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Efficacy of 5-week doxycycline treatment on adult *Onchocerca volvulus*

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Abstract The effects of 5-week doxycycline treatment on the depletion of *Wolbachia* endobacteria from *Onchocerca volvulus*, on the interruption of embryogenesis and on microfilariae production, and with regard to macrofilaricidal activity were studied. In 2003, in an endemic area in Ghana, 22 onchocerciasis patients received 100 mg/day doxycycline for 5 weeks. Two years after the start of the study, 20 treated and ten untreated patients were nodulectomized and skin microfilariae were counted. The onchocercomas were examined by immunohistology for the presence of *Wolbachia*, embryogenesis, and vitality of adult filariae. The latter two parameters were further assessed by alternating logistic regression analysis, taking into account the dependency of worms and nodules in patients. Doxycycline resulted in depletion of *Wolbachia* and in complete interruption of embryogenesis in all worms that were assumed to have been present during treatment. In the treated patients, only 51% of the female worms were alive, compared to 84% in the untreated patients, indicating a moderate but distinct macrofilaricidal activity of doxycycline at this dose. It is concluded that, in areas with ongoing transmission, doxycycline cannot replace regular ivermectin mass treatment because new infections would require repeated rounds of doxycycline. However, doxycycline can be used for the treatment of individuals outside transmission areas, in foci where ivermectin resistance may occur, and in countries where onchocerciasis and loiasis are co-endemic.

Abbreviations

APR	*O. volvulus* aspartic protease
DiWsp	*D. immitis Wolbachia* surface protein
L3	infective larvae of *O. volvulus*
mf	microfilaria (e)

Introduction

Onchocerciasis is endemic in many sub-Saharan countries and in minor foci in Latin America and Yemen (WHO 1995b; Boatin and Richards 2006). The number of

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infections has recently been re-estimated to be more than 37 million (Basañez et al. 2006). The adult filariae, which have developed from the third stage larvae (L3) transmitted by blackflies (*Simulium*), have an average life expectancy of 10 years (Habbema et al. 1990) and produce millions of microfilariae (mf). The mf cause dermatitis, skin atrophy, and inflammation in the eyes, leading to reduced vision and blindness. Apart from blindness, a high mf load has been identified as a factor reducing the life span of infected people (Little et al. 2004). Vector control and mass treatment with ivermectin have been successfully used to regionally reduce the burden of this neglected disease.

Current elimination programmes in America and Africa are based on ivermectin mass treatment. Ivermectin has strong microfilaricidal and temporarily sterilizing activity (Awadzi et al. 1999). It has to be administered for many years until all adult filariae are sterile or dead, because the worms resume their fertility after interruption of ivermectin treatment. After administration of frequent doses of ivermectin every 3 months, moderate macrofilaricidal activity is observed (Duke 2005; Gardon et al. 2002; Kläger et al. 1993). However, the Conference on the Eradicability of Onchocerciasis (Dadzie et al. 2003) concluded that eradication would not be achieved at coverage rates of 65% for 35 or more years. Further, it has been reported that some populations of the intestinal nematode *Haemonchus contortus* in sheep have developed a certain degree of ivermectin resistance (Coles et al. 2005). Since genetic selection of low fertile *Onchocerca volvulus* by ivermectin treatment has been observed, it was hypothesized that *O. volvulus* populations more tolerant to ivermectin may also be selected (Bourguinat et al. 2007). Epidemiological hints for a suboptimal performance of ivermectin against *O. volvulus* populations have been detected in northern Ghana (Awadzi et al. 2004a,b; Osei-Atweneboana et al. 2007). Therefore, alternative drug regimens must be identified before ivermectin resistance may develop and spread. It has been a long-standing aim to develop a safe macrofilaricidal or permanently sterilizing drug.

Doxycycline has been introduced as a novel chemotherapeutic principle, targeting *Wolbachia* endobacteria in the worms (Hoerauf et al. 2000, 2001, 2003). Given for 6 weeks at 100 mg/day, doxycycline led to *Wolbachia* depletion and sterilization of female filariae up to 18 months. There was also a reduction in the proportion of living female and male worms at this point of time, but the number of extirpated worms was too small to allow the definite conclusion of a macrofilaricidal effect. However, a macrofilaricidal activity of doxycycline was demonstrated against *Wuchereria bancrofti* (Taylor et al. 2005; Debrah et al. 2006b), and recently also against *O. volvulus*, albeit at a higher daily dose of 200 mg/day, also after a longer observation period (Hoerauf et al. 2008).

Therefore, the present study was conducted focusing on the assessment of a macrofilaricidal and long-term sterilizing activities after the longer observation time of 21 and 27 months compared to the earlier study (Hoerauf et al. 2000, 2001, 2003). Second, since we knew that a doxycycline treatment shorter than 6 weeks also depletes *Wolbachia* (Debrah et al. 2006a), the regimen was reduced to 5 weeks at only 100 mg/day of doxycycline. Another aim was to analyze the efficacy of doxycycline alone without administration of ivermectin during the trial. Here we report on an open trial in Ghana, comparing treatment with doxycycline at 100 mg/day for 5 weeks against a control group without treatment.

