LACK OF RESISTANCE AFTER RE-EXPOSURE OF CATTLE CURED OF ONCHOCERCA OCHENGII INFECTION WITH OXYTETRACYCLINE

CHARLES K. NFON, BENJAMIN L. MAKEPEACE, LEO M. NJONGMETA, VINCENT N. TANYA, AND ALEXANDER J. TREES

Liverpool School of Tropical Medicine and Faculty of Veterinary Science, University of Liverpool, Liverpool, United Kingdom; Institut de Recherche Agricole pour le Développement, Wakwa, Cameroon

Abstract. Although vector control and ivermectin chemotherapy have led to a dramatic reduction in the incidence of river blindness (onchocerciasis), there is a consensus that additional control tools are required to sustain and extend this success. The recognition of endosymbiotic bacteria (Wolbachia) in filariae and their targeting by antibiotics constitutes the most significant and practicable opportunity for a macrofilaricidal therapy in the short-to-medium-term. Using Onchocerca ochengi in cattle, an analog of human onchocerciasis, we have previously shown that oxytetracycline is macrofilaricidal, and protective immunity exists naturally in a subset of animals termed putatively immune. Here, we report that although 24 weeks of weekly oxytetracycline treatment eliminated adult worms, cured animals remained susceptible to re-infection by natural challenge when compared with putatively immune cattle. However, their susceptibility was not significantly different from that of concurrently exposed, heavily infected animals. Thus, cattle cured by oxytetracycline are neither hypo-susceptible nor hyper-susceptible.

INTRODUCTION

After the Conference on the Eradicability of Onchocerciasis held in Atlanta in 2002, there is a current consensus among researchers and public health professionals that Onchocerca volvulus cannot be eradicated using existing control tools. The obstacles to eradication in Africa include the necessity for decades of annual treatment with ivermectin (since this drug kills microfilariae but not the long-lived adult worms and does not abrogate transmission), re-invasion of post-control areas by infected blackflies, logistical and economic challenges to sustained ivermectin distribution, human migration and civil unrest, severe adverse events after ivermectin treatment in individuals heavily infected with Loa loa, and reduced responsiveness to ivermectin in some populations that may indicate the development of resistance. Although ivermectin, either after or independently of vector control, has been extremely effective in abating the public health impact of onchocerciasis, eradication will require a macrofilaricidal or embryostatic drug that is suitable for mass administration (since suramin and arsenical drugs are too toxic in this context) and/or a vaccine.

In the late 1990s, tetracycline was found to have sub-lethal effects (prophylaxis and embryostasis) against filarial worms in rodent models. Subsequently, oxytetracycline was evaluated in a prolonged intermittent regimen against Onchocerca ochengi in cattle and exhibited potent macrofilaricidal activity. This reinforced the potential of antibiotics for onchocerciasis control because O. ochengi is the closest extant relative of O. volvulus and a well-established tertiary drug screen for anti-filarial compounds. Moreover, field trials in Ghanaian onchocerciasis patients have demonstrated that doxycycline can induce long-term sterilization of adult female O. volvulus, although at least four weeks of continuous daily therapy is required. Numerous in vitro and in vivo studies support the hypothesis that antibiotics act against filarial nematodes in a predominantly indirect manner by disruption of intracellular bacterial symbionts of the genus Wolbachia (see review by Taylor and others). There are several helminthiases for which the existing chemotherapy has serious limitations in terms of efficacy, tolerance, or the prospect of emerging resistance. Conversely, there are no anthelminthic vaccines available for human use, and even the best candidate vaccines under development are extremely unlikely to induce sterile immunity. For these reasons, integrated control combining chemotherapy and vaccination has been advocated for both schistosomiasis and hookworm. Although vaccine research for onchocerciasis has not progressed as far as for these other helminthiases, a recent field evaluation of an irradiated L3 vaccine established proof-of-principle in cattle because immunized animals showed considerable resistance to natural challenge with O. ochengi.

A key question to be addressed in the context of integrated control for onchocerciasis is does chemotherapy facilitate, hinder, or have no effect on protective immunity? Ivermectin treatment restores Th1-type responses to parasite antigen in patentently infected humans, although whether this enhances immunity to L3 challenge is not clear. In addition, there is epidemiologic evidence from the 1960s that indicates that macrofilaricidal therapy of onchocerciasis patients using suramin fails to induce immunity to re-infection. However, because antibiotics target the Wolbachia endosymbionts of filarial parasites (i.e., operate in a fundamentally different manner from conventional anthelminthics), and Wolbachia modify immune responses in the mammalian host, the sequelae of treatment could be quite distinct from those observed with other drugs. Therefore, in the bovine O. ochengi system, we sought to investigate the interaction between chemotherapy and immunity in the specific case of oxytetracycline, administered in a macrofilaricidal regimen. The susceptibility of antibiotic-treated cattle was compared with that of putatively immune (PI) animals (which are naturally resistant to challenge as previously demonstrated); animals treated with the arsenical macrofilaricide, melarsomine (which are fully susceptible to re-infection); and patentently infected control cattle.
MATERIALS AND METHODS

