

Neutrophil accumulation around *Onchocerca* worms and chemotaxis of neutrophils are dependent on *Wolbachia* endobacteria

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ABSTRACT – Unlike in many other helminth infections, neutrophilic granulocytes are major cellular components in the host's immune response against filarial worms. The pathways that drive the immune response involving neutrophils are unclear. This study shows that *Wolbachia* endobacteria (detectable by polyclonal antibodies against endobacterial heat shock protein 60 and catalase and by polymerase chain reaction being sensitive to doxycycline treatment) are direct and indirect sources of signals accounting for neutrophil accumulation around adult *Onchocerca volvulus* filariae. Worm nodules from untreated onchocerciasis patients displayed a strong neutrophil infiltrate adjacent to the live adult worms. In contrast, in patients treated with doxycycline to eliminate the endobacteria from *O. volvulus* and to render the worms sterile, the neutrophil accumulation around live adult filariae was drastically reduced. Neutrophils were absent in worm nodules from the deer filaria *Onchocerca flexuosa*, a species which does not contain endobacteria. Extracts of *O. volvulus* extirpated from untreated patients showed neutrophil chemotactic activity and in addition, induced strong TNF- α and IL-8 production in human monocytes, in contrast to filarial extracts obtained after doxycycline treatment. Thus, neutrophil chemotaxis and activation are induced directly by endobacterial products and also indirectly via chemokine induction by monocytes. These results show that the neutrophil response is a characteristic of endobacteria-containing filariae. © 2001 Éditions scientifiques et médicales Elsevier SAS

Onchocerca / *Wolbachia* / endobacteria / neutrophilic granulocytes / chemotaxis / tumor necrosis factor / interleukin 8

1. Introduction

Filarial nematodes infect more than 150 million people in tropical areas and cause widespread morbidity due to diseases of eyes, skin and lymphatic system, such as elephantiasis and river blindness [1]. Recently, endosymbiotic bacteria of the genus *Wolbachia* known to be hosted by many filarial species were demonstrated to be mutualists given that their depletion by tetracycline antibiotics blocked worm development and rendered female worms sterile [2]. This strong mutualistic inter-relationship

between the worms and their symbionts has been exploited for a novel chemotherapeutic approach in onchocerciasis with doxycycline, leading to long-term worm sterility (Hoerauf A., et al., Lancet (2001) in press ; [3]). Antibacterial responses may be involved in adverse reactions after chemotherapy with the microfilaricidal drugs diethylcarbamazine (DEC) [4] or ivermectin since murine [5] and human [6] monocyte/macrophages can be activated to produce inflammatory cytokines after incubation with extracts from endobacteria-containing filariae. This process is at least in part due to lipopolysaccharide-like molecules (LPS) [5, 6].

In contrast to many other helminth infections, the host's immune responses in filarial infections are characterized by neutrophils in addition to eosinophils [7–9]. Thus,

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neutrophils participate in the formation of *Onchocerca* nodules, being attached to the surface of the worms and constituting the predominant cells in the cysts around males and the anterior ends of female worms [10, 11]. Furthermore, neutrophils constitute a major part of the antimicrofilarial immune response after antifilarial treatment [12, 13]. In the present study we provide evidence that *Wolbachia* symbiotic bacteria represent major triggers of neutrophil accumulation around live filariae.

2. Materials and methods

2.1. Isolation of filariae

Nodules from onchocerciasis patients in south-western Ghana were surgically removed under local anesthesia and stringent aseptic conditions, as described by Albiez et al. [14]. Onchocercomas were obtained either from untreated patients or 4 and 9 months after the end of a 6-week doxycycline treatment (100 mg/day). This treatment has been shown by immunohistology and by polymerase chain reaction (PCR) to strongly reduce *Wolbachia* endobacteria from the adult worms [3]. Nodulectomies for research purposes were approved by the Ethics Commissions of the Medical Board in Hamburg as well as of the School of Medical Sciences in Kumasi, Ghana. Nodules containing *Onchocerca jakutensis* (syn. *O. tubingensis*) and *O. flexuosa* were excised from the carcass of red deer (*Cervus elaphus*) hunted in northern Germany [15]. In a collaborative study with A. Renz (University of Hohenheim, Germany) and V.N. Tanya (Institut de recherche agricole pour le développement, Wakwa, Cameroon) nodules with *O. ochengi* were removed from *Bos indicus* cattle infected in Ngaoundéré in northern Cameroon [6, 16]. Adult *Acanthocheilonema viteae* were kindly donated by R. Lucius (Humboldt University of Berlin, Germany).