Materials and methods

Study area

This open trial was conducted between 8/2003 and 11/2006 in the Assin district, Central Region of Ghana. This focus lies south of the Onchocerciasis Control Programme in West Africa (OCP) and hence transmission is ongoing. In 1999, a rapid assessment of 30 men older than 19 years had identified many villages in Assin as hyperendemic. In the selected study village Awisem, nodule and mf-carrier rates both were 74% (R. Horstmann and D. W. Büttner, Bernhard Nocht Institute). Awisem is situated 1 km west of the River Pra, where *Simulium sanctipauli* vectors breed (map and vector epidemiology see Kutin et al. 2004). Ivermectin mass treatment started in Assin in 1999, but due to the remoteness of the village it proceeded at a low coverage until the end of this study (R. Garms, Bernhard Nocht Institute, unpublished report, 2006). A reduction of the infectivity rates and the loads of L3 in *Simulium* was not observed by Garms and colleagues in 2002 (Kutin et al. 2004) and also not in 2006 (R. Garms, unpublished report, 2006).

Ethical aspects and informed consent

The study was approved by the Ethical Research Committee of the School of Medical Sciences of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The study conformed to the principles of the Helsinki Declaration of 1975 (as revised 1983, 2000, and 2002). To inform the villagers, there was first a meeting held with village elders, where the study procedures and rationale were explained. After general consent obtained by the elders to work in the community, the study procedures were explained to interested people in English by the trial physician and then again in the local Twi language by YMD. Informed consent was obtained from participants and documented by the signatures of two witnesses.

Inclusion and exclusion criteria

Individuals eligible for participation were adult men aged 18–62 years. Women older than 40 years who declared to be in their post-menopause were also included. Participants with a body weight of at least 40 kg, in good health without any clinical condition requiring chronic medication, had to have at least two palpable nodules (onchocercomas) at different sites of the body.

Exclusion criteria encompassed a history of intolerance to doxycycline and of alcohol or drug abuse. Other exclusion criteria were assessed by dipstick chemistry (Reflotron®, Roche, Mannheim, Germany): pregnancy (in case of wrong oral declaration of being post-menopausal), hepatic and renal enzymes above normal values (γ-GT [0–28 IU/L], ALT [0–45 IU/L], and creatinine [53–126 µmol/L]).

Patient treatment and nodulectomies

Twenty-four volunteers were included in the study (Fig. 1). They received 100 mg capsules of doxycycline daily for 5 weeks (Vibramycin®, supplied by Pfizer, Karlsruhe, Germany). Doxycycline was administered after patients had taken a meal. Treatment was monitored daily by a physician (MB). In eight cases of unplanned absence (e.g., due to travelling), the patients were given extra daily doses at the end of the 5 weeks. The maximum of extra doses were three. Two individuals did not complete the treatment. All participants were requested not to take part in ivermectin mass treatments during the study. Fulfillment of this request was checked by questioning. This request was justified since doxycycline leads to long-term sterilization in contrast to ivermectin that causes, after the first doses, only an incomplete and temporary interruption of embryogenesis (Duke et al. 1991, 1999). Thus, the planned doxycycline was expected to supersede the efficacy of ivermectin. Participants received ivermectin from us after the nodulectomies at 27 months (11/2005) and after re-examination at 39 months (11/2006).

At 21 and 27 months after the beginning of doxycycline treatment, nodulectomies were performed aseptically under local anesthesia in the Dunkwa Regional Hospital, as described (Albiez et al. 1988a). Five of the 20 doxycycline patients were operated at both points of time. Wound treatment was begun in the hospital and continued in the village by the trial physician.

In addition to the doxycycline-treated patients, ten patients from the same village, who had not been enrolled in the study and had received neither doxycycline nor ivermectin, were enrolled for nodulectomy at 27 months.
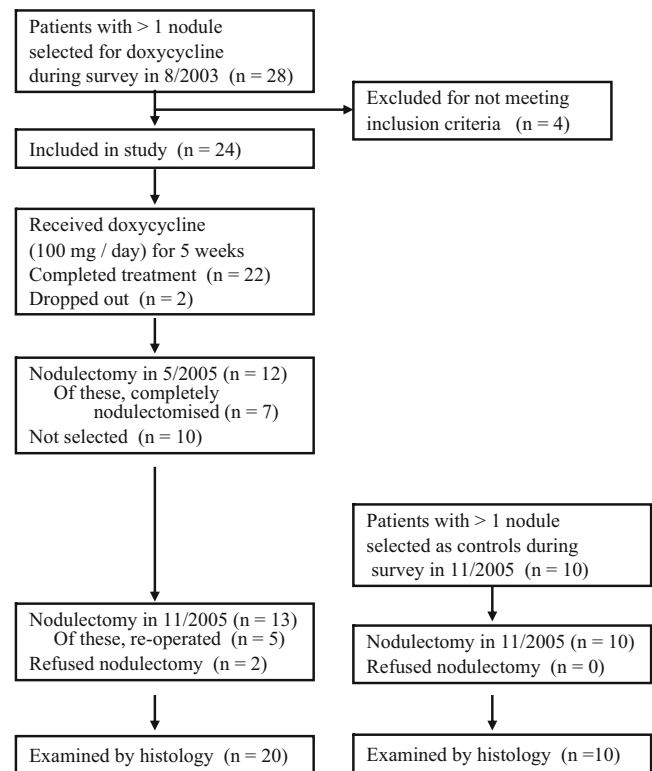

