Bacterial Endosymbionts of *Onchocerca volvulus* in the Pathogenesis of Posttreatment Reactions

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Treatment of onchocerciasis with diethylcarbamazine (DEC) or ivermectin is associated with a posttreatment reaction characterized by fever, tachycardia, hypotension, lymphadenopathy, and pruritus. To investigate the role of the *Wolbachia* bacterial endosymbiont of *Onchocerca volvulus* in these reactions, serum samples collected before and after treatment with either anthelmintic were assessed for evidence of *Wolbachia* DNA. By use of real-time quantitative polymerase chain reaction, *Wolbachia* DNA was detected in both groups—with significantly higher levels in those who received DEC (*P < .0001*). In the ivermectin group, there was a significant correlation between levels of bacterial DNA and serum tumor necrosis factor–α (*P = .013*). Peak DNA levels correlated with reaction scores (*P = .048*). Significant correlations were also seen between *Wolbachia* DNA and the antibacterial peptides calprotectin (*P = .021*) and calgranulin B (*P < .0001*). These findings support a role for *Wolbachia* products in mediating the inflammatory responses seen following treatment of onchocerciasis and suggest new targets for modulating these reactions.

*Onchocerca volvulus* is a filarial nematode transmitted by *Simulium* black flies in endemic regions of Africa and South America. Clinical disease can result from an inflammatory response to the microfilariae (mf) in the skin and in the eyes, progressing in some cases to blindness. Both diethylcarbamazine (DEC) and ivermectin are microfilaricidal, but resistance of the adult worms to drug treatment necessitates long-term annual (DEC) and ivermectin are microfilaricidal, but resistance of the adult worms to drug treatment necessitates long-term annual or semiannual treatment.

The Mazzotti reaction, first described in 1948 [1], is characterized by fever, tachycardia, hypotension, adenitis, pruritus, and arthralgia after treatment of onchocerciasis with DEC. This reaction is thought to represent a response to the sudden release of antigen by dying parasites, a concept supported by the observation that more-severe reactions occur in persons with higher mf densities in their skin [2]. Indeed, it was the severity of such reactions (and the possibility of exacerbating eye disease) that led to the search for a better-tolerated drug. Ivermectin is better tolerated, safer, and more effective, and it is now the drug of choice for onchocerciasis [3]. Nevertheless, even ivermectin can induce serious posttreatment adverse reactions, particularly when mf are present in the skin [4].

A number of studies have sought to characterize the dynamics of the immune response after DEC or ivermectin treatment of onchocerciasis. After treatment with either anthelmintic, the eosinophil count falls initially [2, 5] as a consequence of rapid migration from peripheral blood to the skin. Subsequently, blood eosinophil counts increase as bone marrow eosinophil stores are released and new eosinophil precursors differentiate [2]. The neutrophil count follows inversely, with an increase during the period of eosinopenia followed by a return to baseline [2]. Studies of other immunologic parameters have shown early increases in interleukin (IL)–6 and tumor necrosis factor (TNF)–α [6] and later increases in IL-5 [7] and eosinophil-derived neurotoxin [5]. Serial skin biopsies done early after treatment of onchocerciasis with ivermectin have shown increased expression of eotaxin and RANTES by dermal vascular endothelial cells [8] and infiltration of eosinophils into the dermis with deposition of major basic protein [9]. In other studies, lymph node biopsies after treatment with ivermectin showed a cellular infiltrate rich in eosinophils, neutrophils, and macrophages, with immunohistochemical evidence of macrophage release of calprotectin and lysozyme [10].

The existence of a rickettsia-like endosymbiont in *O. volvulus* has been known since 1977 [11]. The recent demonstration of the ability of doxycycline to clear adult nematodes of their endosymbionts [12], with potentially macrofilaricidal results [13] in *O. volvulus*–infected patients, has drawn increased
attention to these Wolbachiae. Furthermore, the demonstration that extracts of Wolbachia-containing Brugia malayi induce activation of macrophages and production of TNF-α and IL-1β from lipopolysaccharide (LPS)–sensitive but not –insensitive mice suggests a possible influence of these bacteria on the immune response in lymphatic filariasis [14]. Evidence for a role in onchocerciasis comes from the observation that extracts of O. volvulus from skin nodules from untreated patients induce production of TNF-α and IL-8 in monocytic and mononuclear cell cultures, whereas extracts of O. volvulus from doxycycline-treated patients do not [15]. Moreover, because LPS-binding protein levels increase after treatment of B. malayi infections with DEC [16], a link between the released Wolbachiae and posttreatment adverse reactions has been suggested.

