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Studies on the macrofilarial population of *Onchocerca volvulus* in hyper-endemic villages of the Central province of Cameroon

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The population structure of *Onchocerca volvulus* macrofilariae was studied in villages of central Cameroon where onchocerciasis is hyper-endemic. One nodule selected at random was removed from each of 576 adult males, and examined by histology. The numbers of male and female worms/nodule, and the status of the female worms (fecund, non-fecund, or dead) were recorded. The observations were analysed to evaluate whether the mean numbers of worms of each category varied in relation to the patient's age, the level of endemicity in his village, the anatomical localization of the nodule, the weight of the nodule, and the total number of palpable nodules harboured by the patient. The results obtained were very similar to those reported from West Africa. The mean numbers of dead female worms/nodule were relatively high in the villages with the lowest levels of endemicity. The mean numbers of fecund females and of live males were significantly higher in the nodules located around the knees. These results provide information which might be useful in modelling the population dynamics of *O. volvulus*, and also in the context of trials of any potentially macrofilaricidal drugs.

The strategy for onchocerciasis control, previously based upon the control or elimination of the vector blackfly populations, was dramatically modified by the discovery of the safe microfilaricidal activity of ivermectin against *Onchocerca volvulus*, and the subsequent implementation of the African Programme for Onchocerciasis Control (APOC). The objective of this programme, launched in 1996, is to eliminate onchocerciasis as a public-health problem within 12 years, through the implementation of annual, community-directed treatments with ivermectin. The final decision regarding how long this programme will have to continue, in order to ensure its success, will largely depend upon the impact of the successive, large-scale treatments on the fertility and population structure of the adult worms of *O. volvulus*. Although significant progress has been made in modelling the population dynamics of *O. volvulus*, these efforts have mainly focused on the dynamics of the larval populations transmitted to and from the human and blackfly hosts (Plaisier *et al.*, 1990; Davies, 1993; Basáñez and Boussinesq, 1999). Very few attempts have so far been made to describe the population structure of *O. volvulus* macrofilariae, mainly because such studies necessitate performing large numbers of nodulectomies and making a standardized microscopical examination of the nodules and worms obtained (Duke, 1993). The main aim

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of the present study, in Cameroon, was to collect and analyse data on five different factors, any of which might influence the numbers and reproductive status of the male and female adult worms to be found in a given nodule. Such information, as well, perhaps, as advancing the modelling of onchocerciasis, could be useful in the context of trials of any potentially macrofilaricidal drugs. If variations in the population structure of adult worms were to be found in respect of these 'external' factors, the latter would have to be taken into account when selecting those patients in whom nodulectomies are to be performed, and those nodules which are to be removed.

PATIENTS AND METHODS

The nodules investigated in the present study were collected, before treatment, from 576 patients who participated in a trial to investigate the macrofilaricidal potential of high and/or more frequent doses of ivermectin against *O. volvulus*.

Study Area

The trial involved patients living in 20 villages located in the Mbam Valley, in the Central province of Cameroon. Preliminary parasitological and entomological surveys, and a census of blind individuals, had demonstrated that, in this zone of forest–savanna transition, onchocerciasis was hyper-endemic and causing severe ocular disease, and that it was mainly transmitted by *Simulium squamosum* (Boussinesq *et al.*, 1992; Barbazan *et al.*, 1998). Most of the population was involved in cocoa farming and subsistence agriculture.

Patients

The onchocerciasis patients eligible to be included in the trial were males between 18 and 60 years of age, in an otherwise good state of health, who had not received any potentially filaricidal treatment during the previous 5 years, and who were found to have at least two onchocercal nodules each during a preliminary examination session. Those patients chosen to take part were invited to sign a standardized consent form including the objectives and schedule of the trial. All the information concerning the various factors to be analysed was available and a presumed *O. volvulus* nodule was excised from each of 596 patients. However, 20 of these 'nodules' turned out, on histological examination, to be of non-onchocercal origin (mainly lymph nodes, lipomata, or foreign bodies) and had to be discarded, thus leaving 576 patients for analysis.