Fig. 1 Trial profile of patients

Objectives

Previously, we had studied the efficacy of 100 mg/day doxycycline administered for 6 weeks (Hoerauf et al. 2000, 2003). The aim of the present study was to investigate the effects of a shortened regimen of only 5 weeks doxycycline treatment on the depletion of Wolbachia endobacteria from O. volvulus, on interruption of embryogenesis and mf production, and to detect macrofilaricidal activity. We deliberately chose to nodulectomize the patients after a longer period of observation than in the preceding study, namely at 21 and 27 months, since we knew from this previous study as well as from unpublished studies (Hoerauf et al. 2003; in preparation) that the depletion of Wolbachia and the interruption of embryogenesis could still be observed up to 27 months but macrofilaricidal activity would become detectable only later than 11 months, e.g., at 18, 21, or 27 months.

Outcome measurements

The primary outcome measurements were female worm fertility, adult worm survival, and the presence of Wolbachia endobacteria. All parameters were assessed by immunohistology of extirpated nodules. The secondary measurement was the analysis of skin mf from two skin

snips that were taken from the doxycycline-treated patients at study onset as well as at 21 and/or 27 months later.

Sample size

The sample size was planned on the basis of previous histological analyses: accordingly, 50–100 female worms should be available per treatment arm to detect significant differences in embryogenesis and vitality of female worms (Albiez et al. 1988b, Awadzi et al. 1995a, b, 1999; Büttner et al. 1988; Hoerauf et al. 2003). These worm numbers were obtained for both the doxycycline and the untreated group.

Immunohistology

Immediately after extirpation in the operation, theater nodules were fixed in 80% ethanol or in buffered 4% formaldehyde solution. They were embedded in paraffin. Smaller nodules were totally embedded. From medium-sized and large nodules, the outer portions were cut from two sides to obtain the middle portion of the nodule on the level of the two largest diameters. These portions were about 2–4 mm thick, depending on the size of the nodule. From a few larger nodules, two portions were embedded. Because of the damage caused by the cutting, the first sections were discarded. Nodules with many calcified worms were examined using a dissecting microscope, deparaffinized, then decalcified using EDTA, and newly embedded. Sections were stained by hematoxylin and eosin, and some sections by Gomori's method for iron, to differentiate old and young new worms (see below). For immunostaining, the alkaline phosphatase anti-alkaline phosphatase technique was applied according to the manufacturer's instructions (DakoCytomation, Hamburg, Germany). To demonstrate the presence of *Wolbachia*, a rabbit anti-serum against *Dirofilaria immitis Wolbachia* surface protein (DiWsp) was used diluted 1:1,000 (Bazzocchi et al. 2000). Vitality of adult worms, oocytes, and embryos was assessed with rabbit anti-serum against a cathepsin D-like lysosomal aspartic protease of *O. volvulus* (APR) diluted 1:1,000 (Jolodar et al. 2004). This antibody helped also to differentiate the worms and to count them. As secondary antibody, an anti-rabbit mouse monoclonal antibody was used (clone MR12/53, DakoCytomation). Fast Red TR salt (Sigma, Deisenhofen, Germany) was applied as chromogen and hematoxylin as counter stain (Merck, Darmstadt, Germany).

Assessment criteria were used as described (Büttner et al. 1988; Hoerauf et al. 2003). Ten or more sections from each nodule were independently analyzed by two persons. Counting of different female worms was easy since the average numbers of all females per nodule were only 1.5

for untreated and 1.7 for doxycycline-treated patients. For larger nodules, we used the localization in different worm centers divided by connective tissue, the size of the worms and of their organs, the morphology of the worms, the status of embryogenesis, the pigmentation of the intestine and the uterus muscles, the vitality, and the degree of APR labelling. The characteristics for worm death included completely calcified worm portions, remnants of the cuticle of nearly absorbed filariae, loss of body wall integrity, loss of nuclei, and absence of APR staining (Figs. 2, 4, and 5; Jolodar et al. 2004). Degenerated worms still APR positive were considered moribund (Duke et al. 2002; Gardon et al. 2002) and also classified as "dead". However, this criterion was only used for five worms with neoplasms (Duke et al. 2002; Fig. 3) and one other worm after doxycycline. 'Living' means alive at the time of nodulectomy. All embryonic developmental forms from the stage of two cells to the stretched mf in the uterus were classified as 'embryos' (Büttner et al. 1988; Hoerauf et al. 2003). At the time when the nodules were extirpated, 25–45 months had passed since those patients, who had taken ivermectin before the onset of this study, had taken their last dose. It is known that after such a long interval the influence of ivermectin on embryogenesis and mf production is reduced or nearly nil (Albiez et al. 1988b, detailed Duke et al. 1991). For the analyses, the nodules extirpated from doxycycline-treated patients at 21 and 27 months were summarized in Tables 2–5. Regarding the primary objectives addressed in this study, a period of 6 months between the nodulectomies is short and we do not know of any relevant effect of a nodulectomy a few months previously on other adult *O. volvulus* in the patients nodulectomized twice.

