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Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship

Nigel G. Langworthy[1], Alfons Renz[2], Ute Mackenstedt[2], Kim Henkle-Dührsen[3], Mark B. de C. Bronsvoort[1], Vincent N. Tanya[4], Martin J. Donnelly[1] and Alexander J. Trees[1]*

[1]*Liverpool School of Tropical Medicine and Faculty of Veterinary Science, University of Liverpool, Liverpool L3 5QA, UK*
[2]*University of Hohenheim, 70599 Stuttgart, Germany*
[3]*Bernard Nocht Institut fur Tropenmedizin, 20359 Hamburg, Germany*
[4]*Institut de Recherche Agricole pour le Développement, Wakwa, Cameroon*

Filarial nematodes are important and widespread parasites of animals and humans. We have been using the African bovine parasite *Onchocerca ochengi* as a chemotherapeutic model for *O. volvulus*, the causal organism of 'river blindness' in humans, for which there is no safe and effective drug lethal to adult worms. Here we report that the antibiotic, oxytetracycline is macrofilaricidal against *O. ochengi*. In a controlled trial in Cameroon, all adult worms (as well as microfilariae) were killed, and *O. ochengi* intradermal nodules resolved, by nine months' post-treatment in cattle treated intermittently for six months. Adult worms removed from concurrent controls remained fully viable and reproductively active. By serial electron-microscopic examination, the macrofilaricidal effects were related to the elimination of intracellular micro-organisms, initially abundant. Analysis of a fragment of the 16S rRNA gene from the *O. ochengi* micro-organisms confirmed them to be *Wolbachia* organisms of the order Rickettsiales, and showed that the sequence differed in only one nucleotide in 858 from the homologous sequence of the *Wolbachia* organisms of *O. volvulus*. These data are, to our knowledge, the first to show that antibiotic therapy can be lethal to adult filariae. They suggest that tetracycline therapy is likely to be macrofilaricidal against *O. volvulus* infections in humans and, since similar *Wolbachia* organisms occur in a number of other filarial nematodes, against those infections too. In that the elimination of *Wolbachia* preceded the resolution of the filarial infections, they suggest that in *O. ochengi* at least, the *Wolbachia* organisms play an essential role in the biology and metabolism of the filarial worm.

Keywords: *Onchocerca*; *Wolbachia*; macrofilaricide; tetracycline

1. INTRODUCTION

Filarial nematodes are globally distributed parasites of humans and animals, and a number of species, such as *Wuchereria bancrofti* and *Onchocerca volvulus* in humans, and *Dirofilaria immitis* (heartworm) in dogs, are serious pathogens, especially in warm climates. Some 17.7 million humans, mainly in sub-Saharan Africa, are infected with *O. volvulus*, the causal agent of 'river blindness', and hundreds of thousands have been blinded or suffered visual impairment (Molyneux & Davies 1997).

In spite of the huge socio-economic effects of this debilitating disease, no drug safe enough for mass chemotherapy is available to kill adult worms (i.e. is macrofilaricidal). The MACROFIL programme of the WHO, jointly sponsored by Tropical Diseases Research (TDR), the Onchocerciasis Control Programme and the African Programme for Onchocerciasis Control, is dedicated to discovering and developing a macrofilaricidal agent. *O. volvulus* does not infect animals other than humans and chimpanzees, so controlled studies are difficult. However, other species of the genus *Onchocerca* are all parasites of ungulates and we have developed the African bovine species, *O. ochengi*, as an analogue of *O. volvulus* for chemotherapeutic research (Trees 1992; Renz *et al*. 1995). *O. ochengi* is the closest related species to *O. volvulus*, shares the same vector, *Simulium damnosum*, and is common in cattle in northern Cameroon (Bain 1981; Xie *et al*. 1994; Wahl *et al*. 1994; Wahl 1996). Apart from its biological similarities with the target species, *O. volvulus*, *O. ochengi* is a valuable model because adult worms inhabit intradermal nodules and multiple infections occur—thus sequential nodulectomies can be done to reveal the kinetics of the response of the macrofilariae to drugs. In a series of chemotherapeutic studies, we have demonstrated the predictive value of this host–parasite system in experiments with drugs of known effect against *O. volvulus* (Renz *et al*. 1995; Trees *et al*. 1998) but no compound which is safe for mass usage has been efficacious against adults. The avermectins, which are the most potent nematode anthelmintics in veterinary use, are not macrofilaricidal, although they are microfilaricidal and prophylactic (Renz *et al*. 1995; Tchakouté *et al*. 1999).

