Experimental Ocular Onchocerciasis in Cynomolgus Monkeys

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Infection of cynomolgus monkeys with microfilariae (Mf) of *Onchocerca lienalis* was studied as a model for human ocular onchocerciasis. Normal monkeys and immunized monkeys were given intracorneal/subconjunctival, intracameral, or intravitreal injections of Mf or bovine serum albumin (control). Selected animals were given diethylcarbamazine citrate (DEC) orally (15 mg/kg) daily after the ocular infection. Following intravitreal challenge, living Mf and fibrinous exudates were visible by slit-lamp in both the anterior chamber and vitreous. Eosinophils and macrophages surrounded the Mf in the vitreous, with degranulated eosinophils adherent to the Mf. Eosinophils infiltrated the uvea and surrounded the retinal vessels. After intracorneal injection of Mf, living Mf were visible by slit-lamp biomicroscopy in the cornea for 3 days, with minimal inflammation of the corneas occurring over the 7 days after injection. Intracameral injection of Mf induced anterior uveitis. The extent of the inflammatory reactions was not substantially altered by DEC treatment following intraocular injection of Mf. In vitro proliferative responses of peripheral blood leukocytes to a crude Mf antigen were not observed in infected monkeys and proliferative responses to mitogen declined in these animals. Responses to mitogen were inhibited by addition of Mf antigen in vitro in normal monkeys. Circulating IgG antibodies were present in the sensitized, intracorneally, and intravitreally challenged animals. No obvious correlations were present between IgG antibody level and ocular inflammation. Invest Ophthalmol Vis Sci 27:492-499, 1986

Ocular disease caused by *Onchocerca volvulus* infection is a leading cause of blindness in Equatorial Africa and in endemic regions of Central and South America. In regions hyperendemic for *O. volvulus*, blindness rates among adults due to ocular onchocerciasis may approach 30%. Studies on the pathogenesis of the ocular disease in onchocerciasis have been restricted by the lack of suitable animal models.

In previous studies, we have reproduced some aspects of the acute corneal disease of human onchocerciasis produced by inoculation of *Onchocerca lienalis* microfilariae (Mf) into the eyes of guinea pigs. In this model sensitization was needed for ocular lesions to develop, and treatment with the microfilaricidal drug diethylcarbamazine citrate (DEC) increased the immune response to the Mf and the severity of the ocular lesions.

In the present study cynomolgus monkeys were inoculated with *O. lienalis* Mf in order to determine whether lesions more comparable to those of humans might be produced in eyes with greater anatomic resemblance to the human eye. Antibody and cell-mediated immune responses of the animals were followed in order to determine the relationship between the development of these responses and of the ocular lesions. DEC was given to selected animals in order to assess the effect of treatment on ocular inflammatory and immune responses in this model.

Host immune responses were important in producing ocular lesions but in some instances were downregulated by Mf. Inflammatory reactions in the posterior segment were much more severe than those in the cornea, indicating that anatomical factors also affect the ability of Mf to cause ocular damage.

Materials and Methods

Mf of *O. lienalis* were collected from the umbilical skin of cattle as described by Bianco et al. and were cryopreserved using ethanediol as cryoprotectant according to the method of Ham et al. Cryopreserved Mf were thawed in Tyrode's solution with 10% (V/V) normal bovine serum warmed to 38°C, washed three times in cold Tyrode's solution without serum, and used immediately for infections.

Experiments were carried out on seven cynomolgus...
monkeys (*Macaca fascicularis*), divided into two groups. One group, consisting of five animals, received subcutaneous injections of 30,000 freshly thawed *O. lienalis* MF at the beginning of the experiment and again 14 days later. Each inoculum was distributed among six sites on the abdomen and flanks. One week after the 2nd subcutaneous injection these animals received intraocular injections of 10,000 *O. lienalis* MF or 10% bovine serum albumin (BSA) (challenge controls) under ketamine anesthesia. DEC (15 mg/kg) was given orally to two of these animals daily beginning at the time of ocular challenge. The second group, consisting of two animals, received no pre-immunization but only a single intraocular injection of 10,000 MF or 10% BSA. BSA was used as the challenge control to assess the role of bovine serum proteins adsorbed to MF in the inflammatory reactions.