Determination of microfilariae loads

Microfilarial loads of treated patients were assessed before and at 21 and/or 27 months after the start of doxycycline administration. From patients who only attended one of the two follow-up points of time, the available skin mf value was taken for analysis. From those who attended both follow-up times, one value was randomly selected. For mf analysis, two skin biopsies of 1–3 mg were taken from the buttocks using a Holth punch. Each biopsy was immersed in 0.9% NaCl solution in a well of a microtiter plate (Nunc, Roskilde, Denmark). The biopsies were incubated at room temperature for 6 to 20 h. The solution was then transferred onto a slide for microscopic examination. The biopsies were weighed using a Sartorius electronic balance (Göttingen, Germany). Microfilariae per milligram of skin were calculated as median and geometric mean. For the latter, the calculation according to Williams was performed as used by WHO (Remme et al. 1986). Since the geometric mean

Fig. 2 Signs of certain death are entirely calcified worm portions (*arrowhead*) or small cuticular fragments of nearly absorbed filariae (*arrows*)

Fig. 3 Moribund worms with pleomorphic neoplasms as described by Duke et al. (2002) (*arrowhead*) will definitely die even when the APR-positive posterior ends appear undamaged (*arrows*). Untreated patient

Fig. 4 A certainly dead APR-negative worm without organs and surrounded by a strong cellular reaction (*arrowhead*) and a living filaria with an APR-positive intestine (*arrows*) and degenerated embryos in both uterus branches (*u*)

Fig. 5 A dead APR-negative worm with disintegrated organs (*arrowhead*) and a living APR-positive filaria (*arrow*) with empty uterus branches (*u*). All images except Fig. 3 from patients treated 5 weeks with 100 mg/day doxycycline. Immunohistology using serum against a lysosomal aspartic protease (APR). *Scale bar=* 100 μm
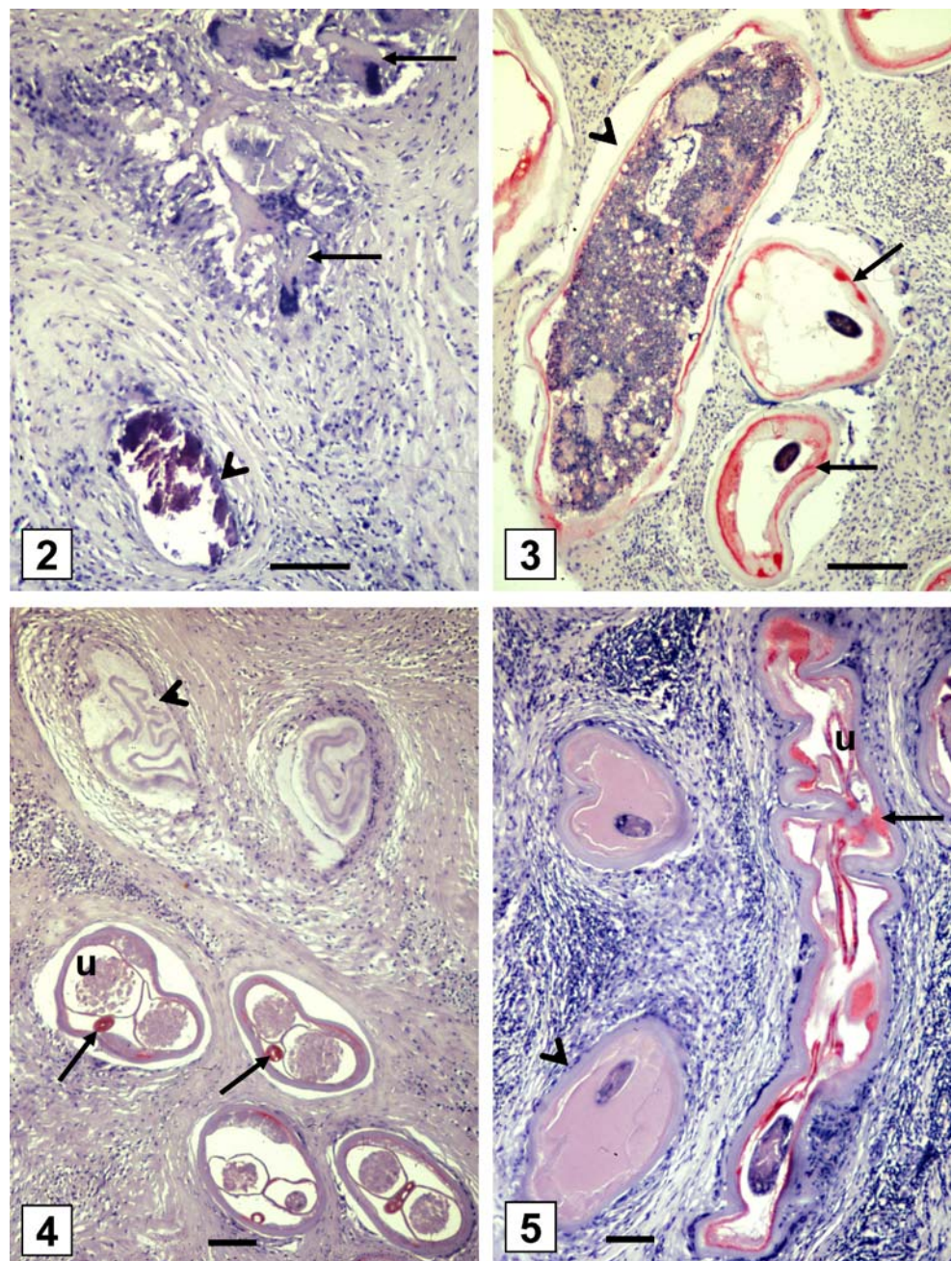
cannot use zero, the calculation uses mf +1 values, and from the calculated mean, 1 is subtracted afterwards.