2.2. Immunohistology

Nodules were fixed in 80% ethanol or in 4% buffered formaldehyde solution and embedded in paraffin using standard methods. For conventional histology hematoxylin and eosin, amidoblack, Giemsa and Movat stains were applied. For immunostaining the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was applied according to the recommendations of the manufacturer (Dako Diagnostika, Hamburg, Germany). A rabbit antiserum against heat shock protein 60 (hsp60) of *Yersinia enterocolitica* [17] was a kind gift of I.B. Autenrieth (University of Tübingen, Germany). It was used as described [3, 18] at a dilution of 1:1 000 for the detection of *Wolbachia* in the filariae. Formaldehyde-fixed sections were also stained using polyclonal antibodies against *Wolbachia* catalase at a dilution of 1:50 [19]. The immunohistological staining of the endobacteria in filariae had been confirmed by polyclonal antibodies against hsp60 from *Wolbachia* in *O. volvulus* using APAAP staining in light microscopy as well as by immunogold labeling in electron transmission microscopy [18, 20]. A polyclonal anti-rabbit IgG antibody (Dako Diagnostika) was used as secondary antibody. Fast red TR salt (Sigma Chemical, Deisenhofen, Germany) was used as the chromogen, and hematoxylin (Merck,

Darmstadt, Germany) served as the counterstain. Monoclonal antibodies against human neutrophil elastase (Dako Diagnostika) diluted 1:150 and defensins (Dianova, Hamburg, Germany) diluted 1:1 000 to 1:4 000 were used as primary antibodies for staining neutrophils [12]. Worms assumed to have been alive at the time of extirpation were designated as live worms. The assessment of vitality was based on worm morphology and detection of proteins (filarial hsp60 and glutathione S-transferase 1) seen in immunohistological sections.

2.3. Preparations of filaria extracts

Adult *O. volvulus* filariae were isolated from nodules of untreated and of doxycycline-treated onchocerciasis patients using the collagenase digestion method [21]. Saline-soluble extracts were prepared as described [11, 22]. Briefly, worms frozen in liquid nitrogen were ground and homogenized in liquid nitrogen and the resulting powder was extracted with phosphate-buffered saline (PBS) overnight at 4 °C. Debris was removed by ultracentrifugation at 10 000 g for 30 min. The supernatant was tested for sterility, the protein concentration adjusted at 1 mg/mL and small aliquots stored at -20 °C until use. Extracts of *O. ochengi* and *A. viteae* were prepared using the same procedures.

2.4. Measurement of neutrophil chemotaxis

Neutrophilic granulocytes were isolated from peripheral blood of healthy Europeans, with a purity of more than 95% using a Percoll gradient [11]. The chemotaxis assay was performed in Boyden chambers (Biorad, Munich, Germany) as previously described [11]. As source for chemoattractants, extract from *O. volvulus* females obtained either from untreated or from doxycycline-treated patients were used at protein concentrations of 12.5–150 µg/mL. For negative control (random migration) the chemotaxis buffer (PBS containing CaCl₂, MgCl₂ and BSA) and as positive control the bacterial peptide formyl-methionyl-leucyl-phenylalanine (Sigma) at 10⁻⁸ M was included. Neutrophils (2 × 10⁵) were allowed to migrate within 1 h at 37 °C in 5% CO₂ through polyvinylpyrrolidone-free polycarbonate filters (pore size: 3 µm; Nuclepore, Tübingen, Germany), the migrated cells were lysed with Triton X-100 (0.1% vol/vol; Sigma), and the lysates were incubated for 18 h with *p*-nitrophenyl-β-D-glucuronide (Sigma) as substrate. For the calculation of the number of migrated cells, a calibration curve was generated with defined numbers of lysed neutrophils. All chemotaxis experiments were performed in duplicate and the chemotactic activity was expressed as chemotactic index (stimulated migration/random migration).

