**Wolbachia-Induced Neutrophil Activation in a Mouse Model of Ocular Onchocerciasis (River Blindness)**

Ilonna Gillette-Ferguson, Amy G. Hise, Helen F. McGarry, Joseph Turner, Andrew Esposito, Yan Sun, Eugenia Diaconu, Mark J. Taylor, and Eric Pearlman

*Department of Ophthalmology and Center for Global Health and Diseases, Case Western Reserve University, Cleveland, Ohio, and Filariasis Research Laboratory, Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom*

Received 5 February 2004/Returned for modification 7 March 2004/Accepted 28 June 2004

Endosymbiotic *Wolbachia* bacteria are abundant in the filarial nematodes that cause onchocerciasis (river blindness), including the larvae (microfilariae) that migrate into the cornea. Using a mouse model of ocular onchocerciasis, we recently demonstrated that it is these endosymbiotic bacteria rather than the nematodes per se that induce neutrophil infiltration to the corneal stroma and loss of corneal clarity (Saint Andre et al., Science 295:1892–1895, 2002). To better understand the role of *Wolbachia* organisms in the pathogenesis of this disease, we examined the fate of these bacteria in the cornea by immunoelectron microscopy. Microfilariae harboring *Wolbachia* organisms were injected into mouse corneas, and bacteria were detected with antibody to *Wolbachia* surface protein. Within 18 h of injection, neutrophils completely surrounded the nematodes and were in close proximity to *Wolbachia* organisms. *Wolbachia* surface protein labeling was also prominent in neutrophil phagosomes. During phagocytosis, numerous electron-dense granules indicated that neutrophils were activated. To determine if *Wolbachia* organisms directly activate neutrophils, peritoneal neutrophils were incubated with either parasite extracts containing *Wolbachia* organisms, parasite extracts depleted of *Wolbachia* organisms (by antibiotic treatment of worms), or *Wolbachia* organisms isolated from filarial nematodes. After 18 h of incubation, we found that isolated *Wolbachia* organisms stimulated production of tumor necrosis factor alpha and CXC chemokines macrophage inflammatory protein 2 and KC by neutrophils in a dose-dependent manner. Similarly, these cytokines were induced by filarial extracts containing *Wolbachia* organisms but not by *Wolbachia*-depleted extracts. Taken together, these findings indicate that neutrophil activation is an important mechanism by which *Wolbachia* organisms contribute to the pathogenesis of ocular onchocerciasis.

*Wolbachia* organisms infect filarial nematodes of importance to human health, including *Wuchereria bancrofti* and *Brugia malayi*, which cause lymphatic filariasis, and *Onchocerca volvulus*, which causes river blindness (10). The presence of intracytoplasmic bacteria in *O. volvulus* was first described by Kozek in 1977, who found *Rickettsia*-like bacteria in hypodermal lateral cords of male and female worms, in female reproductive organs, and in the first-stage larvae (microfilariae) (15). The bacteria appear to have an essential role in embryogenesis in these nematodes, as antibiotic treatment of infected individuals or experimental animals inhibits production of microfilariae (11–13).

Using a murine model of ocular onchocerciasis, we recently demonstrated that *O. volvulus* extracts containing *Wolbachia* organisms induce pronounced corneal inflammation (characterized by neutrophil infiltration and development of corneal haze) when injected into the corneal stroma, whereas parasite extracts depleted of *Wolbachia* organisms by antibiotics do not induce keratitis (24). Similarly, extracts from *B. malayi* (which harbors *Wolbachia* organisms) induce corneal inflammation, compared with extracts from a filarial species that does not have *Wolbachia* organisms (*Acanthocheilonema viteae*) (24). These results are consistent with an essential role for *Wolbachia* organisms in the pathogenesis of this disease.