Statistical methods

The Wilcoxon signed rank test was used to test for a change of mf loads in Table 4. The chi-square test and the Fisher's exact test were applied for qualitative data. In addition, in order to take into account possible dependencies between the nodules achieved from one patient (as described, e.g., by Duerr et al. 2001), alternating logistic regression, as implemented in the SAS procedure Genmod, was used to analyze the rate of normal embryogenesis, living female worms, and the presence of nodular mf. For the calculations, StatView® software version 4.5 and SAS 9.1 were used.

Results

Participant flow

Two of the enrolled 24 patients were excluded because they were absent too often during treatment (Fig. 1). Two patients who had completed the doxycycline treatment

refused nodulectomy. All palpable nodules of the remaining 20 doxycycline and ten untreated patients were excised and analyzed by histology. One year after the last nodulectomies, 12 of the 20 doxycycline patients had no palpable nodules, five had tiny new nodules, and only one patient had one old nodule on the knee that had not been extirpated. Therefore, we consider the examined nodules as representative for these patients.

Baseline data

The characteristics of those nodulectomized patients, who had completed the doxycycline trial or were included as untreated patients, are shown in Table 1. Among the 22 doxycycline-treated participants, 14 declared that they had taken one dose of ivermectin previously; three patients had taken two doses. These patients had taken the last dose(s) of ivermectin four ($n=2$), eight ($n=1$), 18 ($n=11$), or 6 and 18 months ($n=3$) before enrolment. Five doxycycline-treated and all untreated patients had never taken ivermectin. This prior ivermectin intake had affected skin mf loads (Table 1, see different mean mf load in the groups with and without prior ivermectin) However, since the patients had taken ivermectin only once or twice, this drug intake is not considered to have disturbed female worm fertility after another 21–27 months (Gardon et al. 2002; Duke 2005), the times when the nodules were ectomized and analyzed in this study. Therefore, it is concluded that a combined analysis of all doxycycline patients is justified.

Skin manifestations of onchocerciasis were mild. Two individuals had leopard skin.

Effect of doxycycline on *Wolbachia*

For *Wolbachia* assessment, worms were classified as having many, few, or no bacteria (Table 2). In the untreated patient group, only in 2% of living females and in 14% of male worms no endobacteria were detected. In contrast, in nodules from doxycycline-treated patients, 76% female and 62% male worms did not contain *Wolbachia*. However, we did observe 16% of living females and 16% of living males with many bacteria. While we do not formally prove it here, we assume that most or all of the worms with many *Wolbachia* had been acquired after the end of the doxycycline treatment, since they were juvenile or showed all characteristics of young worms. Also, an overall 10% replenishment of worms per year can theoretically be assumed (Schulz-Key and Soboslay 2000; see details in the Discussion section).

Effect of doxycycline on filarial embryogenesis and microfilarial load

Table 3 shows the counted, raw data for the worms and nodules (i.e., not the *calculated* means±confidence interval obtained through multiple logistic regression, as in Table 7). Accordingly, in the untreated group, 45% living female worms showed normal embryogenesis and 34% of the nodules contained living mf in the host tissue. In contrast, after doxycycline, only 7% of the worms presented with normal embryogenesis and 7% of the nodules had living mf in the human tissue. These mf were found only adjacent to young productive *Wolbachia*-positive worms, which had probably been acquired after the end of doxycycline during the follow-up period.

Alternating logistic regression analysis was performed to take into account a possible dependency between nodules in patients. The frequencies estimated with this procedure (Table 7) are only slightly different from Table 3. Significant differences are found between untreated patients and patients treated with doxycycline.