2.5. Production of neutrophil-activating monokines

Peripheral blood mononuclear cells (MNCs) were obtained from healthy donors by Ficoll/Paque (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) density gradient centrifugation of anticoagulated blood on the 1.077 g/mL layer. Monocytes (> 95%) were isolated from MNCs by counterflow centrifugation as described [6]. Monocytes or MNCs were cultured at 4 × 10⁵ or 6 × 10⁵ cells/culture in endotoxin-free RPMI-1640 supplemented with 10% (v/v)

heat-inactivated endotoxin-free fetal calf serum, penicillin (100 IU/mL), streptomycin (100 µg/mL), and glutamine (2 mM) at 37 °C in 5% CO₂ using flat-bottom 96-well plates (Nunc, Roskilde, Denmark) as previously described [6]. Cells were exposed to extracts from *O. volvulus* females obtained from untreated or from doxycycline-treated patients at 20 µg/mL. In a number of experiments extracts of endobacteria-containing *O. ochengi* and endobacteria-free *A. viteae* were used as stimulus.

Cell cultures were harvested after 4 h, 1, 3 and 7 days of incubation at 37 °C, and cell-free supernatants were kept frozen at -28 °C until analyzed for the cytokine content. TNF-α was quantified in cell culture supernatants by ELISAs using a kit from R&D Systems (Minneapolis, MN, USA) according to the instructions of the manufacturer, IL-8 was measured applying the pairs of monoclonal capture antibodies (clone MAB208) and detecting antibodies (clone BAF208) as well as a standard protein, as commercially supplied by R&D Systems. The sensitivity of the TNF-α determination was 2 pg/mL and for IL-8 12 U/mL.

2.6. Statistical analysis

Differences between experimental sets using endobacteria-containing versus endobacteria-depleted filarial extracts were assessed using the Mann-Whitney *U*-test, with *P* < 0.05 taken as the lower limit of significance.

3. Results

3.1. Onchocercomas with endobacteria-containing *O. volvulus* exhibit an accumulation of neutrophils

Several hundred *O. volvulus* males and females extirpated from untreated nodule carriers living in various countries in Africa and America and in Yemen always showed large numbers of *Wolbachia* endobacteria. Using antibodies against *Wolbachia* hsp60 and catalase the bacteria were observed in the hypodermis (figure 1A, C, D) and in the oocytes and all developmental stages during embryogenesis. In the vicinity of the live female filariae, neutrophils were found in many sections. The number of neutrophils varied much. Sometimes only a few neutrophils were seen attached to the cuticle (figure 1D) and sometimes higher numbers were observed adjacent to a layer of cell remnants covering portions of old worms. In many sections large portions of the midbody demonstrated no neutrophils attached to the cuticle. However, the anterior ends of the females and nearly always the entire males were found in small cysts containing a cell suspension of mostly neutrophils that had usually released their defensins (figure 1B). From the analysis of serial sections presenting most portions of female worms it was concluded that neutrophils occur in the vicinity of all or almost all live adult worms in onchocercomas from untreated patients.

3.2. Onchocercomas with *O. volvulus* from hosts treated with doxycycline exhibit few neutrophils

The onchocercomas from untreated patients were compared with 91 nodules excised 4 or 9 months after the end

of a 6-week treatment with doxycycline (100 mg/day) [3]. Using immunohistology it was found that these filariae from doxycycline-treated patients were almost all devoid of bacteria. No *Wolbachia* were found in 70 live male worms. Only five of 99 live females in the 4-month group demonstrated a small number of possibly degenerated endobacteria, and all 66 live females from treated patients in the 9-month group were free of endobacteria (figure 1E, G). However, the anti-hsp60 antibody did stain the filarial hsp60 in the outer zone of the hypodermis (figure 1E), the afibrillar portion of the muscles, oocytes and some embryos, thus indicating correct staining of hsp60. It was concluded from these observations that also the extract from worms excised 5 months after treatment contained only a negligible amount of *Wolbachia* compounds (see 3.4 and 3.5).