In the current study, we examined the interaction between neutrophils and *Wolbachia* organisms in the cornea at the ultrastructural level and also determined if *Wolbachia* organisms directly activate neutrophils to produce cytokines and chemokines associated with pathogenesis. Our results provide a unique perspective on the interaction of neutrophils with filarial nematodes and *Wolbachia* organisms in the cornea and add to our understanding of the early stages of onchocercal keratitis by showing that neutrophils directly associate with microfilariae in the cornea and may contribute to parasite degeneration, neutrophils in the cornea ingest *Wolbachia* organisms, and neutrophils can be directly activated by *Wolbachia* organisms to produce chemotactic and proinflammatory cytokines.

**MATERIALS AND METHODS**

**Preparation of filarial worms.** Live male and female *Brugia malayi* adult worms were obtained from infected Mongolian jirds from the National Institutes of Health filariasis repository (Athens, Ga.). The worms were washed extensively with sterile phosphate-buffered saline and incubated at 37°C in 5% CO₂ in RPMI containing 5% low-endotoxin fetal calf serum and 1% penicillin-streptomycin for 10 to 14 days. The medium was changed daily to ensure that the cultures were free from contaminating bacterial products or endotoxin. After 5 days, medium containing only microfilariae was carefully removed from the adult worm cultures. Worms were washed extensively in sterile Hanks’ balanced salt solution and frozen at –20°C for future experiments. All procedures were performed with strict aseptic technique and endotoxin-free materials. Culture supernatants con-

---

* Corresponding author. Mailing address: Center for Global Health and Diseases, School of Medicine, W-137, 2109 Adelbert Rd., Case Western Reserve University, Cleveland, OH 44106-4983. Phone: (216) 368-1856. Fax: (216) 368-4825. E-mail: Eric.Pearlman@case.edu.
Preparation of soluble extracts. Soluble extracts were prepared from adult B. malayi obtained from the peritoneal cavity of either untreated Mongolian jirds or animals given tetracycline (1.2% in drinking water for 6 weeks). Worms were washed in RPMI 1640 (Gibco BRL) and endotoxin-free phosphate-buffered saline under sterile conditions. Extracts were processed by homogenization and sonication as previously described (25a). Briefly, worms were finely chopped with a sterile scalpel blade and then sonicated in endotoxin-free phosphate-buffered saline (six 30-s pulses), incubated overnight at room temperature, and centrifuged at 10,000 × g for 30 min at 4°C. The protein concentration of the supernatant was determined with Bradford reagent (Bio-Rad). Soluble extracts were frozen at −80°C until required.

Isolation of Wolbachia organisms from B. malayi. Wolbachia organisms were obtained from adult male and female B. malayi worms as has been previously described (17). An immunofluorescent antibody test was used to assess the purity of the preparation as follows. Bacteria were fixed in methanol for 5 min at 4°C, blocked in 5% bovine serum albumin in phosphate-buffered saline for 15 min at room temperature, and then incubated for 1 h with rabbit polyclonal serum raised against Wolbachia surface protein of Wolbachia organisms in B. malayi (diluted 1:300 in block buffer), washed in Tris-buffered saline–0.1% Tween 20, and incubated for 1 h in goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate (FITC; Zymed; Cambridge Bioscience) diluted 1:100 in block buffer. The slide was counterstained with 0.1% Evans Blue and then viewed by fluorescence microscopy. An aliquot of bacteria was incubated with secondary antibody alone as a negative control.

DNA was extracted from 4 μl of the Wolbachia organism preparation by phenol-chloroform extraction (18). Since the Wolbachia organism of B. malayi has one genome and the wsp gene is present in a single copy (17), the number of wsp copies calculated by quantitative PCR can estimate the number of Wolbachia organisms present in the sample. Therefore, amplification of wsp was performed by real-time quantitative PCR on a DNA Engine Opticon thermal cycler (M. J. Research) with the fluorescent double-stranded DNA-intercalating dye Sybr-Green (Quantitect; Qiagen), as described (17). Samples were analyzed in duplicate, and the copy numbers in the starting templates were calculated by reference to standard curves generated from titrations of known copy numbers of a plasmid that contained the wsp insert.