It might follow, therefore, that the release of these intracellular bacteria could induce the antibacterial peptide heterocomplex, calprotectin (also known as L1 protein or designated by its component peptides, mrp8/14 and calgranulin A/B). Calprotectin is produced by neutrophils and macrophages in a variety of infectious and inflammatory conditions [17, 18]. In vitro, it possesses bactericidal and fungicidal activity [19, 20], and an elevated serum level is considered to be a sensitive, although not specific, marker for bacterial infection [21, 22]. A role for calprotectin in posttreatment reactions in onchocerciasis is suggested by immunohistochemical evidence of calprotectin release from macrophages in lymph nodes after treatment with ivermectin [10] and from neutrophils infiltrating the skin after topical treatment with DEC [23]. Thus, we assessed the relationships among Wolbachiae, adverse reactions, and inflammatory mediators, including calprotectin and its subunit calgranulin B, by using archived serum samples from patients with onchocerciasis treated with ivermectin or DEC.

Methods

Serum samples from DEC-treated patients. We used serum samples collected in 1982 from 8 DEC-treated patients with onchocerciasis. All patients were male (ages 15–32 years) and were studied at the Onchocerciasis Chemotherapeutic Center in Tamale, Ghana (table 1). Baseline samples were taken twice before treatment. Posttreatment samples were taken at 1, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h after the first of 7 daily doses of 200 mg of DEC (Ledere). Infection intensity in these patients was quantified by taking the geometric mean (GM) of mf counts from 4 skin snips before treatment. An objective scale for grading clinical severity of posttreatment reactions was used, as described elsewhere [2, 24]. In brief, numeric scores were assigned to each of 6 clinical parameters (fever, heart rate, standing blood pressure, lymph node swelling, joint pain, and itching) on the basis of deviation from baseline observations. The highest scores measured for each parameter from 9 observations over the 48 h after treatment (which were not

| Table 1. Characteristics and Wolbachia DNA levels of patients analyzed in this study. |
|---------------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Patient | Skin, mf/mg | Age, years | Pretreatment Wolbachia DNA levels, fg/μL | Peak Wolbachia DNA levels, fg/μL | Time of peakb | Reaction scoreb |
|-----------------------------|-----------------------------|-----------------------------|--------------------------------|----------------------------|-----------------------------|
| DEC treatment group | | | | | |
| D | 185 | 21 | ND | 0.0018 | 2 | 45 |
| J | 203 | 20 | ND | 0.0018 | 12 | 65 |
| O | 56 | 18 | 0.0535 | 0.0835 | 36 | 35 |
| P | 56 | 15 | ND | 0.0610 | 12 | 70 |
| Q | 104 | 18 | ND | 0.0445 | 36 | 55 |
| T | 34 | 32 | ND | 0.0130 | 12 | 20 |
| U | 18 | 30 | 0.0023 | 0.0020 | 8 | 10 |
| W | 226 | 18 | 0.0016 | 0.0028 | 24 | 55 |
| Ivermectin treatment group | | | | | |
| Iv04c | 112 | 37 | ND | 0.0014 | 8 | 330 |
| Iv08c | 137 | 37 | 0.0012 | 0.0015 | 18 | 18 |
| Iv12d | 70 | 39 | ND | 0.0018 | 36 | 128 |
| Iv13d | 288 | 27 | ND | 0.0012 | 30 | 145 |
| Iv14d | 342 | 21 | 0.0048 | 0.0108 | 12 | 533 |
| Iv19d | 117 | 23 | ND | 0.0028 | 18 | 341 |
| Iv20d | 332 | 30 | ND | 0.0018 | 18 | 350 |
| Iv23e | 380 | 34 | 0.0078 | 0.0120 | 18 | 436 |
| Iv24e | 339 | 33 | ND | ND | — | 232 |
| Iv25e | 53 | 19 | ND | ND | — | 98 |

NOTE. DEC, diethylcarbamazine; fg, femtogram; mf, microfilariae; ND, not detectable.

a Hours after treatment.

b Scoring method differed between the 2 groups; see Methods and [4, 24].
c 150 μg/kg ivermectin.
d 400 μg/kg ivermectin.
e 600 μg/kg ivermectin.
necessarily concomitant with the 10 blood draws made over the same time period) were added to calculate the total reaction score. Aliquots of serum samples were maintained after collection at −70°C.