None of the 230 live adult worms devoid of endobacteria showed an accumulation of neutrophils in the vicinity of the worms or attached to the cuticle (figure 1H) and the cysts around the males and the anterior ends of the female worms only contained very few neutrophils when stained for defensin (figure 1F) or neutrophil elastase. Only small numbers of neutrophils were observed in the nodule tissue (figure 1H), but a few nodules showed large numbers of neutrophils far from the worms in the nodular capsule. The staining of neutrophils in the small blood vessels indicated good staining. These results strongly suggest that doxycycline-sensitive endobacteria are essential sources of signals which result in accumulation of neutrophils adjacent to the filariae. The reduced number of neutrophils in onchocercomas from treated patients compared to those from untreated individuals concerned only live filariae. In the vicinity of dead worms not only macrophages and giant cells were seen but also larger numbers of neutrophils (data not shown). Previous studies [23] had shown that macrophages and giant cells are major cell populations attached to the cuticle of live female *Onchocerca* worms in cattle and deer. Both cells were found adjacent to live worms in onchocercomas from doxycycline-treated (figure 1G) as well as from untreated patients.

3.3. Onchocercomas with *Onchocerca* spp. from red deer exhibit a neutrophil content correlating with the content of endobacteria

The red deer (*C. elaphus*) is the host of several *Onchocerca* species which often live in the same animal. In previous electron microscopic studies endobacteria were observed in *O. jakutensis* but not in *O. flexuosa* worms [15]. Therefore, we compared the occurrence of neutrophils in onchocercomas of these two species. As a first step the presence of *Wolbachia* in *O. jakutensis* (figure 2A–C) and the absence of endobacteria in *O. flexuosa* (figure 2D, E) were confirmed using immunohistology. No endobacteria were found in more than 100 adult *O. flexuosa* from animals living in three different geographic foci. The presence of filarial hsp60 in the ovary (figure 2D) and in the hypodermis proved the correct staining procedure. Neutrophils were found in large numbers adjacent to *O. jakutensis* using hematoxylin (figure 2A–C), Giemsa and Movat stains. No neutrophils were observed in

