No Depletion of Wolbachia from *Onchocerca volvulus* after a Short Course of Rifampin and/or Azithromycin

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Abstract. Endosymbiotic Wolbachia bacteria inside adult *Onchocerca volvulus* worms (causing river blindness) are necessary for female worm fertility. We evaluated whether rifampin and/or azithromycin used in a five-day course could kill Wolbachia. In an open-label trial in Guatemala, 73 patients with 134 palpable onchocercal nodules were randomized into four treatment groups: rifampin, azithromycin, a combination of the two drugs, and controls (multivitamins). After five days of antibiotic treatment, all participants received a single dose of ivermectin on day 6. Nine months after treatment, the nodules were removed and the worms were examined. Skin snips to determine microfilariae were obtained at baseline and nine months. There were no significant differences between any of the treatment groups in the condition of the worms in the nodules, the presence of Wolbachia surface protein, or the number of microfilariae in skin. Short courses with these antibiotics will not clear Wolbachia from *O. volvulus*.

INTRODUCTION

River blindness (onchocerciasis) is caused by an infection with the vector-borne filarial parasite *Onchocerca volvulus*. The microfilariae, released by female worms that reside in fibrous subcutaneous nodules, provoke inflammatory reactions that can cause skin and eye damage. The World Health Organization estimates that 123 million people are at risk of onchocerciasis in an estimated 37 countries in Africa, Yemen, and the Americas.1,2 Ivermectin (Mectizan®) is a potent oral microfilaricidal drug being donated by Merck & Co. (Rahway, NJ) and is now the mainstay of current efforts to control onchocerciasis. Mass drug administration (MDA) programs provide annual or semiannual single dose ivermectin treatment to more than 50 million persons each year.10 However, because ivermectin does not kill the adult worms, annual MDA programs will most likely need to be sustained indefinitely, which is a major challenge for ministries of health and the global public health community. A safe oral medication that could kill or permanently sterilize adult worms (the so-called microfilaricide) would circumvent the sustainability problem and promote the goal of ultimate elimination of the parasite.1,4–6

The recognition of the importance of the rickettsia-like Wolbachia endobacterium to *O. volvulus* fertility has provided a new and promising target in the search for a microfilaricidal drug.7–11 By unknown mechanisms, these intracellular bacteria are required for female worm fertility, and perhaps worm survival itself. A treatment regimen of 100 mg of doxycycline per day for six weeks eliminates Wolbachia from more than 90% of the worms, and is associated with arrested embryogenesis that is believed to be permanent.7 However, less than six weeks of treatment with doxycycline does not appear to be sufficient to obtain these effects.10,11 Shorter antibiotic courses to treat Wolbachia are needed if this approach is to be used in MDA programs.

It is known that Wolbachia are sensitive to the rifampins and azolides.12,13 Rifampin and azithromycin have excellent tissue penetrance and good activity against intracellular pathogens. In addition, they have excellent safety profiles and (unlike doxycycline) may be used in pregnancy and in young children.14 Accordingly, we undertook a study in an onchocerciasis-endemic village of Guatemala to evaluate the efficacy of rifampin and/or azithromycin against Wolbachia in five-day treatment courses that could feasibly be given via MDA programs in Latin America.

MATERIALS AND METHODS

This open label trial was carried out in Finca Santa Isabel, a rural coffee growing plantation in the Department of Suchitepequez, located in the central onchocerciasis-epidemic zone in Guatemala. This finca is currently receiving semiannual ivermectin MDA. The study was reviewed and approved by the Institutional Review Board (IRB) of the Centers for Disease Control and Prevention (CDC-NCID-3843, http://clinicaltrials.gov), the Ethical Review Committee of the Universidad del Valle, Guatemala, and the Guatemalan Ministry of Health (MOH).

After obtaining informed consent from all adult participants and a parent or guardian of all participating children, 82 persons resident of the community and having at least one palpable nodule characteristic of an onchocercoma (nontender, mobile, soft, flat, 0.5–2.0 cm diameter, and not in a lymphatic chain) were enrolled in the study in August 2003. All patients reported having received a dose of ivermectin in February 2003, six months before enrollment, as a part of the ongoing semiannual mass treatment by the Onchocerciasis Elimination Program of the MOH. Excluded were children less than five 5 years of age; persons with a history of allergy or other adverse reaction to ivermectin, azithromycin, or rifampin; use of medication that might interact with any of the study medications; and/or clinical evidence of liver disease, active tuberculosis, alcoholism or stated refusal to accept nodulectomy at the end of the study. At the behest of the CDC IRB, we also excluded females who were pregnant (on the basis of a urine pregnancy test result) or lactating from the study.