Our interest in antibiotic therapy was initiated by the chance observation that, in an animal infected with over

*Author for correspondence (trees@liverpool.ac.uk).
†Present address: Institute for Genetics, Heinrich Heine University, Düsseldorf, Germany.

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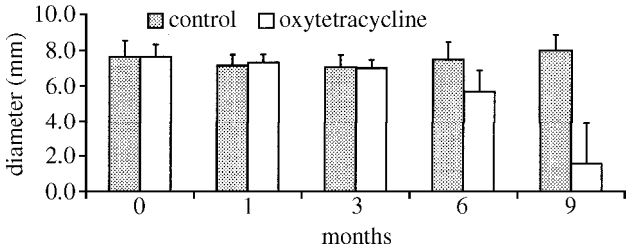

Figure 1. Diameter of *O. ochengi* excised nodules in oxytetracycline-treated and control cattle (each bar is the arithmetic mean of $n = 12$ nodules with standard deviation; except at nine mpt where $n = 4$).

100 *O. ochengi* nodules destined for a chemotherapeutic trial, persistent treatment with oxytetracycline for an incidental but debilitating dermatophytic infection, *Dermatophilus*, led to resolution of all nodules. This observation was intriguing since we have never observed spontaneous resolution of nodules in adult cattle used as controls in chemotherapy experiments. A possible explanation for this observation was the antibacterial activity of the tetracycline on intracellular bacteria. The existence of intracellular bacteria in *O. volvulus* was first reported in 1977 (Kozek & Marroquin 1977) but was somewhat neglected until more recently when various researchers cloned bacterial sequences from worm homogenates (Henkle-Dührsen *et al*. 1998) and increased awareness stimulated research on the phylogenetics of the *Wolbachia* in filariae (Sironi *et al*. 1995; Bandi *et al*. 1998). There is now great interest in the nature of the relationship of *Wolbachia* to their filarial nematode hosts, in their possible contribution to the pathogenesis of filarial disease and in their potential as a chemotherapeutic target (see review by Taylor & Hoerauf 1999). Here we report for the first time that antibiotic treatment is lethal to an adult filarial nematode. Intermittent treatment with oxytetracycline at normal dose rates over a six month period was macrofilaricidal to *O. ochengi* and we relate worm death to the presence of *Wolbachia* bacteria, the elimination of which preceded the death of worms.

2. MATERIALS AND METHODS

(a) *Study site/animals/treatment*

Female *Bos indicus* cattle, each naturally infected with over 20 *O. ochengi* nodules, were assigned to a control or an oxytetracycline treatment group ($n = 3$ per group). Nodule locations were mapped and 20 nodules in each animal were individually tattooed (three points around each nodule) and identified by the subcutaneous implantation adjacently of a microchip transponder (Animalcare Ltd, York, UK). Animals were kept at the Institut de Recherche Agricole pour le Développement station at Wakwa, near Ngaoundere, northern Cameroon, and the trial commenced in August 1997. Oxytetracycline was administered by intramuscular injection as follows; twice weekly for three weeks at 10 mg kg^{-1} (Terramycin[1] Q100; Pfizer, Sandwich, UK), then after a six week pause twice weekly for two weeks at 20 mg kg^{-1} (Terramycin[1] Long Acting; Pfizer, Sandwich, UK), then after a one month pause once monthly for three months. In addition, six infected cattle were treated with the avermectin, moxidectin (Cydectin; Fort Dodge, Southampton, UK) at 200 µg kg^{-1} either with a single dose or monthly for seven months.