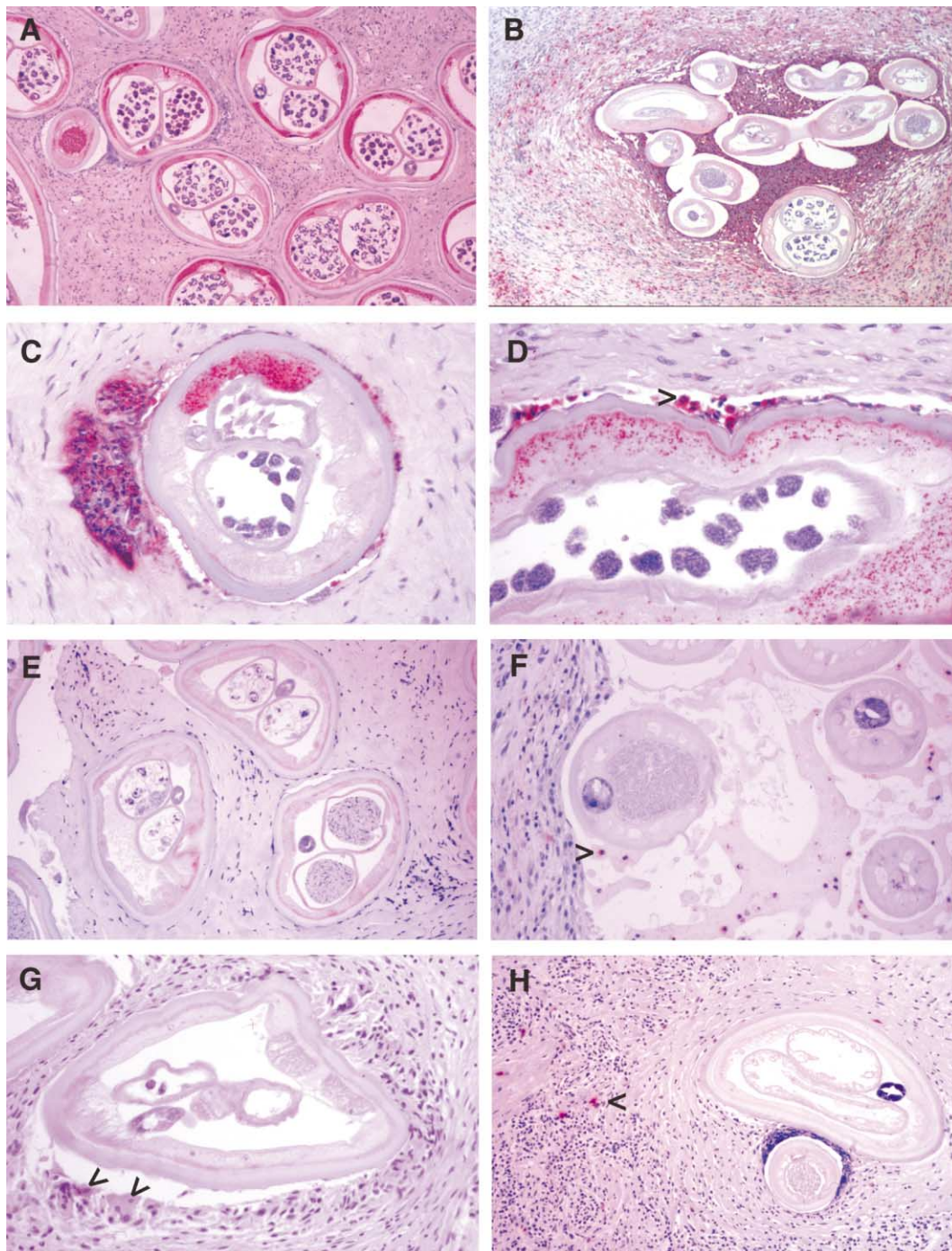


Figure 1. Sections of live *O. volvulus* worms from untreated (A–D) and doxycycline-treated patients (E–H). In worms from untreated patients many hsp60-positive endobacteria (red stained) are detected in the hypodermis (A, C, D), whereas no hsp60 staining is detectable in worms recovered from doxycycline-treated patients (E, G). In the vicinity of worms from untreated patients many defensin-positive neutrophils are observed (B, C, D arrowhead), but few neutrophils are seen adjacent to worms from treated patients (F, H arrowheads). This difference is very obvious in the cysts containing male worms (B versus F). Macrophages and small giant cells are detected adjacent to the cuticle (G arrowhead) of many worms from treated patients. The bluish cells around the male worm in H probably are disintegrating macrophages. APAAP, primary antibodies against hsp60 (A, E, G), hsp60 and defensin (C, D), defensin only (B, F), and elastase (H). Magnification, $\times 50$ (A, B), $\times 100$ (C), $\times 190$ (D), $\times 80$ (E, H), $\times 120$ (F, G).

onchocercomas of *O. flexuosa*. In the vicinity of *O. flexuosa* filariae only macrophages and giant cells were seen

(figure 2E). The lack of neutrophils was not due to host factors, since one deer (no. 5) presented neutrophils adja-