Injection of Brugia microfilariae into the corneal stroma and immunoelectron microscopy. Microfilariae were isolated from sterile adult B. malayi worm cultures as described above. With a 25-gauge needle, a single abrasion was made in the corneal epithelium of 6-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine), and 2,500 microfilariae in 5 μl of Hank's balanced salt solution were injected into the corneal stroma with a 33-gauge needle. After 4 or 18 h, the mice were sacrificed, and the corneas were dissected and placed into a 1% paraformaldehyde–0.1% glutaraldehyde phosphate buffer fixative solution. Thin sections were immunostained with a 1:700 dilution of polyclonal rabbit antiserum specific for Wolbachia surface protein and visualized with goat anti-rabbit immunoglobulin conjugated to 15-nm gold particles (Amersham). Sections were counterstained with uranyl acetate and lead citrate following standard protocols. Control sections were stained with immunoglobulin secondary alone and counterstained with uranyl acetate and lead citrate in parallel with sample sections, allowing the specificity of gold staining to be determined. In some experiments, mice were immunized with two subcutaneous injections of 100,000 B. malayi microfilariae 1 week apart prior to intrastromal injection. This procedure induces a pronounced T-cell and antibody response (19).
In vitro neutrophil activation. Neutrophil isolation was based on the method described by Gregory et al. (7). C57BL/6 mice received two intraperitoneal injections with 1 ml of 9% casein at 0 h and after 16 h, and mice were sacrificed 3 h later. The peritoneal cavity was lavaged with Hanks’ balanced salt solution, and cells were washed and layered on a 90% Percoll gradient (Pharmacia Biotech, Piscataway, N.J.). After centrifugation (31,000 rpm, 20 min at 4°C), a >95% pure neutrophil population was recovered from the second layer. The cells were washed in RPMI and incubated for 2 h at 37°C in the presence of 50 ng of granulocyte-macrophage colony-stimulating factor or with medium alone. Cells were then stimulated for 16 h with isolated granulocyte-macrophage colony-stimulating factor or with medium alone. Cells were then stimulated for 16 h with isolated Wolbachia organisms or filarial extract, and production of KC, macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor alpha (TNF-α) into the culture supernatants was determined by two-site enzyme-linked immunosorbent assay (RD Systems, Minneapolis, Minn.). The viability of neutrophils was >95% as determined by trypan blue exclusion.

RESULTS

Neutrophils surround microfilariae in the corneal stroma. In individuals infected with O. volvulus, the parasite larvae migrating through the skin can invade ocular tissue and induce an inflammatory response that results in visual impairment and eventual blindness. To characterize the early events of this process after invasion of microfilariae into the cornea, we injected parasites directly into the corneal stroma and examined the response in the tissue in the first 24 h. (As O. volvulus microfilariae cannot be obtained in sufficient numbers for these experiments, we injected microfilariae from a related species, B. malayi, into the corneal stroma. B. malayi is similar to O. volvulus in terms of having Wolbachia organisms and inducing keratitis [24].)

Corneas were dissected 18 h after injection and processed for electron microscopy by standard methods. As shown in Fig. 1, neutrophils were recruited to the corneal stroma after injection of microfilariae and were observed in close proximity to more than 67% of the 34 worms in the fields that we examined. Neutrophils were found to completely surround the microfilariae in the corneal stroma, with neutrophil membranes interdigitated with the nematode cuticle. Corneas of mice that were immunized prior to intracorneal injection of microfilariae (Fig. 1D) were similar in appearance to those of mice not immunized (Fig. 1A to C), indicating that an adaptive immune response is not essential for this process.

Neutrophils ingest Wolbachia organisms in the corneal stroma. To identify Wolbachia organisms in the cornea, sections from three separate experiments totaling nine eyes were immunostained with rabbit antibody to Wolbachia surface protein and detected with anti-rabbit immunoglobulin G conjugated to 15-nm gold particles. Sections were counterstained with uranyl acetate and lead citrate and examined by electron microscopy. (A and B) At 4 h after injection. Wolbachia surface protein was clearly detected inside microfilariae in the corneal stroma (arrows). mf, microfilariae. (C to E) At 18 h after injection, microfilariae containing Wolbachia organisms were surrounded by neutrophils (PMN). Wolbachia surface protein labeled with gold particles (arrows) was present in the microfilariae adjacent to the neutrophils in both unimmunized (C and D) and immunized (E) mice. Magnifications: A, ×4,800; B, ×8,400; C, ×5,300; D, ×16,000; E, ×14,500.