Table 1 Characteristics of patients who completed 5 weeks of doxycycline treatment

Characteristics Baseline data[a]	Not treated No ivermectin	Doxycycline No ivermectin	Doxycycline Ivermectin before treatment
No. of individuals	10	5	17
Sex ratio, m/f	5/5	5/0	11/6
Mean age, years	34 (18–53)	35 (25–49)	41 (25–60)
Mean weight in kg	54 (40–60)	56 (46–65)	56 (44–68)
Median of nodules	3 (2–7)	4 (3–5)	4 (2–10)
No. of mf carriers	10	4	14
Median of mf/mg	55 (5–597)	51 (0–127)	3 (0–121)
Ivermectin before recruitment months, mean[b]	None	None	14 (4–18)
No. with complete treatment	Not applicable	5	16
Less days of treatment			1× only 34 days

[a] Numbers in brackets denote the range
[b] Three persons received ivermectin 6 and 18 months before recruitment

Table 2 Effects of 5 weeks doxycycline treatment on *Wolbachia* endobacteria

Treatment group	No. of patients/nodules	No. of living female worms				No. of living male worms			
		All	With *Wolbachia*			All	With *Wolbachia*		
			Many	Few	None		Many	Few	None
Not treated	10/41	51	46	4	**1**	20	14	3	**2**
Total for doxycycline	20/96	83	13	7	**63**[a]	37	6	8	**23**[a]

[a] Significant difference between the total doxycycline and the untreated group regarding the distribution between many, few, and no *Wolbachia* ($p<0.0001$, chi-square test)

The decrease of the skin mf loads is shown in Table 4. Skin mf levels were determined before doxycycline treatment and at 21 and 27 months thereafter. From patients who only attended one of the two follow-up points of time, the available skin mf value was taken for analysis. From those who attended both follow-up times, one value was randomly selected. Thus, data from 21 doxycycline-treated patients were collected. According to this analysis, 13 of the 21 doxycycline-treated patients had no mf after 21/27 months and the mf load had decreased to 0.2 mf/mg skin, supporting an interruption of embryogenesis by doxycycline alone since the last ivermectin intake was by then 31–43 months ago. Since transmission in this area was not interrupted (R. Garms, Bernhard Nocht Institute, unpublished report, 2006), we assume that the producing worms may have been acquired after the end of doxycycline treatment (see 'Study area', Materials and methods section, and Discussion section). The data therefore suggest that doxycycline treatment has led to a complete interruption of embryogenesis in those worms that had been present at the time of drug administration.

Macrofilaricidal activity of doxycycline

Dead and living worms were assessed using the criteria described in the Materials and methods section (Figs. 2, 3,

4, 5) and discussed in the Discussion section. Table 5 shows the detailed data for the dead worms. Nodules from untreated patients contained 16% dead female and 5% dead male worms. Four moribund female worms with pleomorphic neoplasms, as described by Duke et al. (2002) and Gardon et al. (2002), were included in the category 'dead'. The proportions of dead worms are in accordance to those from other studies in West Africa (Table 6). These data, in comparison with the data from other endemic areas throughout Africa, suggest a high and constant degree of regulation in acquisition and decay of worms, which is taking place in a very similar way in different *O. volvulus* strains from different endemic areas. In contrast, nodules from doxycycline-treated patients contained a significantly higher proportion of dead worms, as 49% dead females and 27% dead males were observed. Of the dead females after doxycycline, 72% were calcified and/or nearly absorbed, 27% were diagnosed as dead based on morphological criteria or APR-negative reactions, and only one was moribund.

Results from alternating logistic regression analysis are shown in Table 7. In the untreated group, 85% of nodules contained living female worms, as opposed to 56% of nodules from doxycycline-treated patients. When the doxycycline subgroup without ivermectin before the start of the study (see Table 1) was analyzed separately, only

Table 3 Effects of 5-week doxycycline treatment on embryogenesis and production of microfilariae

Treatment group	No. of patients/nodules	No. of living female worms				No. of nodules	
		All	Embryogenesis			All	00
			None	Inter-rupted	Normal[a]		
Not treated	10/41	51	19	9	**23**	41	**14 (34%)**
Total for doxycycline	20/96	83	46	31	**6**[b]	96	**7 (7%)**[c]

[a] "Normal embryogenesis" refers to the younger embryos. Degenerated stretched mf assumed to be due to ivermectin prior to study onset are not considered

[b] Significant difference between the doxycycline and the untreated group regarding the frequency of interrupted and normal embryogenesis ($p<0.0001$, chi-square test)

[c] Significant difference between the doxycycline and the untreated group regarding the proportions of nodules with intact mf ($p<0.0001$, chi-square test)

Table 4 Microfilarial loads in skin snips before and 21 or 27 months after doxycycline treatment

Treatment group	Time of skin snip	Months after study onset	No. of patients examined	No. of mf carriers	mf levels (mf/mg)			
					GMI[a]	(Range)	Median	p value
Not treated	11/2005	27	10	10	43	(5–597)	55	n.a.
Doxycycline treated	8/2003	0	22	18	6	(0–127)	4	0.024[b]
	5 or 11/2005	21 or 27	21	8	0.9	(0–58)	0.2	0.005[c]

[a] Geometric mean intensity (GMI) was calculated using the log $(x+1)$ method according to Williams [Remme et al. 1986]

[b] Changes in median skin mf levels from baseline (at 0 months) to 21 or 27 months were assessed by Wilcoxon signed rank test (in cases where patients attended both time points, one value was randomly selected)

[c] Difference in the proportion of skin mf carriers before doxycycline treatment and at 21 or 27 months (Fisher's exact test)

40% in the group without previous ivermectin were calculated to harbor living females (not shown). A significant difference to the untreated group was seen compared to the doxycycline subgroup without prior ivermectin, indicating that the macrofilaricidal effect observed was due to doxycycline and not to a potential effect of an ivermectin intake several years before nodulectomy.