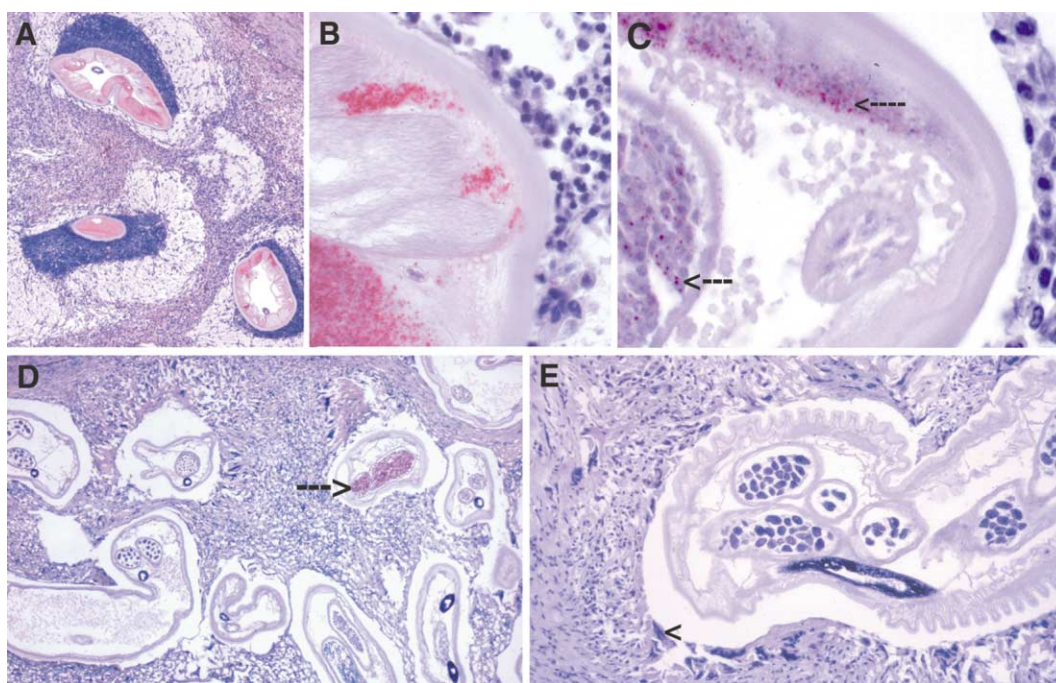


Figure 2. Sections of live female *O. jakutensis* worms (A–C) with many red hsp60 stained endobacteria in the hypodermis and ovary (arrows in C) and bluish neutrophils in their vicinity, whereas *O. flexuosa* filariae (D, E) show no endobacteria and only macrophages and giant cells attached to the cuticle but no neutrophils (arrowhead). The *O. flexuosa* and the *O. jakutensis* worms in C were excised from the same animal. The red staining of the ovary in D (arrow) is due to a weak cross-reaction of filarial hsp60. APAAP, primary antibodies against bacterial hsp60. Magnification, $\times 30$ (A, D), $\times 280$ (B), $\times 500$ (C), $\times 70$ (E).

cent to *O. jakutensis* (figure 2C) but none in several *O. flexuosa* onchocercomas (figure 2D, E).

3.4. The neutrophil chemotactic activity detected in *O. volvulus* extracts is sensitive to doxycycline treatment

The extracts of *O. volvulus* females obtained from untreated or doxycycline-treated patients were examined for the presence of chemoattractants for neutrophils. Extracts of worms recovered from untreated patients induced dose-dependent chemotactic responses with chemotactic indices (C.I.) > 3.0 at a concentration higher than $50 \mu\text{g/mL}$. The half maximal dose (ED_{50}) was obtained at $70 \mu\text{g/mL}$ (figure 3). In comparison to the endobacteria-positive extract, the chemotactic response of neutrophils was found reduced to levels between 50 and 70% when extracts of endobacteria-depleted females were applied. Significant differences were observed for 100 and $150 \mu\text{g/mL}$, respectively ($P < 0.01$; 0.05).

3.5. Upon exposure to *Onchocerca* spp. extracts human monocytes/macrophages release the neutrophil activator TNF- α and chemoattractant IL-8

The two *O. volvulus* extracts from untreated and doxycycline-treated patients also differed in their capacity to induce neutrophil-activating cytokines. Thus, extracts containing bacterial components induced the production of TNF- α and IL-8 in 24-h cultures of monocytes as well as of mononuclear cells from healthy blood donors, while extracts devoid of bacteria only weakly stimulated the secretion of TNF- α ($P < 0.01$). Kinetics experiments

revealed that TNF- α peaked between 4 and 24 h of cell cultures, while IL-8 showed increasing concentrations during 1–3 days of culture (figure 4A, B). The association