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On day 1, the locations and sizes of all suspected onchocercal nodules were recorded using a standard body atlas diagram, and quantitative superficial skin biopsies for *O. volvulus* microfilariae (mf) were obtained. The skin biopsy specimens (snips) were obtained from the left shoulder and the left iliac crest using a 2.0-mm corneoscleral punch. Between sampling each patient, the instruments were washed sequentially with full-strength bleach (30 seconds), 95% ethanol (30 seconds), distilled water (30 seconds), 95% ethanol (30 seconds), and then air-dried. The skin snips were placed in polystyrene microtiter plates with 0.1–0.2 mL of normal saline, covered, and incubated for 24 hours at room temperature. Skin snips were removed from the wells after 24 hours and placed in corresponding wells of another microtiter plate containing 2% formalin. Two drops of 2% formalin were also added at that time to the fluid in each original well to fix any mf that were present. Skin snips were labeled in a blinded fashion with the code remaining unbroken until the laboratory work and data analysis were completed.

The participants were randomized into three treatment groups and a control group. Children ≤ 10 years of age were randomized separately because they were likely to have younger *O. volvulus* worms exposed to less ivermectin treatment. Treatment groups received a single daily dose of medication on days 1–5. The first group (RIF) of 21 patients received a five-day course of rifampin (Rifampicina; Macleods Pharmaceuticals Ltd., Mumbai, India), 20 mg/kg orally (maximum 600 mg/day). The second group (AZT) of 21 received a five-day course of azithromycin (Zithromax®; Pfizer Ltd., New York, NY), 12 mg/kg orally (maximum 500 mg/day). The third group (COMB) of 20 received a five-day course of rifampin, 20 mg/kg (maximum 600 mg/day) and azithromycin 12 mg/kg (maximum 500 mg/day), administered orally and simultaneously. The fourth group of 20 served as a control group and was given a five-day course of one multivitamin tablet per day. The day after the last day of treatment (day 6), all participants received a single oral dose of ivermectin (Mectizan®; Merck & Co.) (150 μg/kg). All treatments were directly observed. The patients were monitored for any complaints or problems daily for days 1–6 and for three days thereafter (days 7–9) by physicians (B.A. and C.B.). In such cases, results from a focused history and physical examination were recorded and treatment was offered free of charge when appropriate.

All other eligible members of Santa Isabel were offered ivermectin treatment on days 7–9, and those with nodules who were not enrolled were offered nodulectomy at that time. The 82 enrollees agreed to forego nodulectomy for nine months, and not to take the next six monthly doses of ivermectin when offered by the MOH in February 2004. This procedure was needed to enable worms to recover from the well-described 4–6-month halt in uterine mf release provoked by ivermectin prior to nodulectomy.16–18 Study monitors and authors (B.A. and C.B.) were present in the community when the six monthly ivermectin treatments were offered to remind drug study participants not to take ivermectin at that time.

In May 2004, nine months after their antibiotic course was completed, nodules that were previously recorded on entry to the study (e.g., that had received drug exposure) were removed under local anesthesia by experienced MOH medical personnel using standard techniques.19 No participant refused nodulectomy, which was widely practiced in Guatemala by the MOH as a control measure for onchocerciasis a decade ago and remains welcomed by the population. Immediately after removal, each nodule was placed in a capped bottle and fixed in 20 times its volume of a mixture of 70% ethyl alcohol, 10% glycerol, and 20% distilled water. The fixative was changed after 24 hours and nodules were stored until sectioning. Two skin biopsy specimens for *O. volvulus* mf were obtained again from the left shoulder and left iliac crest using the same methods as before. The nodules and skin snips were labeled in a blinded fashion with the code remaining unbroken until the laboratory work and data analysis were completed. The patients were all treated with ivermectin (the missed six-month dose), and for three days afterward they were seen daily (by B.A. or C.B.) for any complaints or problems.

**Laboratory analysis.** The plates with skin snips were transported to the laboratory in Guatemala City where the skin snips were blotted dry on smooth filter paper and weighed individually on an analytical balance. The fluid was examined directly with an inverted microscope at 400× and all visible mf were counted. Geometric mean microfilarial skin densities (mmfd) were calculated for each treatment group using the formula mmfd = anti-log ([(Σlog(x + 1))/n] – 1, where x is mf/mg of skin and n = the number of people in the respective treatment group.