Adverse reactions of doxycycline

Doxycycline treatment was well tolerated by the participants. None of them complained or presented severe adverse reactions such as bloody diarrhea. The dipstick tests for liver enzymes and creatinine, repeated after 3 weeks of treatment, remained normal. For a generalized judgment on doxycycline safety, the study group was too small.

Discussion

In the present study, three new findings were observed: (1) *Wolbachia* depletion and interruption of embryogenesis was achieved after reduction of the treatment duration from 6 (Hoerauf et al. 2003) to 5 weeks at 100 mg/day of doxycycline. (ii) *Wolbachia* depletion and sterility were observed for a longer time of 21 and 27 months than before and also without accompanying ivermectin treatment. (iii) Doxycycline showed a macrofilaricidal activity.

The results in Tables 2, 3, and 5 are presented after analysis per worm or per nodule (Table 3, right column). This analysis was done to make the results comparable to earlier studies from this (Albiez et al. 1988b; Büttner et al. 1988; Hoerauf et al. 2003, 2008) and other groups (e.g., Awadzi et al. 1995a, b, 1999). It also facilitates tracking down real individual worms in their different read-out characteristics (*Wolbachia* content, embryogenesis, mf in the human nodule tissue, and vitality), something that is not possible with logistic regression, because numbers are not counted but calculated. However, in order to account for the issue of interdependency of worms in nodules (e.g., aggregation as described by Duerr et al. 2001) and nodules in patients, we used alternating logistic regression to compare the rate of nodules with normal embryogenesis, the rate of nodules with mf, and the rate of nodules with living and dead female worms (Table 7). It confirmed the analysis from Tables 2, 3, and 5. Interestingly, the macrofilaricidal effect in the patients without prior ivermectin (see Table 1) appeared to be even higher (only 40% nodules with living female worms, data not shown) than in the group that had received prior ivermectin (61%). Together with the finding that only multiple, frequent doses of ivermectin (which were not administered here) show only a moderate macrofilaricidal effect (Duke 2005), our findings allows the conclusion that doxycycline is macrofilaricidal on its own.

In addition to interdependency, the dynamics of new infections of the study patients is another variable that has

Table 5 Macrofilaricidal activity of 5-week doxycycline treatment

Treatment group	No of patients	No. of all female worms			No. of all male worms		
		All	Dead no. (%)	Calcified or almost absorbed	All	Dead no. (%)	Calcified or almost absorbed
Not treated	10/41	61	**10 (16%)**	6	21	**1 (5%)**	1
Total for doxycycline	20/96	162	**79 (49%)**[a]	57	48	**13 (27%)**[a]	5

[a] Significant difference between the doxycycline and the untreated group regarding the proportion of dead female worms ($p<0.0001$, chi-square test) and dead male worms ($p<0.034$)

Table 6 Proportion of dead *O. volvulus* after 5 weeks of doxycycline and in untreated patients as observed in several studies in West Africa

Treatment/country	No. of patients/nodules	No. of all female worms		No. of all male worms		Reference
		All	Dead in %	All	Dead in %	
Doxycycline	20/96	162	49%	48	27%	This study
Untreated	10/41	61	16%	21	5%	This study
Untreated West Ghana	29/85	202	15%	91	3%	Hoerauf et al. 2003
Untreated East Ghana [a]	25/107	255	26%[a]	79	4%	Awadzi et al. 1995b
Untreated, Burkina Gaoua	33/217	500	6%	207	4%	Büttner et al. 1988
Untreated, Burkina Diebougou	22/162	242	20%	143	4%	Büttner et al. 1988
Untreated, Liberia	16/62	115	20%	69	0%	Büttner et al. 1988

[a] Increased proportion of dead female worms after 2 years of vector control in 1988

influenced the reported numbers of living and fertile female worms. Obviously, ongoing transmission leads to the acquisition of new worms after the administration of doxycycline, some of which in our study have already become fertile during the 21–27-month observation period. As detailed in the Materials and methods section, no reduction of transmission compared to an area without onchocerciasis control was observed in two surveys in 2002 (Kutin et al. 2004) and again in 2006 (R. Garms, Bernhard Nocht Institute, unpublished report, 2006). In summary, in the latter survey, infection pressure was still at an average of at least one infectious bite per person per week, or at least 50 L3 per year. Other factors to consider in the dynamics of new infection are that the calculated average adult worm life span is 10 years (Habbema et al. 1990) and that in an area with ongoing transmission the adult worm loads are rather stable in an adult patient (Duerr et al. 2003). Worm loads are maintained despite a very large observed variance of L3 infection pressures, which range over 100-fold when different areas are compared, so that between 0.1% and 10% of L3 were calculated to develop into adult worms (Schulz-Key and Soboslay 2000). Therefore, it is reasonable to assume a yearly turnover of approximately 10% of the total adult worm load in areas with normally ongoing transmission like in our study area. In the present study, 16% of female worms displayed high wolbachial loads after 21 and 27 months ("many *Wolbachia*", Table 2). This number is within the expected range of newly acquired worms after a 2-year observation period, and the infection rate with L3 estimated for this area can maintain such a level of re-infection (i.e., one to two adult worms per person per year, from at least 50 transmitted L3).