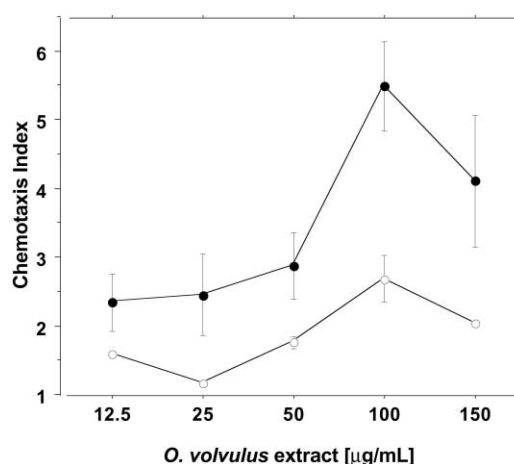


Figure 3. Dose-dependence of the chemotaxis response of neutrophils ($n = 5$) exposed to extracts from *O. volvulus* females recovered from untreated patients (black circles) containing endobacteria and from worms from patients after doxycycline treatment (white circles) depleted of bacteria. The figure shows the chemotactic indices for 12.5– $150 \mu\text{g/mL}$ *O. volvulus* protein. Significant differences between both extracts exist for 100 and $150 \mu\text{g/mL}$, respectively ($P < 0.01$, $P < 0.05$).

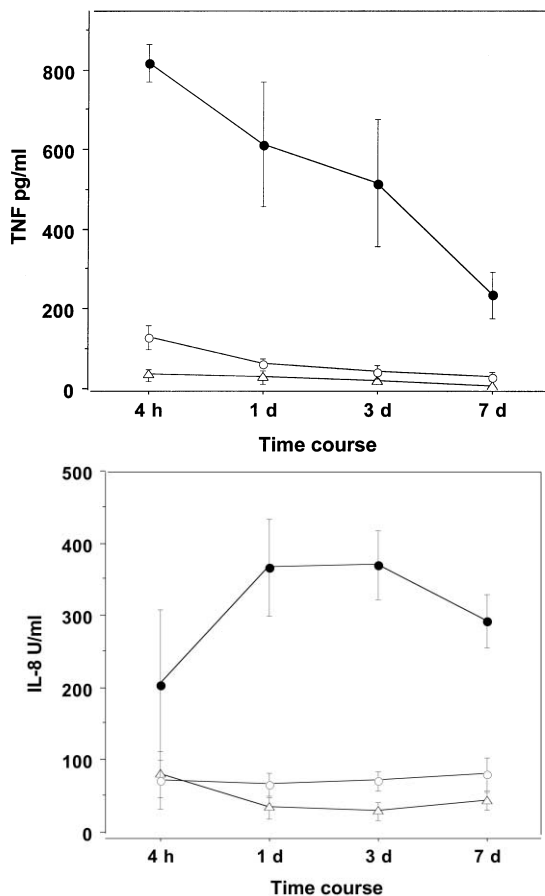


Figure 4. Kinetics of TNF- α ($n = 3$; A) and IL-8 ($n = 5$; B) production by monocytes/macrophages exposed to extracts from *O. volvulus* females recovered from untreated persons (black circles) and from worms of doxycycline-treated patients (white circles). Significant differences between both extracts exist for the TNF- α production after 4 h and 1, 3 and 7 days ($P < 0.05$) and for the IL-8 production after 1, 3, and 7 days of incubation ($P < 0.01$). The triangles indicate the background level in the absence of stimulus.

between IL-8 induction and the presence of endobacteria in filariae was strengthened by the finding that the extract of the closely related endobacteria-positive species *O. ochengi* induced strong IL-8 responses in contrast to a negligible IL-8 production induced by an extract of *A. viteae* containing no endobacteria (data not shown).

4. Discussion

Granulocytes represent important participants in the host's innate defense system against helminth parasites. Filarial infections are characterized by the fact that in addition to eosinophilic granulocytes, neutrophils also play a major role in the attack on adult worms and microfilariae [7–12]. In addition, neutrophil chemotactic molecules have been detected in extracts of *O. volvulus* females and it has been hypothesized that one reason for

this may be the presence of endobacteria in many filarial species but not in other helminths [11, 24].