FIG. 2. Proximity of neutrophils to Wolbachia organisms in the nematode hypodermis. The corneal stromata of C57BL/6 mice were injected with microfilariae, corneas were removed after 4 or 18 h, and thin sections were immunostained with anti-Wolbachia surface protein (WSP) and visualized with immunoglobulin G conjugated to 15-nm gold particles. Sections were counterstained with uranyl acetate and lead citrate and examined by electron microscopy. (A and B) At 4 h after injection. Wolbachia surface protein was clearly detected inside microfilariae in the corneal stroma (arrows). mf, microfilariae. (C to E) At 18 h after injection, microfilariae containing Wolbachia organisms were surrounded by neutrophils (PMN). Wolbachia surface protein labeled with gold particles (arrows) was present in the microfilariae adjacent to the neutrophils in both unimmunized (C and D) and immunized (E) mice. Magnifications: A, ×4,800; B, ×8,400; C, ×5,300; D, ×16,000; E, ×14,500.
kines are important in onchocerca keratitis (9, 24) and that neutrophils can produce CXC chemokines (5, 6), we determined whether Wolbachia organisms induce neutrophil production of chemotactic and proinflammatory cytokines. A 95% pure population of neutrophils was derived from peritoneal exudate cells as described in Materials and Methods and incubated with either Wolbachia organisms isolated from filarial worms, filarial extracts containing Wolbachia organisms, or filarial extract depleted of Wolbachia organisms by antibiotic treatment. Culture supernatants were collected after 18 h of incubation in the presence or absence of granulocyte-macrophage colony-stimulating factor, and production of KC, MIP-2, and TNF-α was measured by enzyme-linked immunosorbent assay.

As shown in Fig. 4, isolated Wolbachia organisms stimulated neutrophil production of TNF-α, MIP-2, and KC in a dose-dependent manner that was greatly enhanced if neutrophils were first incubated with granulocyte-macrophage colony-stimulating factor. Similarly, incubation with filarial extract containing Wolbachia organisms stimulated production of these cytokines at a level similar to that induced by 1,200 Wolbachia organisms. In contrast, the soluble extract from antibiotic-treated parasites induced significantly lower tumor necrosis factor alpha, MIP-2, and KC production, indicating that production of these cytokines is induced by Wolbachia endobacteria rather than by filarial antigens.

**DISCUSSION**

The findings from the present study reveal the early inflammatory response after microfilariae containing Wolbachia endobacteria are introduced into the cornea and are likely indicative of degenerating *O. volvulus* microfilariae in the cornea, which release Wolbachia organisms. Neutrophils recruited from peripheral, limbal vessels surround the microfilariae and may contribute to their degeneration. The presence of Wolbachia surface protein in neutrophil granules in the cornea is consistent with their ingestion by neutrophils, and our findings that neutrophils produce chemotactic and proinflammatory cytokines in response to Wolbachia organisms indicate that neutrophils in the cornea are activated in response to Wolbachia stimuli. Although not demonstrated in the current study, the release of cytotoxic neutrophil mediators such as nitric oxide, oxygen free radicals, and matrix metalloproteinases is likely to have a cytotoxic effect on resident cells that maintain corneal clarity, contributing to visual impairment and eventual blindness (27).

Neutrophils in the corneas of chronically infected individu-
als have been described (20) and are predominant in onchocerca skin abscesses (8, 28). Furthermore, the number of neutrophils in nodules from doxycycline-treated patients (depleted of Wolbachia organisms) is significantly lower than that in nodules from untreated individuals (containing Wolbachia organisms) (3), indicating that neutrophils migrate to the tissue in response to Wolbachia organisms. Consistent with that observation, treated nodule extracts have decreased neutrophil chemotactic activity compared with nodule extracts from untreated individuals (3).