(b) *Parasitology*

Four nodules (or nodule sites if the nodule had resolved) were removed from each animal per group at each time-point (12 nodules per group per time-point). Previous studies have shown that each nodule almost invariably contains one female worm; numbers of male worms average one per nodule but vary since males appear to migrate between nodules (Trees *et al*. 1992, 2000; Wahl *et al*. 1994). After nodulectomy under local anaesthesia, nodules were trimmed of extraneous tissue and measured, then the adult worms examined as previously described (Renz *et al*. 1995) for motility, ability to reduce the tetrazolium salt monotetrazolium (MTT) to formazan (Comley *et al*. 1989*a*,*b*) and state of embryogenesis (Schulz-Key 1988). Mid-body segments of worms were removed for electron microscopy (EM) and DNA studies, prior to homogenization of the remaining female body for embryogenesis determination. For EM, fragments of male and female worms, pre- and at intervals post-treatment, were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in graded ethanol and embedded in epoxy resin of low viscosity. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 902 (Carl Zeiss, Oberkochen, Germany). To determine the identity of the micro-organisms in *O. ochengi*, fragments of female worms were preserved and homogenized in guanidinium thiocyanate. From DNA, a fragment of the bacterial 16S rRNA gene was amplified using endobacterial primers as previously described (Henkle-Duhrsen *et al*. 1998). Skin microfilarial

Table 1. *Effects of oxytetracycline treatment of cattle on* O. ochengi *worm number and motility*

	control				oxytetracycline			
	no. of worms[a]		motility[b] (median)		no. of worms		motility (median)	
months	female	male	female	male	female	male	female	male
0	12	12	2	2	13	20	2	2
1	12	6	2	2	12	24	2	2
3	12	24	2	2	12	17	2	2
6	12	23	2	2	12	7	0	0
9	12	9	2	2	2	2	0	0

[a]From 12 nodules or nodule sites.
[b]Classification of motility scores: 0, no movements; 1, slow, occasional movement; 2, normal vigorous movements after 30 min incubation at 36° C.

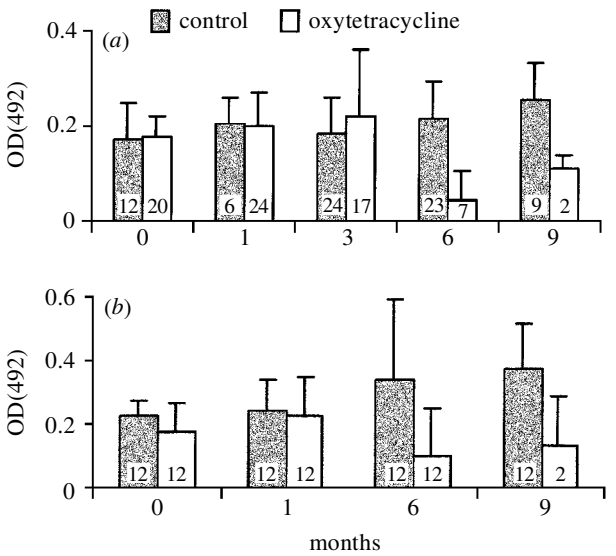

Figure 2. Viability of *O. ochengi* male (*a*) and female (*b*) worms based on reduction of the tetrazolium salt, MTT (dimethylthiozol to formazan). Each bar is the arithmetic mean (with standard deviation) of optical densities (at 492 nm) of formazan from worms or worm fragments adjusted to constant unit length (10 mm). Numbers of worms are shown in each bar.

densities were determined as previously described (Renz *et al.* 1995). Some additional nodules were excised, fixed in formol saline, sectioned and stained by haematoxylin and eosin for histological examination.

(c) *Statistical analysis*

The data were analysed using the Statview[1] program (SAS Institute, Cary, NC, USA). Treatment effects on mean nodule size and on MTT reduction activity were assessed using a two-way ANOVA test with treatment group and month as factors. A Tukey–Kramer *post hoc* test was performed to determine at which time-points there were significant differences between groups.

3. RESULTS

The first effects of treatment were observed at three months post start of treatment (mpt), when three out of 29 worms were immobile and another three showed reduced motility. At six mpt, nodules were significantly smaller in treated animals (ANOVA, $p < 0.001$; Tukey–Kramer *post hoc* test, $p < 0.001$) (figure 1), and fewer male worms were recovered (table 1). Five out of seven males recovered were immotile, one showed limited movement and only one was fully motile (table 1). Three out of 12 nodules contained only fragments of dead female worms and other female worms recovered were immotile and MTT reduction was significantly reduced (ANOVA, $p < 0.001$; Tukey–Kramer *post hoc* tests, $p < 0.05$; figure 2*b*). By nine mpt most nodules had resolved, were not palpable and their position could only be located by reference to mapping, transponder location and tattoo marks. Two immobile males were recovered. Two females could be recognized in skin biopsies and both were disintegrated, immotile and non-viable based on MTT reduction.