In the present study we provide evidence that *Wolbachia* endobacteria are essential components of the neutrophil response in vitro and in vivo. Thus, the chemotactic activity observed in extracts of *O. volvulus* isolated from untreated patients was found significantly reduced in extracts from bacteria-depleted filariae. The latter were isolated from onchocercomas excised 4 months after the end of a 6-week course of doxycycline treatment which was sufficient to deplete the worms of their endobacteria (Hoerauf A., et al., Lancet (2001) in press ; [3]). In accordance with these in vitro findings, histological observations documented a stringent association between the presence of endobacteria in live adult *O. volvulus* and the presence of neutrophils in their vicinity, and conversely, between the absence of endobacteria and neutrophils after antibiotic treatment. This association of neutrophil accumulation with intracellular bacteria was also confirmed in other filarial species. Of major interest is the observation that massive neutrophil infiltration was observed near the endobacteria-positive deer filaria *O. jakutensis*, while it was absent around the endobacteria-free deer filaria *O. flexuosa*. Since onchocercomas of both species were studied from the same animal, variation of host reactivity in different deer can be excluded as a cause for the different neutrophil chemotaxis.

In addition to direct chemotactic effects of endobacteria-containing filarial extracts on purified neutrophils, we found that these extracts can also indirectly provide neutrophil chemotaxis and activation via induction of the cytokines IL-8 and TNF- α by monocytes. Macrophages, monocytic tissue counterparts, are abundant in the vicinity of live adult filariae in an onchocercoma and therefore can provide chemotactic and activating signals for circulating neutrophils. They were also observed adjacent to filariae after doxycycline treatment.

The depletion of *Wolbachia* in the filariae results in a lack of endobacteria-derived chemoattractive molecules for neutrophils and secondly in the lack of host monocyte-derived neutrophil chemokines as a consequence of a depletion of endobacteria-derived activators of monocytes. Thus, multiple mechanisms may be operative in neutrophil accumulation in the proximity of the worms. Bacterial LPS as well as lipoprotein have been shown to be major microbial mediators of macrophage activation inducing TNF- α and IL-8 formation [25]. Recently, LPS-like molecules were demonstrated in endobacteria-containing *O. volvulus* extracts [5, 6]. LPS exhibits strong macrophage- and neutrophil-activating but no neutrophil-chemotactic activity. Owashi et al. [26] cloned a neutrophil chemotactic factor from the *Wolbachia*-containing filaria *Dirofilaria immitis*, which contains a motif related to known bacteria-derived neutrophil chemotactic peptides [27, 28].

In tissue, numerous chemoattractants are thought to be operative and recently it has been demonstrated that leukocytes can integrate competing chemoattractant signals [29, 30]. Thus, leukocytes navigate to their destination through complex chemoattractant arrays in a step-by-step fashion thereby prioritizing newly arising attractants. This

process leads to neutrophil promotion by sequential chemotaxis to one attractant after another and allows cells to find their final target efficiently. While IL-8 is supposed to provide a more general recruitment signal to attract effector cells into the site of infection, the final orientation to the invading pathogen may be achieved by the 'end target-derived' chemoattractants. In the infected tissue, IL-8 can not only be produced by activated macrophages but also by TNF- α -exposed epithelial cells, fibroblasts and endothelial cells [28]. Taking these data into account, it is likely that in onchocerciasis, different chemoattractants are necessary for the fine-tuning of neutrophil accumulation around filariae.

The question arises whether neutrophil accumulation around filariae is only to be considered a defense mechanism beneficial to the host. This may be not the case, since despite encapsulation by neutrophil-containing tissue, adult worms live up to more than 15 years in an immunocompetent host environment. Possibly, neutrophils also contribute to the generation of a cyst observed at the anterior end of females which is filled with a semiliquid cell suspension. This cyst may aid the females to uptake soluble constituents of the host as nutrients and furthermore may facilitate mating with males. Thus, neutrophils recruited by pathogen-associated chemoattractants may contribute to the survival of the parasites. Furthermore, neutrophils have been incriminated in causing pathological manifestations in onchocerciasis [10, 12, 13, 31]. Pearlman [31] reported of a recruitment and involvement of neutrophils apart from eosinophils in experimental onchocercal dermatitis. It is of major interest to investigate a potential role of *Wolbachia* in neutrophil-derived pathogenesis in onchocerciasis.

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