Serum samples from ivermectin-treated patients. Serum samples from 10 ivermectin-treated patients with onchocerciasis, obtained from samples collected in 1994, were also used. These patients were otherwise healthy men, aged 19–39 years, who had been studied at the Onchocerciasis Chemotherapy Research Center in Hohoe, Ghana (table 1). Baseline samples were drawn twice before therapy, and posttreatment samples were collected at 2, 4, 6, 8, 12, 18, 24, 30, 36, 44, and 48 h after a single oral dose of 150 µg/kg (2 patients), 400 µg/kg (5 patients), or 600 µg/kg (3 patients) of ivermectin (Merck). Infection intensity in these patients was quantified by taking the GM of mf counts from 4 skin snips before treatment. An objective scale for grading clinical severity in these patients has been described elsewhere [4]. Aliquots of collected serum samples were maintained at −70°C.

Measurement of antibacterial peptides. Measurements of calprotectin and calgranulin B were done with a commercial EIA kit (BMA Biomedicals) according to the manufacturer’s instructions. Normal serum levels for calprotectin and calgranulin B are 0.5–3.0 µg/mL and 0–100 ng/mL, respectively.

DNA extraction from serum. DNA from serum samples was extracted with the QIAamp DNA minikit (Qiagen) according to the manufacturer’s instructions. We used 200-µL serum aliquots. The DNA obtained was eluted in 200 µL of sterile water and was frozen at −70°C until use.

Quantitative PCR. DNA encoding the 16S ribosomal protein of the Wolbachia endosymbiont was cloned from O. volvulus genomic DNA, using a nested polymerase chain reaction (PCR). The PCR product was ligated into pCR 2.1-TOPO and was used to transform OneShot Escherichia coli cells, according to the kit manufacturer’s instructions (Invitrogen). We grew a single transformed colony in antibiotic selective media overnight and purified the plasmid with the CONCERT High Purity Plasmid Purification System (Invitrogen). Plasmid DNA was quantified by using a spectrophotometer and was aliquoted for use in generating a standard curve for the quantitative PCR reactions (figure 1). Forward and reverse primers and a probe for the quantitative PCR reaction were designed with PrimerExpress software (PE Biosystems Custom Oligonucleotide Synthesis Service) and were synthesized as follows: forward primer, 5'-GAAGATAATGACGGTACTCACAGAAGAA; reverse primer, 5'-TTACGCCCAATAATTCCGAATAA; TaqMan probe, 6-carboxyfluorescein (6-FAM)–AACTCCGTGCCAGCAGCCGC–6 carboxytetramethylrhodamine (TAMRA). The probe was labeled both with the fluorescent dye 6-FAM and with the quencher TAMRA. When polymerization progresses through the probe, the dye is released from the quencher and fluorescence can be detected. The amount of starting template is proportional to the cycle number at which the amount of detectable fluorescence exceeds a predetermined threshold [25]. Master mix consisted of

![Figure 1](https://journals.oxfordjournals.org/)
Results

Table 1 shows the characteristics of the study population. As a group, the ivermectin-treated patients were older (29.2 vs. 20.8 years; \( P = .013 \)) and had higher GM mf densities in the skin (176 vs. 80 mf/mg; \( P = .062 \)). *Wolbachia* DNA was detectable in the serum at baseline in 3 patients in each group. Within 48 h of treatment, *Wolbachia* DNA was detectable in the serum of all patients treated with DEC and in 8 of 10 patients treated with ivermectin. The peak levels (table 1) were significantly above baseline for the DEC group and nearly so for the ivermectin group (\( P = .016 \) for DEC and \( P = .064 \) for ivermectin), occurring at various times after therapy (median time, 18 h). The levels of *Wolbachia* DNA detected were significantly higher in the DEC group than in the ivermectin group at 2, 12, 24, and 48 h after treatment (\( P = .028 \), .008, .019, and .049, respectively). Peak levels and levels over all posttreatment time points were both significantly higher in the DEC group (\( P = .014 \) and \( P < .0001 \), respectively). For both groups, a large number of data points for the detection of *Wolbachia* DNA were near the limits of detection. In all cases, a minimum of 1.3 cycles separated the values used from the corresponding control water blanks. There was no correlation between pretreatment skin mf density and either baseline or peak *Wolbachia* DNA levels in either treatment group (data not shown).