Changes in embryogenesis paralleled but preceded effects on adult worms (figure 3). There was a decline in the quantity of all embryonic stages by three mpt and an increase in the proportion of stages showing abnormal morphology. Normally developing embryos were not detected at or after six mpt. Histologically, early pathological changes were seen in adult worms at two mpt, with the formation of vacuoles in the hypodermis and in the oocysts (figure 4), the malformation of embryonic stages and the degeneration of hypodermic nuclei. Eosinophils invaded the nodules and were attracted in large number close to the worms. Microfilarial densities in skin progressively declined; very few microfilariae were found at six and nine mpt and none were detected at 12 mpt (figure 5). In contrast, in untreated control animals there were neither macro- nor microfilaricidal effects nor significant changes in embryogenesis. In addition, in the six cattle concurrently treated with moxidectin there was no effect on nodule diameter, worm number, worm motility or MTT reduction up to 12 mpt, although microfilariae were eliminated from the skin and embryogenesis showed changes characteristic of avermectin treatment.

EM revealed the abundant presence of micro-organisms in worms before treatment, especially in the hypodermis (figure 6*a*,*b*), but also in different developmental stages in embryogenesis. The size of the oval-shaped bacteria ranged from 0.3 to 0.6 µm. They divided within vacuoles, such that some of them contained up to seven bacteria. To determine their identity, a segment of the bacterial 16S rRNA gene from pre-treated worms was isolated, cloned and sequenced (GenBank accession AF 172401). The sequence of this segment of 858 nucleotides confirmed the bacteria as *Wolbachia* as previously sequenced and described for *O. ochengi* (Bandi *et al.* 1998) and showed only one nucleotide difference from the homologous sequence of the *Wolbachia* of *O. volvulus* (Henkle-Dührsen *et al.* 1998).

Sequential EM examination of transverse sections of the hypodermis in the mid-body of the male and female worms showed degenerating bacteria in the hypodermis (figure 6*c*,*d*) at one and two mpt, respectively. Initially, the bacteria reduced in size and assumed an irregular shape. Finally, vacuoles contained only remnants of bacteria, as electron-dense material and membrane whirls. Some of the vacuoles did not show any bacterial remnants. The hypodermis was virtually free of bacteria at two mpt.

4. DISCUSSION

These data demonstrate an unequivocal macrofilaricidal effect of oxytetracycline *in vivo* against *O. ochengi*, the most closely related parasite to *O. volvul*us. Worms from established, gravid infections were killed and their nodules resolved. Although the number of oxytetracycline-treated cattle was small ($n = 3$), their entire worm burdens were eliminated. In contrast, no macrofilaricidal effect was seen in three, untreated controls, and none was observed in six cattle concurrently treated with moxidectin and observed over the same period (data not shown). Moreover, spontaneous resolution of nodules has never been seen by us in a total of 24 untreated control cattle observed in eight experiments conducted over a period of five years (Trees *et al.* 2000). The activity of oxytetracycline is hypothesized to be due to its effects on the *Wolbachia* organisms