Field site. The field site has been described in detail elsewhere. Briefly, the herd was assembled at the Institut de Recherche Agricole pour le Développement, Regional Center of Wakwa, located in the Adamawa Province of northern Cameroon. At this site, the transmission of *O. ochengi* is negligible. For continuous exposure to high levels of natural challenge, the entire herd was moved approximately 10 km to pasture bordering the Vina du Sud River, which has numerous breeding sites for *Simulium squamosum*, the local vector of *O. ochengi*. In this hyper-endemic area, the annual transmission potential for *O. ochengi* has been estimated as 74,000 L$_v$/animal.

Animals, parasitology, and ethical considerations. Female Gudali-breed cattle (*Bos indicus*) > 5 years old on the basis of dentition and general physical condition were obtained from ranches alongside the Vina du Sud River. Putatively immune cattle were identified by absence of *O. ochengi* parasitosis in conjunction with evidence of exposure to infection as previously described. The herd was divided into four groups (identified hereafter by the abbreviations listed in Table 1) in which all animals (except PI) had ≥ 9 nodules each. Parasitologic observations (performed at predetermined intervals) entailed the enumeration of intradermal nodules by palpation and quantification of microfilaridermia by microscopic examination of ventral skin biopsy specimens, as previously detailed. Individual nodules were permanently marked in situ using tattoo ink and their position was recorded on a hide map for each animal; consequently, at every time point nascent nodules could be distinguished from those present during previous examinations. If necessary for parasitologic analysis, nodules were surgically removed under local anesthesia. Although Cameroon has no national legislation to regulate the experimental use of animals, procedures conducted at the field site conformed with those authorized by a Home Office Project Licence [Animals (Scientific Procedures) Act 1986] for experiments performed in the United Kingdom.

Chemotherapy. Chemotherapy was conducted during the pre-exposure phase. Animals in the Mel group received a regimen of melarsomine hydrochloride (Cymeralsan®; Merrial, Lyon, France) with previously verified macrofilaricidal activity; the dose was 4 mg/kg administered by slow intravenous injection every other day for three days. Cattle in the Tet group were treated with a long-acting formulation of oxytetracycline (Terramycin® L.A., Pfizer, Tadworth, United Kingdom) administered weekly at a dose of 20 mg/kg by intramuscular injection for 24 weeks. The interval between termination of chemotherapy and exposure of the animals was 5 months for the Mel group and 4.5 months for the Tet group. Infected control (IC) and PI animals did not receive chemotherapy.

Statistical analysis. Statistical analyses were performed with SPSS 13.0 software (SPSS Inc., Chicago, IL). Parasitologic time-course data [normalized by the log$_{10}$ (x + 1) transformation] were analyzed by the repeated measures function of general linear model, with time (months of exposure) as the within-subjects factor and group as the between-subjects factor. The interaction between time and group was analyzed by polynomial contrasts, and the significance of differences between groups was determined by Tamhane’s T2 post-hoc test. Pairs of means were also compared between time points within a group using paired t-tests. Group differences in median time to first development of parasitosis were assessed using the Mann-Whitney U test with exact significance, whereas frequency data (prevalence) were analyzed using Fisher’s exact test. In all analyses, a critical probability $P < 0.05$ was considered significant.

RESULTS

Filaricidal activity of oxytetracycline and melarsomine. Oxytetracycline and melarsomine exhibited potent macrofilaricidal activity (Table 1 and Figure 1A). For both drugs, the overall reduction in mean nodule loads as a result of chemotherapy was significant relative to the IC group ($P < 0.05$), as was the rate of change in mean nodule load (linear contrast, $P < 0.001$). At time 0 (5 months after melarsomine treatment and 4.5 months after termination of oxytetracycline therapy), a subset of five nodules each was removed from the Mel and Tet groups. These contained fragmented worm remnants, or in the case of a single nodule from the Tet group, a moribund but intact female. However, complete elimination of all palpable nodules was not apparent until 16 months post-exposure (mpe) for the Tet group (Figure 1A); 10 nodules persisted in one animal from the Mel group at the termination of the experiment, although these contained fragments of dead worms only.

