature. Error bars denote ±SEM. Dashed line denotes minimum positive OD$_{492}$ value for autoantibody at 25°C. Subconjunctival injections of 5000 Mf were given on days 0, 27, 68 and 84; DEC, DEC plus betamethasone, or no treatment, were given on days 31–35, 71–75 and 85–90. (B) Anti-$O$. lienalis and anti-guinea pig cornea autoantibody following subcutaneous and intracorneal injection of $O$. lienalis Mf. IgG, ELISA OD$_{492}$ (X1000) for undiluted serum tested against 3 M KCl extract of guinea pig cornea. Mean ELISA OD$_{492}$ (X1000) for serum diluted 1:50 and tested against $O$. lienalis Mf extract. Incubations were carried out at room temperature. Error bars denote ±SEM. Dashed line denotes minimum positive OD$_{492}$ value for autoantibody at 25°C. Subcutaneous injections of 5000 Mf were given on days 0 and 14, and an intracorneal injection of 5,000 Mf was given on day 28 (arrows).

Fig. 2. Anti-cornea autoantibody versus time for the three drug treatment groups in the experiment shown in Figure 1A. ELISA OD$_{492}$ (X1000) for undiluted serum tested against 3 M KCl extract of guinea pig cornea. DEC only; DEC plus betamethasone; no treatment. Incubations were carried out at room temperature. Subconjunctival injections of Mf were given on days 0, 27, 68 and 84; drug treatments were given on days 31–35, 71–75, and 85–90. Dashed line denotes minimum positive OD$_{492}$ value for autoantibody at 25°C.
with cornea is released from collagenous tissues dur-
also is possible that an autoantigen cross-reacting
against simple cross-reactivity between host cornea
IgG antibody (eg, day 70 in Fig. 1A) argues
response.
The absence of detectable anti-cornea autoantibody
autoantibodies against corneal antigens is unknown.
The origin of the immunogens which induced the
autoantibodies against corneal antigens is unknown.
Discussion. In the present study we have examined
potential role of serum IgG autoantibody reactive
with a 3 M KCl extract of guinea pig cornea, induced
infection with Mf of Onchocerca lienalis, in acute
corneal inflammatory responses to Mf. No apparent
correlations were found between the presence of au-
toantibody in the serum and the magnitude of local-
ized acute inflammatory reactions around intracor-
neal Mf, estimated by counting of punctate opacities
and by measurement of corneal neovascularization.
The magnitude of the autoantibody response corre-
lated with the titer of anti-O. lienalis IgG antibody,
and the proportion of animals expressing autoanti-
body increased with successive injections of Mf. Nei-
ther DEC nor Ivermectin treatment increased the au-
toantibody response, and betamethasone given after
the injection of Mf did not affect the autoantibody
response.

The origin of the immunogens which induced the
autoantibodies against corneal antigens is unknown.

Fig. 3. Anti-cornea autoantibody versus time for guinea pigs injected subconjunctivally with Mf and given no treatment, DEC, or ivermectin. ELISA OD492 (×1000) for undiluted serum tested against 3 M KCl extract of guinea pig cornea. Incubations were carried out at 37°C. •, No treatment; ○, DEC; ■, Ivermectin. Dashed line denotes minimum positive OD492 value for autoanti-
body at 37°C. Subconjunctival injections of Mf were given on days 0, 32 and 87, and drug treatments were given on days 37–39 and 92–96 (DEC), or 37 and 92 (ivermectin).

Discussion. In the present study we have examined
potentially significant effect of drug treatment could be
demonstrated.

Key words: onchocerciasis, experimental model, cornea, autoantibody, punctate keratitis

Acknowledgments. Ivermectin was a gift from Dr. M. A. Aziz of the Merck Institute for Therapeutic Research (Rahway, NJ). Rene A. Morris and Steven C. Levin provided expert technical assistance.

From the *Department of Ophthalmology, Scheie Eye Institute, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, and the †Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. Supported by the Onchocerciasis Chemotherapy Project of the World Health Organization Onchocerciasis Control Programme of the Volta River Basin, by USPHS NIH grants EY-03984, EY-06616, and AI-19995, and by the Harold G. Scheie Teaching and Research Fund. Submitted for publication March 2, 1987; accepted December 7, 1987. Reprint requests: John J. Donnelly, PhD, Scheie Eye Institute, Myrin Circle, 51 North 39th Street, Philadelphia, PA 19104.

References
2. Donnelly JJ, Rockey JH, Bianco AE, and Soulsby EJL: Aqueous humor and serum IgE antibody in experimental ocu-

ing migration of Mf through skin or conjunctiva, poss-
ibly by proteolytic enzymes of microfilarial origin.

Human onchocercal keratitis may appear as re-
vulsive local acute inflammatory foci around individ-
ual Mf (punctate keratitis), or as a permanent fi-
brovascular pannus which progresses centripetally
from the limbus (sclerosing keratitis). Sclerosing
keratitis is the leading cause of blindness due to on-
chocerciasis in savanna regions of West Africa. Pun-
culate keratitis and sclerosing keratitis are not gen-
erally seen in the same individuals, with sclerosing
keratitis being associated with more advanced disease
of longer duration. The present studies suggest that
autoantibody to corneal antigens may not contribute
to the acute punctate keratitis-like lesions of the
guinea pig model. The importance of autoimmune
mechanisms in the chronic inflammatory processes
of sclerosing keratitis remains to be determined. The
ability of Mf in extracocular sites to induce autoanti-
body reactive with a component of cornea suggests
that significant autoimmune corneal disease could
occur in human onchocerciasis.