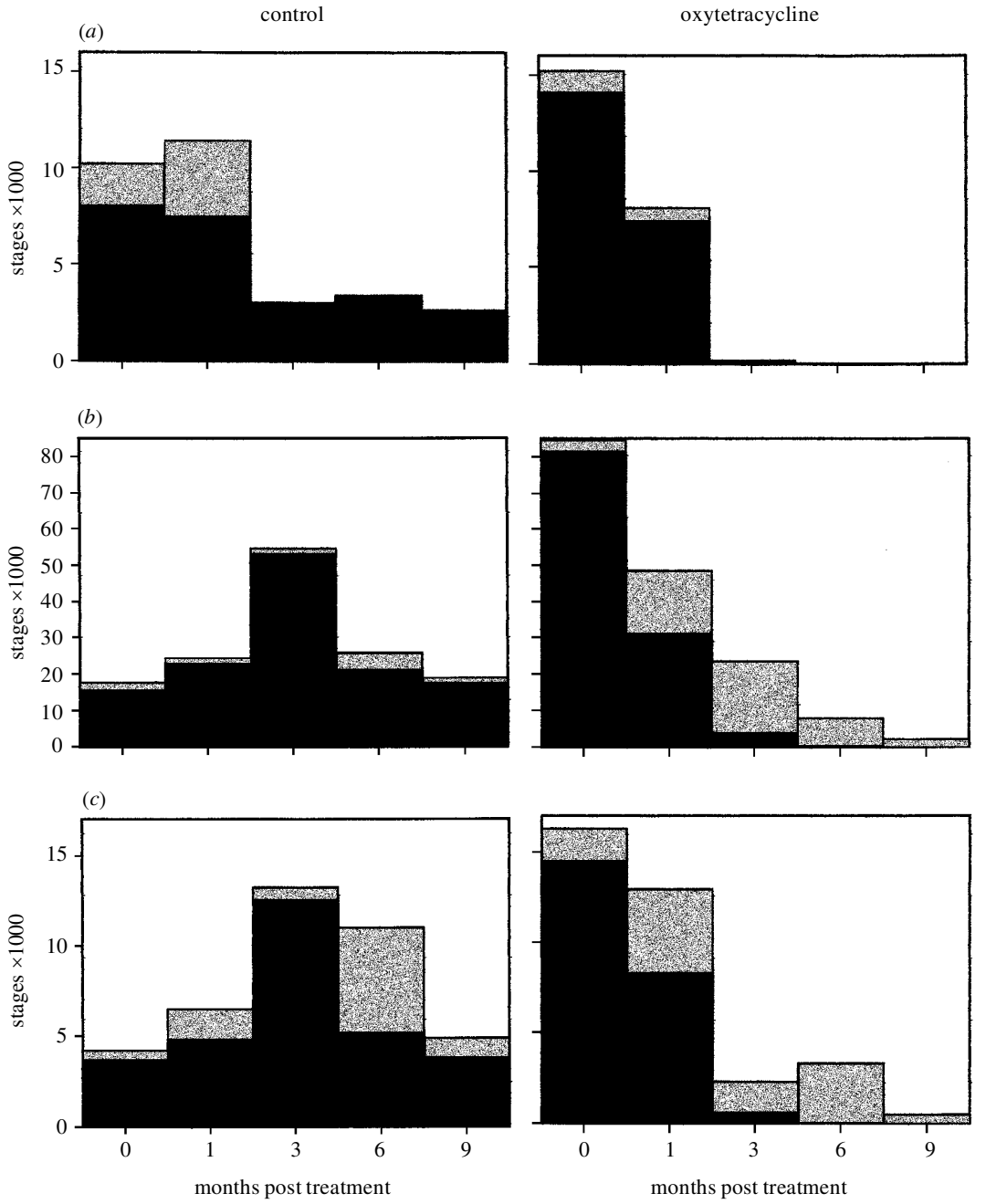

Figure 3. Effects of oxytetracycline treatment on embryogenesis in *O. ochengi* female worms. For each time post treatment, the mean number (× 1000) of morphologically normal (solid) and abnormal (shaded) forms in each of three developmental stages are shown. (*a*) Oocytes, (*b*) developing stages, (*c*) intrauterine microfilariae. In each case $n = 12$, except in oxytetracycline-treated animals at nine mpt, where $n = 2$.

common in *O. ochengi* tissues, although we cannot completely eliminate the possibility that there is some direct effect of oxytetracycline on worm viability. However, the sequential changes in the physical integrity of the *Wolbachia* organisms observed by EM, which preceded the death of worms, support this hypothesis. The effects on adults, intrauterine stages and microfilariae were concurrent and progressive. This is consistent with the fact that *Wolbachia* organisms are transovarially transmitted and, in *O. volvulus*, are present in all stages including oocytes, microfilariae and adults (Kozek & Marroquin 1977; Henkle-Dührsen *et al.* 1998). Based on small subunit rRNA gene sequencing, the *O. ochengi Wolbachia* is closely related to that of other *Onchocerca* spp. (as shown by Bandi *et al.* 1998). Moreover, the existence of an almost identical organism in *O. volvulus* suggests that tetracycline treatment may also be macrofilaricidal in humans. Moreover, *Wolbachia* organisms have been identified in a number of filarial species including *Dirofilaria immitis*, *Brugia malayi*, *B. pahangi*, *Litomosoides sigmodontis* and *Wuchereria bancrofti* (Sironi *et al.* 1995; Bandi *et al.* 1998; Hoerauf *et al.* 1999; Taylor *et al.* 1999), as well as a number of *Onchocerca* spp. (Henkle-Dührsen *et al.* 1998; Bandi *et al.* 1998), suggesting the possibility of a new approach to the chemotherapy of a range of filarial infections. In experimental infection of jirds with *Brugia*, tetracycline was found to be prophylactic (although not curative) (Bosshardt *et al.* 1993). Recent studies on oxytetracycline therapy *in vivo* with *L. sigmodontis* in mice demonstrated filarial growth arrest and infertility (Hoerauf *et al.* 1999). Crucially, tetracycline