Each preserved nodule was bisected; half of the nodule was embedded in paraffin wax and 6-μm sections were cut from the face of the cut surface. Three hematoxylin and cosin–stained sections were evaluated by two independent observers (J.G. and M.E.) for fertility (presence of mf in the female reproductive tract) and vitality of female worms (live worms were defined as those that had distinctly stained cytoplasmic and nuclear features; dead worms stained only with eosin) following a classification system developed by Duke and others.20,21 Presence of *Wolbachia* antigens in adult *O. volvulus* worms was studied using an immunohistochemical assay that processed in the DAKO autostainer (DakoCytomation, Carpinteria, CA). Sections were deparaffinized and incubated for 20 minutes with a monoclonal antibody specific for *Wolbachia* surface protein (WSP) that was produced at CDC (unpublished data). Colorimetric detection of the antigen antibody reaction was performed using the LSAB2 universal alkaline phosphatase kit (Dako, Glostrup, Denmark). Positive controls were formalin-fixed adult male and female *Brugia pahangi* worms (obtained from the Filariasis Repository at the University of Georgia, Athens, GA). A negative control for each section consisted of the sequential section incubated with an unrelated isotype control antibody instead of antibody to WSP. Three sections and controls were examined by two independent observers (G.P. and M.E.) and read semiquantitatively (negative, 1+, 2+, 3+) for the presence of WPS stain in the reproductive organs and lateral cords of female worms.

**Statistical analysis.** The study design and sample size would detect a 75% decrease in *Wolbachia* or worm vitality in one of the antibiotic groups compared with control (ivermectin alone), with type I error of 1% and a power of 80%. This expectation was based on previous reports of doxycycline impact on *Wolbachia*.7,9 Poisson regression (SAS version 9.1 Proc Genmod; SAS Institute, Cary, NC) was used to investigate the effect of various treatment groups on *Wolbachia* (WSP) in living female worms, vitality of worms, and the
presence of mf in the reproductive organs of the female worms. Because patients frequently contributed more than one nodule, the regression implemented the generalized estimating equations procedure to adjust for the lack of independence between observations. Wilcoxon signed-rank test was used to compare mmfd between groups. Chi-square and Fisher’s exact test was used to compare proportions. A P value < 0.05 was considered significant.

RESULTS

Seventy eight (95%) of the 82 persons enrolled completed the protocol. The four patients who dropped out (two women, one man, and one eight-year-old girl) successfully completed the treatment phase, but left Santa Isabel during the nine-month follow-up period and could not be found for follow-up. A total of 139 study nodules were removed from the remaining 78 patients in May 2004; however, five nodules (from five patients) were found to not be onchocerual nodules on histologic examination, but benign lipomas (2) and neuromas (3). These five patients were excluded from the final analysis, leaving 73 patients: 17 in the rifampin group (RIF), 20 in the azithromycin group (AZT), 20 in the combination rifampin and azithromycin group (COMB), and 16 in the control group. The results from these 73 are summarized in Table 1. Eighty-five percent were male, with a median age of 12 years (range = 5–61 years). Of the 134 onchocerual nodules, 41 were from the RIF patients, 32 from AZT patients, 31 from COMBO patients, and 30 from controls. There were no statistically significant differences between groups at the start of the study. No adverse experiences were recorded during the administration of study antibiotics (days 1–5). Typical and mild Mazzotti reactions (headache, rash, edema) occurred in approximately 20% of patients 24–48 hours after each of the ivermectin doses. These reactions were treated with analgesics and antihistamines and promptly resolved.

Wolbachia. Eighty seven (65%) of the 134 nodules examined contained only live female worm segments and so were included in the WSP analysis. We excluded the seven (6.2%) nodules that contained both live and dead worm segments from concern that these segments might be from the same coiled degenerating female worm, and that degeneration might influence the presence of WSP in other sections of the worm’s histologic examination. Overall, 93.1% of living female worms contained WSP (Figure 1), and less than 10% of positive samples were weakly staining (1+). Combined reproductive and lateral cord WSP results ranged from a low of 85.7% in the RIF group to 100% in the AZT and COMB groups, but these differences were not statistically significant when analyzed qualitatively and semiquantitatively.

Histologic analysis. Overall, 26.1% of nodules contained only dead worms (range = 20–31.3%). Male worms were found in 27.6% of nodules (range = 21.9–40%); 97% of male worms were living, and 94% of those that could be assessed contained Wolbachia. Differences between groups were not statistically significant. As in the WSP analysis, we limited our analysis for reproductive activity to the subset of 87 nodules that contained only live female worm segments. Overall, 27.6% of female worms were fertile, ranging from 25% (RIF) to 33.3% (AZT) and differences between groups were not statistically significant.