The intraocular injections of MF were given intraconearly, intracameraly, and intravitreally and were distributed among the animals in both groups as shown in Table 1. Intracameral midstromal injections of 10,000 MF in 10−20 μl volume were given into the central cornea with a Hamilton syringe equipped with a 30-gauge needle. An additional 10,000 MF were injected subconjunctivally in these eyes. Intracameral and intravitreal injections of 10,000 MF in 0.1 ml volume were given with a 1-ml tuberculin syringe equipped with a 30-gauge needle. Anterior chamber paracentesis was performed prior to the intracameral and intravitreal injections to relieve intraocular pressure. All monkeys were killed 7 days after the intraocular injection of MF or BSA. Bleedings were performed before immunization, at the time of intraocular injection, and at the time of death. Anterior chamber taps were performed on selected animals at the time of intraocular injection and at the time of death. All studies were conducted in accordance with the ARVO Resolution of the Use of Animals in Research.

Eyes were prepared for light and electron microscopy by fixation in cacodylate-buffered 2.5% glutaraldehyde (pH 7.4) and postfixation in Millonig’s buffered Osmium tetroxide, as described.4 Thick (1 μm) sections of Polybed-812 embedded tissues were stained with toluidine blue. Thin (70 μm) sections were stained with uranyl acetate-lead citrate and examined with a Zeiss EM10 electron microscope (Carl Zeiss, Inc.; Oberkochen, West Germany).

*O. lienalis* extract antigen was prepared from MF which had been separated by Percoll density gradient centrifugation. Gradients were formed by centrifugation of 12 ml of 55% Percoll (Sigma Chemical; St. Louis, MO) for 50 min at 27,000 × g (16,000 RPM) in an SW-27 rotor. Suspensions of 2−5 × 10⁵ MF were layered over each gradient and centrifuged for 10 min at 2,000 × g. Living MF were recovered from the bottom of the gradient (density ≥ 1.076) while particulate debris and dead MF remained at lower density levels. MF recovered from gradients were washed three times in Tyrode’s solution, homogenized by sonication, and extracted as previously described.3

Enzyme-linked immunosorbent assays (ELISA) for specific IgG antibody were carried out as described by Khatami et al8 with minor modifications. Polyvinyl chloride microtiter plates (Cooke) were coated with 10−25 μg/well of *O. lienalis* extract. Sera were diluted in PBS containing 0.5 mg/ml BSA to absorb anti-BSA antibody. Optical density at 492 nm was determined using a Multiskan MC ELISA reader with Apple (Cupertino, CA) IIe computer interface.

Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood by Ficoll-Hypaque density gradient sedimentation.9 The mononuclear cell-rich fraction was washed and resuspended in Eagle’s Minimal Essential Medium (MEM, GIBCO; Grand Island, NY) with 2 mM glutamine, 25 mM Heps buffer, and 10% fetal bovine serum. Cells were cultured at 1.5 × 10⁵ cells/well in 96-well round bottomed microtiter plates with or without Concanavalin A (Con A) (50-100 μg/ml), *O. lienalis* extract (10−100 μg/ml), or both. The cells were pulsed with 1 μCi [³H]-methyl thymidine per well after 72 hr, harvested 18 hr later, and counted in a liquid scintillation counter. Results are expressed as ΔCPM, ie, counts per minute of stimulated cells minus counts per minute of nonstimulated (control) cells.

Arithmetic means, standard deviations, and standard errors of the mean (SD), were calculated as described by Brown and Hollander.10 Changes in the lymphocyte proliferative response to Con A over time were analyzed by one-way analysis of variance with replication as a Model 1 anova as described by Sokal and Rohlf.11 Lymphocyte proliferative responses to Con A with and

| Table 1. Immunization and challenge of experimental animals |
|-----------------|-----------------|-----------------|
| **Subcutaneous** | **Intraocular**  | **DEC**         |
| pre-immunization | challenge        | treatment       |
| (days 0 and 14)  | (day 21)         | (15 mg/kg, 21–28) |
| OD               | OS              |
| + Anterior       | Cornea          |
| chamber          |                 |
| + Vitreous       | —               |
| + Cornea         | Vitreous        |
| + Anterior       | Cornea          |
| chamber          | (BSA)           |
| + Vitreous       | Vitreous        |
| — Vitreous       | Cornea          |
| — Vitreous       | Anterior        |
| — Anterior       | chamber         |
V of the retina (Fig. 1). Cuffs of infiltrating eosinophils and mononuclear cells were present around the retinal vessels and appeared to be migrating toward the vitreous (Fig. 2). Fibrin strands were present in the inner retina. Focal areas of vacuolation and duplication were present in the RPE, and pigment-containing cells were scattered through the inner retina in some areas (Fig. 3).