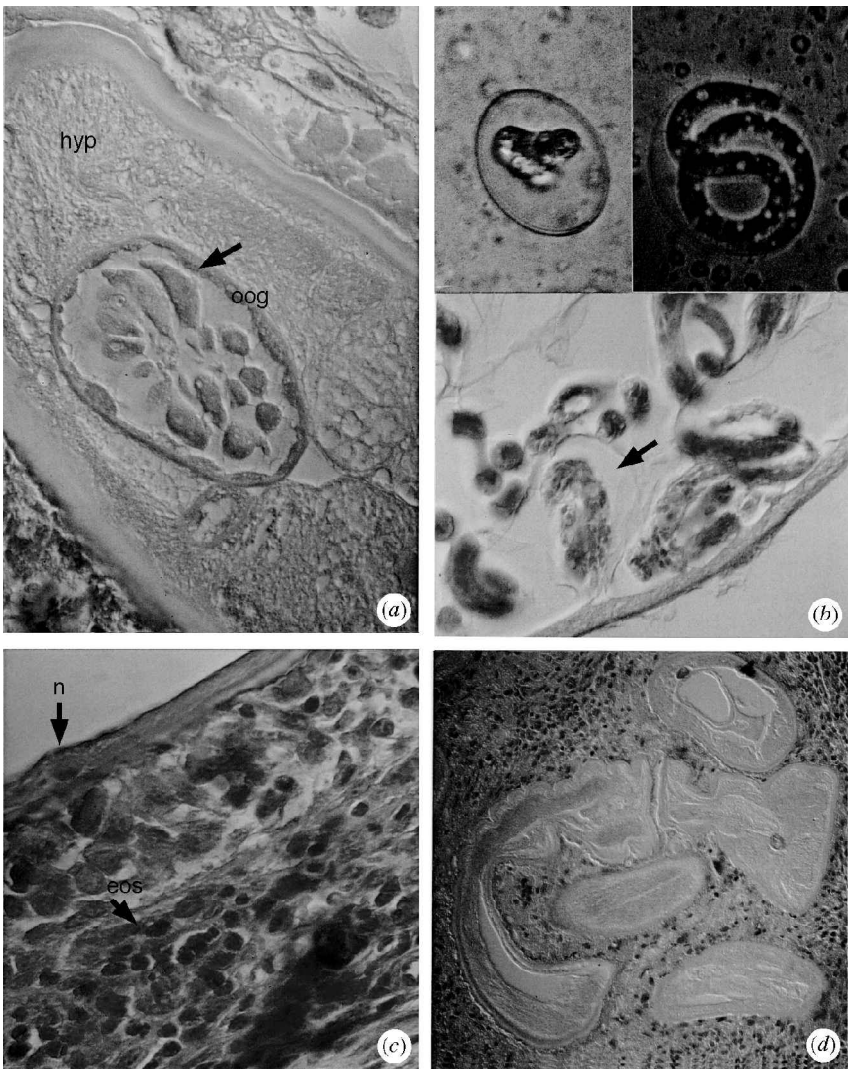

Figure 4. Light microscopy of uterine embryonic stages (*b*, upper left and upper right insets) and histological sections (*a*, *b* bottom image, *c*, *d*) of tetracycline-treated worms. (*a*) Enlarged hypodermis (hyp) and formation of vacuoles in oogonia (oog), at two mpt (× 600). (*b*) Vacuolated (upper left inset) and degenerating horseshoe-shaped and coiled microfilariae (upper right inset) at two mpt (phase contrast); below, degenerating intrauterine microfilariae (differential interference contrast) (× 630). (*c*) Invasion of eosinophils (eos) near degenerating female at three mpt. Inner worm is top left with thin hypodermis containing a degenerating nucleus (n) and below it cuticular debris is being shed into the hypercellular nodule tissue (× 440). (*d*) Collapsed, dying or dead female with empty uteri at six mpt (differential interference contrast) (× 150).