Macrofilaricidal chemotherapy also resulted in a gradual decrease of microfilariae (Table 1 and Figure 2), which was

<table>
<thead>
<tr>
<th>Group*</th>
<th>Time (months)</th>
<th>Nodules (per animal)</th>
<th>Microfilariae (per 100 mg of skin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC, n = 5</td>
<td>−12</td>
<td>24.7 (17–49)</td>
<td>3836.7 (1,778–5,986)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>27.2 (20–50)</td>
<td>2,433.8 (902–6,253)</td>
</tr>
<tr>
<td></td>
<td>+21</td>
<td>96.2 (53–153)</td>
<td>444.5 (80–4,452)</td>
</tr>
<tr>
<td>PI, n = 5</td>
<td>−12</td>
<td>0.0 (0–0)</td>
<td>0.0 (0–0)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.4 (0–2)</td>
<td>0.0 (0–3)</td>
</tr>
<tr>
<td></td>
<td>+21</td>
<td>2.2 (0–7)</td>
<td>1.6 (0–279)</td>
</tr>
<tr>
<td>Mel, n = 4</td>
<td>−12</td>
<td>14.8 (9–39)</td>
<td>852.7 (0–280)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2.4 (0–2)</td>
<td>74.6 (0–16)</td>
</tr>
<tr>
<td></td>
<td>+21</td>
<td>18.7 (0–59)</td>
<td>156.3 (0–1,269)</td>
</tr>
<tr>
<td>Tet, n = 6</td>
<td>−12</td>
<td>33.6 (21–64)</td>
<td>1814.1 (283–4,904)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2.7 (0–10)</td>
<td>25.8 (0–830)</td>
</tr>
<tr>
<td></td>
<td>+21</td>
<td>25.9 (7–68)</td>
<td>210.7 (1–943)</td>
</tr>
</tbody>
</table>

* IC = infected control group; PI = putatively immune group; Mel = melarsomine-treated group; Tet = oxytetracycline-treated group.
statistically significant relative to the IC group for both drugs up to 12 mpe (linear contrast, \( P < 0.001 \)). Accordingly, the overall mean microfilarial loads were significantly lower in the chemotherapy groups compared with the IC group up to 12 mpe (\( P < 0.01 \)), at which point microfilariae became undetectable in most treated animals (Figure 2).

**Susceptibility to re-infection.** The mean nodule load in the IC group increased significantly during the pre-exposure period (Table 1 and Figure 1A; \( P = 0.014 \)), presumably as a result of the maturation of previously acquired \( L_4 \) larvae coupled with low-level transmission of \( L_3 \) at Wakwa. After transfer to the Vina du Sud River, nascent nodules were first detected in the IC group at 6 mpe (Figure 1B), and the geometric mean nodule burden in these animals increased by 17 during the full period of exposure (Figure 2). In the Tet group, new nodules were detected at 6 mpe (Figure 1B), and the median time to nodule appearance was significantly shorter in these animals compared with the Mel and PI groups (\( P < 0.05 \)). Furthermore, the prevalence of nodules was significantly greater in the treated groups than in the PI group at 9 and 12 mpe (\( P < 0.01 \)), and this was related to a significantly faster rate of increase in nodule load from 6 mpe until the termination of the experiment (Figure 1B; linear contrast, \( P = 0.001 \)). Thus, the Tet group was fully susceptible to re-infection, attaining a significantly higher mean nodule burden than the PI group between 6 and 21 mpe (Figure 1B, \( P < 0.001 \)) and a significantly higher mean microfilarial load between 12 and 21 mpe (Figure 2, \( P = 0.015 \)). However, there was no evidence that cattle in the Tet group were any more susceptible to infection than animals in the IC group because there were no significant differences between these groups in mean nodule loads or the rate of increase in nodule acquisition during the overall period of exposure (Table 1 and Figure 1B).

**DISCUSSION**

In previous studies, we have established that oxytetracycline induces macrofilaricidal effects (60–100% efficacy) against *O. ochengi* when administered as monthly long-acting
IM injections over six months, with or without an initial loading dose of daily intravenous treatment. However, in the current experiment, we evaluated a weekly intermittent regimen for the first time, and this amounted to at least twice the total dose of oxytetracycline compared with our earlier trials, and therefore might have been expected to accelerate the killing of adult worms. Although in the present study we did not conduct a detailed analysis of worm viability (limiting our observations to nodule resolution), by comparisons with our previous experiments there was no evidence that the weekly regimen caused a more rapid disappearance of nodules relative to monthly or daily plus monthly chemotherapy because substantial reductions in nodule diameter were apparent from six months post-treatment in all cases. Nonetheless, it is possible that weekly treatment eliminated the target of antibiotic chemotherapy (i.e., Wolbachia endosymbionts) at an increased rate compared with alternative regimens, but that nodule clearance is an intrinsically slow process. If so, the weekly dosing could have been terminated after a much shorter period of time with an ultimately equivalent outcome. In this context, the comparably slow resolution of nodules after melarsomine treatment is noteworthy because arsenical drugs have a direct mode of action on filarial worms, which is different from the indirect mechanism exhibited by antibiotics.