A significant positive correlation between *Wolbachia* DNA levels and peripheral neutrophil count was seen in both patient groups (\( P = .041 \) for ivermectin patients and \( P = .032 \) for DEC patients; table 2). A significant positive correlation was seen between *Wolbachia* DNA and serum TNF-\( \alpha \) levels over the entire 48-h analysis period (\( P = .013 \); table 2) in the ivermectin patients (TNF-\( \alpha \) levels were not available for the DEC-treated patients). A positive correlation was also found between peak *Wolbachia* DNA levels and reaction scores in the ivermectin group (\( P = .048 \); figure 2).

Serum calprotectin levels before treatment were significantly higher (\( P = .023 \)) in the ivermectin group (GM, 4.31 \( \mu \)g/mL) than in the DEC-treated group (GM, 2.11 \( \mu \)g/mL; figure 3A) and were slightly higher than the normal range (0.5–3 \( \mu \)g/mL). Despite this, calprotectin levels in the DEC group increased to a peak of nearly 3 times the pretreatment values 24–48 h after the first dose, whereas calprotectin levels in the ivermectin group did not significantly change from baseline (figure 3B). Calprotectin levels were positively correlated with neutrophil counts in both groups (\( P < .0001 \); table 2). Calprotectin levels did not increase in 2 uninfected patients who were treated as part of the DEC study (data not shown).

Unlike calprotectin, calgranulin B levels at baseline were significantly higher (\( P = .026 \)) in the DEC group (GM, 13.569 ng/mL) than in the ivermectin-treated group (GM, 5.443 ng/mL).

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**Table 2.** Spearman’s rank correlations over all available time points among serum levels of *Wolbachia* DNA, calprotectin, calgranulin B, and tumor necrosis factor (TNF)-\( \alpha \) and peripheral cell counts.

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Diethylcarbamazine</th>
<th>ivermectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PMNL</td>
<td>.032</td>
<td>.041</td>
</tr>
<tr>
<td>EOS</td>
<td>NS</td>
<td>.042</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>NS</td>
<td>.013</td>
</tr>
<tr>
<td>Calgranulin B</td>
<td>NS</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>NA</td>
<td>.013</td>
</tr>
</tbody>
</table>

Statistical comparisons were done with StatView software (version 5.0; SAS Institute). Paired comparisons have been reported elsewhere [5–7, 26].

**Cytokine levels.** Levels of TNF-\( \alpha \), IL-5, IL-1\( \beta \), IL-6, and RANTES in the ivermectin patients and IL-5 in the DEC patients have been reported elsewhere [5–7, 26].

**Statistical methods.** Statistical comparisons were done with StatView software (version 5.0; SAS Institute). Paired comparisons of nonparametric data were done by Spearman’s rank analysis. Unpaired comparisons were made using the Mann-Whitney *U* test.

25 \( \mu \)L of 2X Universal Master Mix (PE Applied Biosystems), 2 \( \mu \)L of 10 \( \mu \)M primers, and 1 \( \mu \)L of 1 \( \mu \)M probe per 50-\( \mu \)L reaction. Extracted DNA (10 \( \mu \)L) or serial dilutions of standard were combined with 40 \( \mu \)L of master mix in 96-well MicroAmp optical plates. Thermal cycling and data analysis were done on the ABI Prism 7700 (PE Applied Biosystems).

**Figure 2.** Reaction scores (Y-axis) correlate with peak *Wolbachia* DNA levels (X-axis) in ivermectin-treated patients with onchocerciasis. Each dot represents an individual patient (\( \rho \) statistically significant by Spearman’s rank correlation; \( P = .048 \)). fg, femtogram.
Although calgranulin B levels did not correlate with neutrophil counts or calprotectin levels in either group, they correlated strongly with both TNF-α and Wolbachia DNA levels in the ivermectin group \((P < .0001; \text{table 2})\). No significant associations were seen between Wolbachia DNA levels and IL-5, IL-6, IL-1β, or RANTES \((\text{data not shown})\).

**Discussion**

Posttreatment reactions in onchocerciasis have been largely assumed to result from the host’s immune system reaction to the sudden exposure to large quantities of parasite antigen \([27]\). Some of the clinical manifestations resemble acute bacteremia \((\text{fever, tachycardia, and hypotension})\), while others \((\text{e.g., pruritus and lymphadenopathy})\) do not.