had no effect on *Acanthocheilonema viteae*, which does not carry *Wolbachia* endosymbionts, and other antibiotics, ineffective against rickettsial bacteria, did not affect *Litomosoides* development (Hoerauf *et al.* 1999), evidence that the anti-filarial effects were mediated through action on the *Wolbachia* bacteria. Tetracycline has also been shown to adversely affect embryogenesis in *B. pahangi* and *D. immitis* (Genchi *et al.* 1998; Bandi *et al.* 1999), and to be prophylactic against mite-induced infections of *L. sigmodontis* in jirds (McCall *et al.* 1999).

The lack of macrofilaricidal effect reported in these host–parasite systems may partly reflect the difficulty in continuing therapy over a prolonged period. Our results with *O. ochengi* in cattle described here (and of subsequent work, J. Gilbert, A. Renz, V. Tanya and A. J. Trees, unpublished data) indicate that filarial death is slow and only follows sustained periods of chemotherapy. This may indicate that worm attrition is mediated by indirect rather than direct effects of bacterial elimination. Moreover, differences in filarial response to antibiotic therapy may reflect different degrees of mutualism between the nematodes and *Wolbachia*. Some species, e.g. *O. flexuosa* and *A. viteae*, appear not to be infected and in others, e.g. *Brugia*, the intensity of infection in male worms may be low (Taylor & Hoerauf 1999). In contrast, our observations here and other studies by EM or polymerase chain

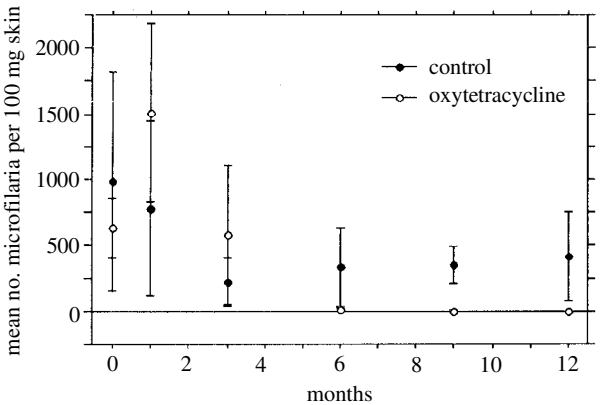

Figure 5. Effect of oxytetracycline treatment on *O. ochengi* skin microfilarial density. Mean microfilarial density s.e. per 100 mg of skin; three cattle per group.

reaction (see Trees *et al.* 2000) have indicated that *Wolbachia* are both abundant and prevalent in 100% of *O. ochengi*. Taken together with the effect of antibiotic chemotherapy, the results suggest that in *O. ochengi*, the *Wolbachia* play an essential role in worm survival. This may involve synthesis of detoxifying enzymes, e.g. catalase (Henkle-Dührsen *et al.* 1998), necessary to modulate host immune molecules. Alternatively, they may have a pivotal role in nutrition, since a primary route of uptake