Large numbers of Mf and microfilarial fragments were present in the vitreous (Figs. 4–7). Some Mf were embedded in densely packed masses of degranulated eosinophils and other inflammatory cells (Figs. 5, 6), while others were relatively free of adherent cells (Fig. 7). The ciliary body and pars plana choroid were densely infiltrated with eosinophils and mononuclear cells, especially in areas adjacent to the masses of cells in the anterior vitreous. Administration of DEC to these animals did not appreciably alter the severity of the ocular inflammatory reactions.

Intracorneal injection of Mf or BSA in normal or immunized animals induced mild transient anterior uveitis of 2 to 3 days duration, but no clear differences

without *O. lienalis* antigen were compared by the one-tailed Student t-test for paired variates.

**Results**

Intravitreal injection of Mf in a normal monkey produced anterior chamber flare and cells on days 1 and 3, and formation of a large fibrin clot in the anterior chamber that prevented funduscopic examination. Histologically, a very few inflammatory cells were present over the inner surface of the retina. Intravitreal injection of BSA in both normal and pre-immunized animals induced mild anterior uveitis and minimal histologic changes. Intravitreal injection of Mf in pre-immunized monkeys produced severe uveitis with hypopyon, fibrin, and hemorrhages in the anterior chamber, keratic precipitates, and corneal edema, precluding viewing of the fundus after day 3. Mf were observed in the anterior chamber on day 1 and the vitreous on day 1 and 3. Histologically a dense fibrinous exudate approximately 2 mm thick, containing many extensively vacuolated inflammatory cells, and Mf with adherent inflammatory cells, covered the inner surface of the retina (Fig. 1). Cuffs of infiltrating eosinophils and mononuclear cells were present around the retinal vessels and appeared to be migrating toward the vitreous (Fig. 2). Fibrin strands were present in the inner retina. Focal areas of vacuolation and duplication were present in the RPE, and pigment-containing cells were scattered through the inner retina in some areas (Fig. 3).

Intracorneal injection of Mf or BSA in normal or immunized animals induced mild transient anterior uveitis of 2 to 3 days duration, but no clear differences
were seen between the different treatments in this limited number of animals. Mf were found in histologic specimens from intracorneally injected eyes. Intracameral injection of Mf induced anterior uveitis in both normal and immunized animals, with the immunized animals having slightly more severe reactions. By day 7 after injection Mf were no longer visible in the aqueous by slit lamp.

The serum and aqueous IgG antibody responses to *O. lienalis* microfilarial extract of the five pre-immunized and intraocularly challenged monkeys are shown in Figure 8. Seropositivity was defined as an ELISA optical density of 3 or more standard deviations above the mean ELISA optical density of normal non-immunized monkey serum at the same dilution. Two of the animals seroconverted following the two subcutaneous injections of Mf, and a third seroconverted after the intraocular challenge. The remaining two animals did not show ELISA activity above background levels. Of the two pre-immunized animals that received intravitreal injections of Mf, only one developed detectable serum IgG antibody (Fig. 8, open circles). No obvious differences were seen in the severity of the intraocular inflammation in these animals. Of the two
animals given DEC after the intraocular infection, one was already seropositive at the time DEC was initiated and did not show any change in IgG antibody thereafter. The other DEC-treated animal seroconverted following intracameral (OD) and intracorneal (OS) challenge and DEC treatment. Aqueous humor IgG antibody was detected following intraocular challenge in animals positive for serum IgG antibody. Animals lacking serum IgG antibody also did not demonstrate aqueous IgG antibody.