In addition, the female worms harboring many *Wolbachia* showed the characteristics of young worms that had been newly acquired, such as having a small diameter, thin cuticle, prominent lateral cords and somatic muscles, lack of inclusion bodies or other signs of degeneration in hypodermis, muscles, and epithelia, many of them being still nulliparous (Büttner et al. 1988; Franz 1988; Schulz-Key 1988; Duke 1991; Hoerauf et al. 2003). They also showed little or no brown or (using Gomori's method) blue staining in the gut because of less iron deposition (Wildenburg and Henkle-Dührsen 1999). Therefore, we assume that these worms have been acquired after the end of doxycycline treatment. However, this is not critical for the overall message of this study, as discussed in the next paragraph. In addition to those worms being nulliparous, the other worms from this probably newly acquired group showed normal embryogenesis and fully encompassed the 7% (6/83) female worms in the doxycycline groups observed with normal embryogenesis in Table 3.

One can further conclude that, if those probably newly acquired worms had been subtracted from the data for Tables 5 and 7, the macrofilaricidal effect of the 5-week regimen of doxycycline would have been higher than the counted 49% (Table 5), and vice versa that fewer than the

Table 7 Multiple logistic regression analysis

Treatment group	Females with normal embryogenesis		Nodules with mf		Nodules with living females	
	% (CL)	*p* value	% (CL)	*p* value	% (CL)	*p* value
Not treated	43.5 (27.1–61.4)		36.1 (19.1–57.5)	0.005[a] 0.078[b]	84.8 (59.1–95.6)	
Total for doxycycline	7.9 (3.7–16.4)	<0.0001[a]	8.3 (3.6–17.0)	0.003[a]	56.3 (43.1–68.8)	0.046[a]

[a] Not treated against doxycycline treated

calculated 56% of nodules (Table 7) would have contained living female worms. However, it is also clear that, after subtraction of these newly acquired worms, the macro-filaricidal activity would not exceed approximately 60% in female worms and thus, doxycycline has not killed all doxycycline-treated worms at this dose. But also a macro-filaricidal effect of less than 100% is meaningful for the patient. For example, suramin does not kill all worms; 34% of 101 female worms or 0.87 females per nodule were still alive 12 months after a full course of suramin in an area without ongoing transmission (Awadzi et al. 1995b) compared to 51% of 162 living female worms or 0.86 females per nodule after 5 weeks of doxycycline. Further-more, WHO accepts cure rates of 60–90% for schistosomes (WHO 1995a). Still, more studies are needed to define the optimal macrofilaricidal regimen of doxycycline.

The effects of doxycycline on the mf densities were found to be slow compared to ivermectin and diethylcar-bamazine. This feature is important when potential adverse reactions due to the antifilarial activity of doxycycline are considered. The decay of mf induced by doxycycline is apparently too slow to induce severe adverse reactions. Similarly, the macrofilaricidal activity of doxycycline is slow and does not show characteristics of fast killing of adult worms, such as nodule perforation as observed after suramin (Büttner, unpublished). The general adverse reac-tions to doxycycline, such as bloody diarrhea, are known from numerous applications, because the drug has been registered and widely used since long time. Serious adverse effects were found to be very rare, so that doxycycline is being used already for other indications by populations in onchocerciasis-endemic areas, even in those with a low standard of medical care. Doxycycline is readily available in pharmacy shops in these areas.

The long doxycycline treatment, the slow decay of mf, and the appearance of newly acquired worms after doxycycline in a focus with ongoing transmission show clearly that ivermectin remains the drug of choice for the areas which are currently covered by the African Programme for Onchocerciasis Control (Boatin and Richards 2006), because doxycycline would have to be frequently re-administered to people that have already been treated in order to cure new infection. Indications for doxycycline have been described recently in detail (Hoerauf and Pfarr 2007; Hoerauf 2008). They apply to onchocerci-asis patients who are no longer exposed to re-infection, because they left the endemic area. Further, in the USA, the elimination of onchocerciasis has reached 'end-game' scenarios, where transmission has apparently been inter-rupted and only a limited number of people are infected with onchocerciasis in few foci. Administration of doxycy-cline to these people has been suggested (WHO 2007) on the basis of its long-term interruption of embryogenesis.

The new data on the macrofilaricidal activity may further underscore this argument.

Doxycycline may also be developed as a reserve drug if the concerns regarding the potential development of ivermectin resistance (Bourguinat et al. 2007; Osei-Atweneboana et al. 2007) are proven to be correct and quick action is needed to prevent spreading of resistant worms. Finally, anti-wolba-chial chemotherapy may become an option for areas that are co-endemic for loiasis and where ivermectin treatment has resulted in the occurrence of encephalopathy (Thomson et al. 2004). Studies in co-endemic foci are currently being performed.

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