A failure of macrofilaricidal therapy to induce immunity to re-infection has been previously reported for *O. ochengi*-infected cattle treated with melarsomine and in human onchocerciasis patients who had been cured using suramin, a drug that targets the carbohydrate and folate metabolism of the worm. Thus, the indirect activity of tetracycline on filariae does not appear to generate an enhanced immune response compared with drugs that operate on the worm itself, irrespective of the gradual elimination of Wolbachia endosymbionts over many months and the deleterious effect this process would be expected to incur on parasite defenses. However, chemotherapy can have immunoprototecting effects against parasitic infections in certain circumstances. The co-administration of tetracycline with *Theileria* sporozites attenuates infection in cattle and forms the basis of an effective vaccine against East Coast fever, although in this case the parasite does not become fully established in the host prior to chemotherapy. In addition, praziquantel treatment facilitates the development of a protective immune response in schistosomiasis, despite the prior establishment of patent infection and the associated immune evasion strategies used by the worms.

Currently, there is a major gap in knowledge between the recent advances in understanding the hypo-responsive state in filarial infections and the effect of chemotherapy on host susceptibility. The critical role of regulatory T cells in allowing filarial maturation is now well established. However, the fact that ivermectin therapy reverses antigen-specific immunosuppression does not constitute direct evidence for increased protective immunity against *L*. In treated individuals; this effect may be transient or restricted in terms of the number of responsive individuals and the antigenic subfrctions recognized. Thus, there is an urgent need to investigate the relative duration of hypo-responsiveness after microfilaricidal and macrofilaricidal treatments in tractable animal models in addition to humans, particularly since existing computer simulations of onchocerciasis control do not incorporate the immunologic sequelae of chemotherapy. An epidemiologic study of human onchocerciasis concluded that adult worms facilitate super-infection of their hosts, and in murine filariasis there are the beginnings of a theoretical framework that could explain the persistence of filarial immunomodulation after worm death, in which Th2 cells are postulated to be conditioned towards an anergic phenotype even after the removal of T regulatory cells elicited by the parasite. This has implications for the design of vaccines in terms of the relative timing of chemotherapy and immunization and the potential for combining both methods to enhance parasite killing.

It is important to emphasize that for a number of reasons, the findings presented here do not detract from the promise of antibiotic chemotherapy for the control of onchocerciasis. First, we found no evidence that tetracycline chemotherapy increased susceptibility to ongoing infection compared with that exhibited by control animals, even though the latter already harbored heavy parasite burdens (approximately 40–150 nodules) at the onset of exposure. Second, in contrast, a previous study in *O. ochengi*-infected cattle suggested that ivermectin significantly interferes with the development of immunity to *L* in some contexts because frequent administration in uninfected calves prevented the development of patent infection but induced a state of hyper-susceptibility once the drug was withdrawn. Third, depletion of *Wolbachia* from *O. volvulus* leads to a prolonged (perhaps permanent) block of microfilarial output from female worms and may prevent the development of residual microfilariae to the infective *L* stage in the vector. These effects have the potential to reduce transmission considerably below levels achieved with ivermectin alone, thus reducing the incidence of re-infection in treated individuals to the point where eradication is feasible. Finally, antibiotics do not affect the *Wolbachia*-negative filaria *L. loa* which can cause serious adverse events after ivermectin therapy, supporting the view that they should be evaluated in areas co-endemic for loiasis and onchocerciasis.

Received July 27, 2006. Accepted for publication September 17, 2006.

Acknowledgments: We thank the herdsmen, watchmen, and technical staff of the Institut de Recherche Agricole pour le Développement for invaluable assistance. We are grateful to Pfizer (Tadworth, United Kingdom) and Merial (Lyon, France) for donating Terramycin LA and Cyemelarsan free of charge, respectively.

Financial support: This study was supported by the European Union (INCO-DEV contract no. ICA4-CT-1999-10002), Leo M. Njomena received a research training grant from WHO-TDR (No. M8/181/4 N.194).

Authors’ addresses: Charles K. Nfon, Benjamin L. Makepeape, Leo M. Njomena, and Alexander J. Trees, Liverpool School of Tropical Medicine and Faculty of Veterinary Science, University of Liverpool, Liverpool, L3 5QA, United Kingdom, Telephone: 44-151-705-3118, Fax: 44-151-705-3373, E-mails: nfonck@yahoo.fr, blm1@liv.ac.uk, lmnjomena@utmib.edu, and trees@liv.ac.uk. Vincent N. Tanya, Institut de Recherche Agricole pour le Développement, Regional Centre of Wakpa, BP 65 Ngoundéré, Cameroon, Telephone: 237-223-7720, E-mail: vtanya@yahoo.com.

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