Peripheral blood mononuclear leukocyte (PBML) proliferative responses to 100 μg/ml Con A declined steadily during the course of subcutaneous immunization and intraocular challenge (Fig. 9) (mean ± SEM of ΔCPM × 10^{-3} for day 0, 25.8 ± 5.76; for day 21, 13.0 ± 3.53; for day 28, 9.4 ± 2.29, P < 0.05 by one-way analysis of variance). Normal monkey PBML assayed at the same time as cells from immunized monkeys showed no comparable changes in their Con A responses.

Incubation of normal or immunized monkey PBML with *O. lienalis* microfilarial extract antigen showed neither antigen-specific nor nonspecific blastogenesis over a dose range of 10–100 μg/ml. However, addition of 10 μg/ml *O. lienalis* extract and 100 μg/ml Con A simultaneously to cultures of normal monkey PBML reduced the proliferative responses to Con A by one third to one half in 4 of 5 animals (Fig. 10) (mean ± SEM of ΔCPM × 10^{-3} for Con A only, 25.8 ± 5.8; for Con A + *O. lienalis* antigen 16.0 ± 3.6, P < 0.01 by the t-test for paired variates). One pre-immunized monkey that had been given DEC after intracameral (OD) and intracorneal (OS) challenge showed costimulation by antigen and mitogen on day 28 (Fig. 10). This animal's response to Con A in combination with antigen was twice as great as its response to Con A.
alone, while no proliferation above background was observed with antigen alone.

**Discussion**

Severe vitritis, retinitis and uveitis was observed in pre-immunized animals intravitreally challenged with Mf. In some instances, vitreal Mf were found free within the vitreous with few cells adherent, while other Mf in the same eye were surrounded by dense masses of inflammatory cells. These may represent stages in the destruction of the Mf. The principal sources of the infiltrating cells appear to be the retinal vessels and the choroid of the pars plana. This pattern of cellular infiltration is markedly different from that occurring in guinea pig eyes intravitreally injected with ascarid larvae, in which inflammatory cell infiltration of the retina was minimal, even around intraretinal parasites, unless the larvae also had penetrated the choroid. These complementary models allow the relative contributions of retinal vascular and choroidal inflammatory components to be studied independently and emphasize the importance of the retinal vasculature in posterior segment inflammation in the primate eye. The mechanisms by which the eosinophil perivascular sheaths are induced are unknown. *Baylisascaris procyonis* infection of cynomolgus monkey eyes also induces eosinophil perivascular sheaths even though the parasites reach the retina by a different route (via the retinal or choroidal circulation or the optic nerve) than was used in our study (intravitreal injection). The occurrence of eosinophil vascular sheaths under such widely different experimental conditions suggests that similar infiltrations could occur when *Onchocerca* Mf enter
the posterior segment transclerally or hematogenously in the normal course of human onchocerciasis.

The RPE changes (focal vacuolation and proliferation) seen in the present study parallel those occurring in guinea pigs intravitreally infected with ascarid larvae. In the guinea pig system, action of mediators from choroidal eosinophils across Bruch’s membrane is thought to contribute to the RPE changes. The occurrence of similar RPE changes in the monkey model in combination with choroidal eosinophil and plasma cell infiltrates suggests that similar mechanisms may be at work. Such mechanisms could produce the patchy retinal depigmentation with surrounding hyperpigmented areas seen in human onchocerciasis.

The minimal inflammatory response to intracorneal injection of MF in both normal and pre-immunized monkeys in the present study is in marked contrast to the reactions previously observed in laboratory rodent models of onchocerciasis. The reactions to subconjunctival and intracorneal injections of *O. lienalis* MF in the corneas of pre-immunized guinea pigs, and *O. volvulus* in rabbits, included both punctate opacities and generalized interstitial keratitis. Anatomical differences between species may account for a portion of this difference, since the guinea pig and rabbit corneas lack a well-developed Bowman’s membrane and are readily vascularized. The monkey cornea, like the human, possesses a substantial Bowman’s layer and is relatively resistant to neovascularization. Onchocercal punctate keratitis in humans generally develops after several years of patent systemic *O. volvulus* infection and is seen particularly after microfilaricide treatment.