Microfilariae in skin. There were no statistically significant differences between treatment groups in prevalence of mf in skin or the mmfd at the start of the study or at 9 months. However, the overall prevalence increased significantly between the start (mean = 15.1%) and end (28.8%) of the study. Similarly, the mmfd significantly increased overall (from 0.14 mf/mg skin to 1.25 mf/mg skin) and in RIF and COMB groups (Table 1).

DISCUSSION

The recognition that antibiotics can be used to kill Wolbachia and permanently sterilize O. volvulus meant that for the first time patients with onchocerciasis could be essentially cured of their infections (because morbidity is related to mf

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* RIF = rifampin; AZT = azithromycin; NS = not significant; mf = microfilariae; mmfd = geometric mean microfilarial density.
† Calculated using only nodules with only live females (n = 87).
‡ Significant (P < 0.05) compared with initial mf prevalence or mmfd using paired tests for year-to-year comparisons.
production) with one course of safe oral treatment. However, delivering prolonged doxycycline treatment courses within current onchocerciasis programs that use mass community annual or semiannual single dose drug administration is at best challenging and at worst impossible. The longest successfully deployed MDA effort in the Americas has been five days of chloroquine and primaquine in past malaria eradication efforts. A further complication to large-scale doxycycline deployment is that the medicine cannot be given to children less than eight years of age or to pregnant women. The discovery of a short treatment course for Wolbachia using safer antibiotics would provide a programmatically feasible approach to efforts in the Americas, where the goal to eliminate transmission has been embraced by national programs. Large-scale treatment for Wolbachia would result in considerably less time to regional elimination compared with current estimates of 6–14 years of twice per year ivermectin treatments.

Unfortunately, our study in Guatemala found that five days of rifampin and/or azithromycin, the best commercially available alternatives to doxycycline, will not clear Wolbachia from O. volvulus worms. Our study was adequately powered to detect the 94% rate of clearance of Wolbachia from female worms (examined after treatment with six weeks of doxycycline) reported by Hoerauf and others. Our results in all treatment groups replicated the findings of the untreated control group of Hoerauf and others, in which 93% of living female worms had Wolbachia.

The only statistically significant finding in our study was in the increase between baseline and the nine-month measurement of skin mf prevalence and mmfd. This finding is best explained by the temporary fertility loss caused by ivermectin on O. volvulus female worms that lasts approximately six months. The baseline mf counts were measured six months after ivermectin treatment, whereas the second (higher) observation occurred nine months after ivermectin treatment. Our study design predicted that stable or increased microfilarial parameters at nine months could in fact be interpreted as a lack of antibiotic effect on Wolbachia.

A report by Cupp and others studied nodules from different Guatemalan fincas under prolonged ivermectin therapy and noted significantly lower numbers of male worms (19.7% of nodules) obtained in 2001 compared with historical Guatemalan controls (73% of nodules). They speculated that male O. volvulus worms have a heightened sensitivity to macrofilaricidal ivermectin effects compared with female worms. Our findings that only 27.6% of nodules examined having male worms seem to reflect these findings that there is a paucity of male worms. However, because of the limited number of sections examined and the small size of coiled male worms, our data are only suggestive and do not provide confirmation of these observations.

Further research in short-course treatment of Wolbachia is needed. Rifampin and/or azithromycin in combination with doxycycline should be evaluated, as should newer long-acting rifamycins (rifapentine), or combinations of antibiotics with anthelmintics (such as ivermectin and/or albendazole). Identifying new antibiotics more active against Wolbachia should also be undertaken. In the meantime, prolonged doxycycline treatment courses could be used now as an adjunctive end-game strategy after large onchocerciasis programs have operated with ivermectin distribution for many years. Such a strategy would require identification of the few persons still infected, using a diagnose and treat approach rather than provision of presumptive mass treatment. Rapid diagnostic tests (e.g., antigen detection) would need to be developed and deployed to allow a point of care determination of patients with active infections. Treatment could be delivered following models developed for administering prolonged treatment courses in rural areas of developing countries (e.g., directly observed therapy for treatment of tuberculosis). Wolbachia treatment using doxycycline in this way could accelerate elimination efforts in the Americas and Africa, halt recrudescence if it is identified in the countries in West Africa in the former Onchocerciasis Control Program, or slow ivermectin resistance should it emerge.

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