Although eosinophils are a hallmark of filarial and other helminth infections and are recruited to the cornea in onchocerca keratitis (1, 10, 21), we demonstrated that development of corneal opacification is most strongly associated with the presence of neutrophils. Inhibiting recruitment of neutrophils but not eosinophils to the corneal stroma results in significantly reduced corneal disease (9, 14).

Our previous studies demonstrated that development of an adaptive immune response is essential for interleukin-5 and eosinophil production and enhances neutrophil and eosinophil recruitment to the corneal stroma (22, 23). However, we have also shown that neutrophils are recruited to the corneal stroma in the absence of an adaptive immune response and that this is dependent on the presence of Wolbachia organisms (24). In the present study, neutrophils were found to infiltrate the cornea and surround the parasites in immunized and nonimmunized mice, indicating that an adaptive immune response is not essential for this process.

A specific adaptive immune response is also not required for Wolbachia-induced cytokine production by neutrophils, as neutrophils isolated from unimmunized animals produced tumor necrosis factor alpha and the CXC chemokines MIP-2 and KC in response to Wolbachia organisms. These findings are consistent with our previous observations that CXC chemokine-receptor interactions are important in onchocerca keratitis (9) and with reports that Wolbachia surface protein induces interleukin-8 production by neutrophils (2). In the present study, it is likely that chemokine production by neutrophils themselves led to further recruitment of neutrophils, thereby perpetuating the inflammatory response.

In summary, the results of the present study add to our previous observations (with soluble parasite extracts) by examining an earlier stage in the pathogenesis of onchocerca keratitis consistent with the following sequence of events: microfilariae invade the cornea, where they eventually die and release Wolbachia endobacteria into the confined environment of the corneal stroma; Wolbachia organisms and parasite antigens stimulate resident corneal fibroblasts to produce chemotactic cytokines that mediate initial neutrophil recruitment; infiltrating neutrophils surround the microfilaria, possibly contributing to their degeneration; ingestion of Wolbachia organisms by neutrophils stimulates production of chemokines and proinflammatory cytokines, which mediate further neutrophil recruitment and activation; and the release of cytotoxic neutrophil mediators such as nitric oxide, oxygen free radicals, and matrix metalloproteinases has a cytotoxic effect on resident cells that maintain corneal clarity, contributing to visual impairment and eventual blindness.

ACKNOWLEDGMENTS

This work was supported by NIH grants EY10320 (E.P.), AI07024 (L.G.-F.), K08 AI054652 (A.G.H.), and EY11373 and by the Research to Prevent Blindness Foundation and the Ohio Lions Eye Research Foundation. M.J.T. thanks the Wellcome Trust for Senior Fellowship support and the EC (ICA4-CT2002-10051).

REFERENCES

11. Hoerauf, A., K. Nissen-Pahle, C. Schmetz, K. Henkle-Duhrsen, M. L. Blax-
ter, D. W. Buttnner, M. Y. Gallin, K. M. Al-Quond, R. Lucius, and B. Fleis-
symbiont Wolbachia of Dirofilaria immitis and Brugia pahangi using a poly-
19. Mehlora, R. K., L. R. Hall, A. W. Higgins, I. A. Dreshaj, M. A. Hazhiu, J. W. Kazura, and E. Pearlman. 1998. Interleukin-12 suppresses filaria-induced pulmonary eosinophilia, deposition of major basic protein and airway hyper-
25. Taylor, M. J. 2000. Wolbachia bacteria of filarial nematodes in the patho-
25a. Taylor, M. J., H. F. Cross, and K. Bilo. 2000. Inflammatory responses induced by the filarial nematode Brugia malayi are mediared by lipopolysac-
29. Wiiko-Sarsat, V., P. Rieu, B. Descamps-Latscha, P. Lesavre, and L. Halb-
wachs-Mecarelli. 2000. Neutrophils: molecules, functions and pathophysio-

Editor: W. A. Petri, Jr.