Sclerosing keratitis may occur after a further period of years. Thus prolonged repeated invasion of the primate cornea may be an essential prerequisite for the development of onchocercal-type reactions. The generalized decrease of cell-mediated immunity observed in the present study also might contribute to the relative lack of responsiveness to corneal challenge in the monkeys.

The circulating IgG antibody response to microfilarial antigens varied among individuals, with some animals failing to demonstrate antibody during the experiment. The presence of circulating IgG antibody did not appear to correlate with the severity of the ocular inflammatory response to challenge with MF. The site of the ocular challenge (eg, intracorneal vs intravitreal) did not appear to influence the expression of circulating IgG antibody. The expression of circulating IgG antibody following DEC treatment of one animal suggests this may be a stimulus to antibody production, as has been described in the guinea pig/0 lienalis model. The expression of aqueous IgG antibody was restricted to inflamed eyes of animals with circulating IgG antibody, suggesting that leakage from the serum was the principal source of the aqueous IgG antibody.

Subcutaneous injection of MF exerted an inhibitory effect on cell-mediated immunity, as estimated by the proliferative response of PBML to Con A. In *Onchocerca volvulus* infection of humans, cell-mediated immune responses to parasite antigens and to unrelated antigens are depressed in vivo and in vitro in some individuals. In human *Brugia malayi* infection, cell-mediated unresponsiveness to parasite antigens is maintained by antigen-specific suppressor T cells and by a serum suppressor factor. In both infections the anergy may be reversed by treatment with DEC. The present results suggest that MF or microfilarial products may contribute to this phenomenon.

The inhibitory effect of *O. lienalis* microfilarial antigen on blastogenic responses of normal monkey PBML to Con A is consistent with the depressive effect of MF on blastogenesis in vivo in the present study. The induction of nonspecific suppressor cells in vitro by an extract of *O. volvulus* adults, and the inhibition of Con A and Phytohemagglutinin (PHA)-induced blastogenesis by a prostaglandin-like substance in culture supernatants of *O. gibsoni* MF, have recently been reported. In our studies, outright killing of the cells by the extract of MF may not be a principal mechanism since the ability of the cells to exclude trypan blue was not affected (data not shown). The ability of the extract to co-stimulate in some instances also argues against simple toxicity as the mechanism. Other potential mechanisms may be the induction of nonspecific suppressor cells, or of the secretion of an inhibitory substance, such as prostaglandins, by monocytes or lymphocytes.
One monkey injected with *O. lienalis* Mf demonstrated co-stimulation of lymphocyte proliferation by microfilarial extract and Con A, following intracamer (OD) and intracorneal (OS) challenge and treatment with DEC. Thus significant cell-mediated immune responses to microfilarial antigen may occur, but it may be that Mf alone do not provide a sufficient sensitizing stimulus to overcome the inhibitory effects described above. Previous studies on the guinea pig/O. lienalis model have demonstrated that DEC treatment of animals injected with Mf may increase IgE antibody production. The possibility exists that DEC treatment may likewise stimulate cell-mediated responses, and it might be that these responses are qualitatively different from those induced by Mf alone.

The present study allows some generalizations to be made about the relatively low influence of anatomical and immunological factors and microfilarial load on the development of onchocercal eye disease in different tissues of the eye. Corneal lesions resembling the human disease did not occur even in pre-immunized monkeys from a single corneal injection, suggesting that chronic or repeated invasion may predispose to the development of such lesions, possibly by increasing the development of new corneal blood vessels. Intravitreal injection of Mf in pre-immunized monkeys produced a dense acute cellular reaction, while non-immunized monkeys did not mount such a reaction in the 7 days following injection. The severity of the vitreoretinitis induced by 10,000 Mf suggests that invasion of the posterior segment of a sensitized primate host by a very few Mf might be sufficient to produce the slowly progressive lesions seen in humans. Thus sensitization of the host may be a key determinant of whether Mf are tolerated or produce ocular disease in the uvea, retina, and vitreous. Mf may down-regulate as well as induce immunological responses, and host immune responses may be further modified by DEC treatment. Further studies of the roles of these factors in onchocercal eye disease are needed.

**Key words:** onchocerciasis, monkeys, *Onchocerca lienalis*, intracorneal, intracameral, intravitreal, ELISA, lymphocyte proliferation, cell-mediated immunity

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