This study demonstrates that DNA from the Wolbachia endosymbiont of *O. volvulus* can be detected in the serum of patients after treatment of onchocerciasis with either DEC or ivermectin. The use of a quantitative PCR technique enabled us to demonstrate a relationship between the peak levels of bacterial DNA present in the serum and the severity of the posttreatment reaction in ivermectin-treated patients. This is not explained entirely by an increased intensity of infection, as skin mf density and peak Wolbachia DNA levels were not directly correlated.

A role for bacterial products in modifying the immunologic profile of posttreatment reactions is supported by the correlation between bacterial DNA levels and peripheral neutrophil counts, as well as serum TNF-α levels, in ivermectin-treated patients over all available time points \((\text{table 2})\). The rapid release of TNF-α into the bloodstream after experimental intravascular inoculation and the rapidity with which it is degraded \([28]\) suggest it to be a suitable parameter for paired comparisons with similarly transient levels of bacterial DNA. As mentioned, macrophages from LPS-sensitive strains of mice increase production of TNF-α when exposed to extracts of filarial parasites known to harbor *Wolbachia* \([14]\), as do monocytes and mononuclear cells exposed to extracts of *O. volvulus* from patients not treated with doxycycline \([15]\). The current study supports a causal role for bacterial products in production of this cytokine in human filarial infections and provides an explanation for why some of the clinical manifestations associated with treatment of onchocerciasis resemble acute bacteremia.

Serum levels of the antibacterial peptide calprotectin were closely associated with peripheral neutrophil counts in both study groups \((P < .0001; \text{table 2})\), consistent with the notion that calprotectin reflects activation and turnover of this cell type in acute inflammatory states \([22]\). Although the time course and magnitude of the neutrophilia were similar in both groups \((\text{figure 3B, inset})\), a significant increase in calprotectin levels...
was seen only in the DEC patients, suggesting that neutrophils were more activated in this group (figure 3B). An earlier study of neutrophil activation in onchocerciasis after ivermectin therapy showed an increase in serum elastase that correlated with reaction severity but showed no change in serum lactoferrin. This implies selective degranulation of azurophilic granules [29] and suggests that qualitative as well as quantitative differences in neutrophil activation must be considered.

Of interest, the reaction scores did not correlate with peak DNA levels in the DEC group. It should be noted, however, that the system for scoring reaction severity differed between the 2 groups. The reaction scores for the DEC patients did not include parameters for headache, muscle aches, or rash and had only one parameter for blood pressure and heart rate, whereas scores for the ivermectin patients reflected both standing and recumbent blood pressure and heart rate. Aside from differences in data collection, it is probable that the host response to low levels of bacteremia determines the clinical response more than any objective measurement of the magnitude of bacteremia. Since quantitative PCR is a relatively new technique, there are not significant amounts of data on how the magnitude of bacteremia determined in this fashion correlates with the clinical manifestations of bacteremia. Further studies of our patients, including assessment of toll-like receptor–4 polymorphisms that may alter the response to bacterial LPS, are ongoing.

Few studies have explored the differences between the microfilaricidal mechanisms of action of ivermectin and DEC. In our study, DEC therapy was associated with higher levels of detectable bacterial DNA. This could be due to a more rapid rate of mf killing, to a mechanism of action that causes more bacteria to be released per parasite, or to killing at an anatomic site (e.g., skin vs. lymph node) that allows more released bacteria access to the host bloodstream. An increased level of bacteremia may be one reason why posttreatment reactions are more severe with DEC than with ivermectin, although the available data do not permit us to compare reaction scores between our 2 groups directly. This study also suggests differences in the nature or magnitude of neutrophil activation between the 2 treatment groups. Whether this is a result of the greater degree of bacteremia or other, yet undefined factors remains to be discovered.

In lymphatic filariasis, in which the mf are bloodborne, one might predict similar degrees of bacteremia regardless of whether DEC or ivermectin is used. Indeed, the clinical severity of posttreatment reactions in lymphatic filariasis does not appear to differ significantly between anthelmintics [30]. We hope to elucidate the role of Wolbachia in posttreatment reactions in lymphatic filariasis in the near future.

The demonstration that endosymbiotic bacteria play a prominent role in posttreatment reactions implies that adjunctive use of antibacterials may decrease the severity of these reactions. If such a strategy were to prove successful and easy to implement, the morbidity of treatment could be reduced and the compliance with large-scale eradication campaigns enhanced.

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References