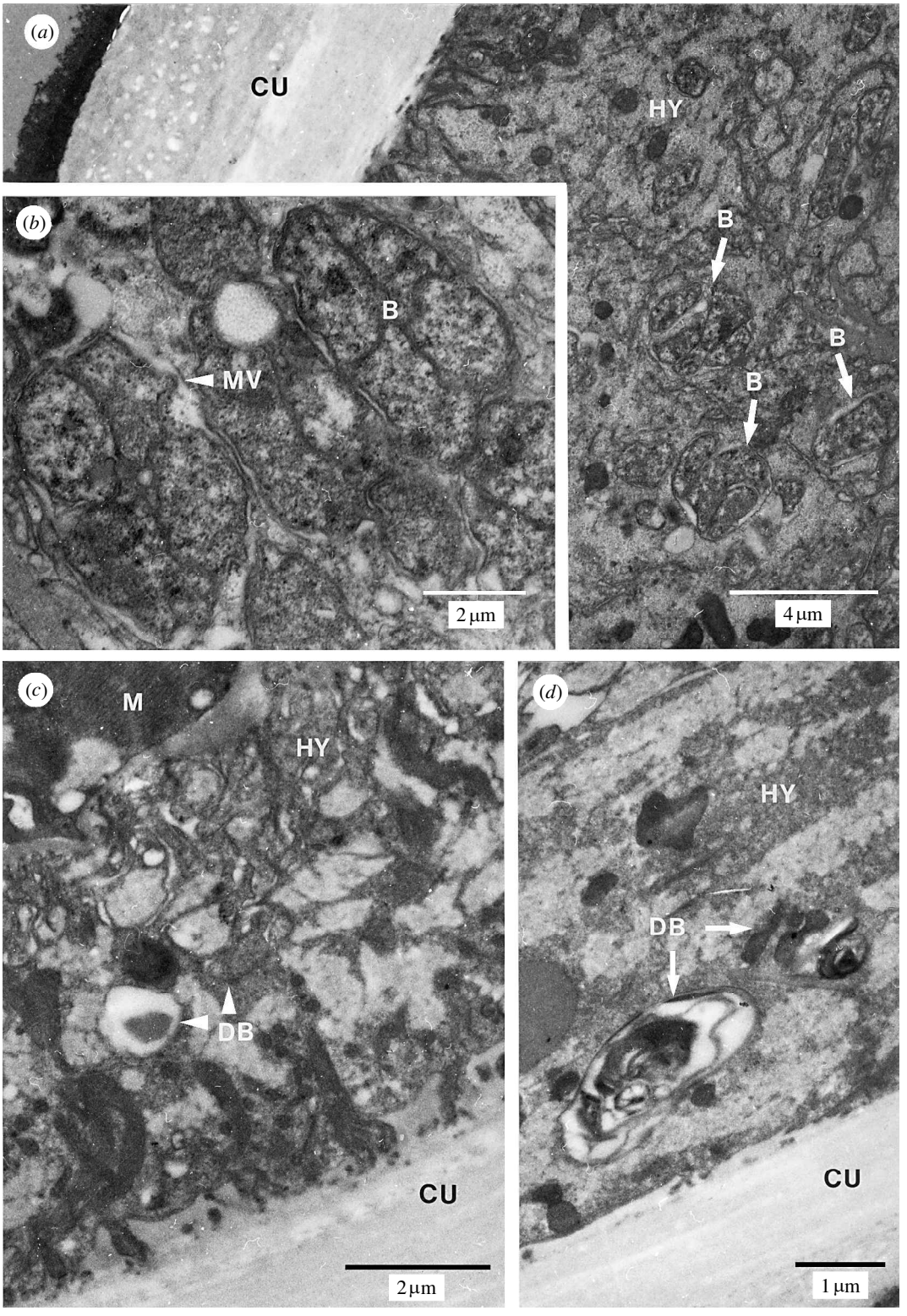

Figure 6. Transmission electron micrographs of *O. ochengi* adult worms. (*a*) Control; hypodermis of *O. ochengi*. Note the numerous bacteria distributed in the hypodermis. (*b*) Control: bacteria are enclosed by a membrane. (*c*) Hypodermis of *O. ochengi* at one mpt: bacteria are shown at different stages of degeneration (arrow heads). (*d*) Hypodermis of *O. ochengi* at two mpt: vacuoles contain remnants of degenerating bacteria. Note the membrane whirls in the vacuoles. Key: CU, cuticle; B, bacteria; HY, hypodermis; DB, degenerating bacteria; M, muscle; MV, membrane of vacuole.

of nutrients in filariae is transcuticular (Howells & Chen 1981) and the *Wolbachia* organisms are concentrated in hypodermal tissue.

That the macrofilaricidal effects were observed only after repeated treatment over several months explains why an incidental effect on human filariasis following anti-microbial chemotherapy has not been reported previously. Whether or not a practicable and efficacious treatment regimen can be achieved using current antibiotics requires further research, but these data, and other results (Hoerauf *et al.* 1999; Bandi *et al.* 1999) indicate an important new target for anti-filarial

chemotherapy, and hold promise of a safe, effective macrofilaricide. In 1977, the original description of intracytoplasmic bacteria in *O. volvulus* (Kozek & Marroquin 1977) concluded in discussion that 'if the . . . endosymbionts are essential for the survival of the filarid, this . . . could be exploited for chemotherapeutic purposes, suggesting the development of macrofilaricidal compounds the action of which would be directed against [them]'. That neglected statement may yet prove prophetic.

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