Nodulectomies

Just prior to the nodulectomy, each patient was stripped down to a loin cloth and again carefully examined for nodules, whose distribution was checked. Excluding a few nodules which were considered to be unduly difficult to remove because of their proximity to large nerves or blood vessels (e.g. at the elbows, wrist etc), an affected site on each patient was selected for excision using a random procedure. Nodulectomies were performed in the operating theatre of two local hospitals, under local anaesthesia with optimum aseptic precautions. When more than one nodule was found in a site at operation, all of them were removed. Each excised nodule was then placed in about 20 times its volume of fixative containing 70% ethanol, 10% glycerol and 20% water and was subsequently stored in its own, separate, labelled tube. The fixative was changed after 24 h and the nodule itself was then stored in the fresh fixative until subsequent histological examination.

Processing of the Nodules

The nodules which had been fixed in the ethanol–glycerol mixture were cleaned of unwanted connective tissue and weighed with a precision of 0.1 mg. When more than one nodule was removed from a given site, only one of them, randomly selected, was further processed for histological examination. They were embedded in paraffin wax, and sections were cut at 6 μm. All of the larger nodules, with a diameter exceeding 1 cm, were cut at three levels (at the first quarter, at the middle, and the last quarter of the most appropriate dimension) in order to get optimum coverage of the contained worms (Awadzi *et al.*, 1997).

The sections were stained with haematoxylin and eosin and examined 'blind' by two independent parasitologists (B.O.L.D. and M.B.) who had no information on their origin. The results of the first examinations were compared and, when there was some discordance for a given nodule, the slides were re-examined until a consensus was reached.

The results were recorded on standardized forms according to the system developed by Duke *et al.* (1990), slightly amended as follows. The female worms in the nodules were split into three main categories: F—fecund worms, inseminated or re-inseminated, producing embryos of any stage up to microfilariae [categories FF, FO and IA of Duke *et al.* (1990)]; NF—live non-fecund worms, including potentially fertile worms which were not currently inseminated but were shedding oocytes which were transforming into unfertilized ova, and then degenerating [categories SF, SO and IZ of Duke *et al.* (1990)], and empty senescent worms no longer producing oocytes [category E of Duke *et al.* (1990)]; or MD—moribund or dead worms, almost or completely void of internal organs and showing loss of turgor, collapse of the body wall, and invasion by giant cells [categories M and D of Duke *et al.* (1990)]. A sub-category, MDc [category C of Duke *et al.* (1990)], was defined by the calcification of at least five sections of the worm. Males were recorded as being either alive or dead.

Factors Potentially Associated with Variability in Population Structure in the Nodule

Five factors were examined for their possible association with variation in the population structure of the macrofilariae in the nodules: (1) the level of endemicity of onchocerciasis in the patient's village of residence, as assessed by the community microfilarial load (CMFL) (Remme *et al.*, 1986) measured in the total population during previous surveys (Boussinesq *et al.*, 1992; unpubl. obs.); (2) the weight of the excised nodule; (3) the anatomical site of the nodule excised; (4) the age of the patient; and (5) the total number of sites where nodules were found in the patient at the examination performed before nodulectomy.

The CMFL were divided into four classes, of 10–40, 41–60, 61–70 and 71–114 microfilariae per skin snip (mff/ss). The weights of the nodules were analysed using classes of <0.1, $0.1-<0.2$, $0.2-<0.3$, $0.3-<0.5$, $0.5-<1.0$, $1.0-<1.5$ and ≥ 1.5 g. The anatomical sites of the excised nodules were divided into five groups: (1) head and upper limbs; (2) thorax; (3) iliac crests; (4) greater trochanters; and (5) knees and legs. The ages of the patients were divided into four age-groups: 18–29, 30–39, 40–49, and ≥ 50 years of age. The total numbers of sites where nodules were found were analysed in six classes: two or three, four, five, six, seven or eight, or more than eight nodules/patient.

Data Analysis

The analysis focused on the population structure of the adult worms in the nodule (i.e. the mean numbers of worms in each category) after processing for histological examination. As, in most cases, the frequency distributions of the numbers of nodules, or of worms of a given category, showed a marked over-dispersion, the spread of variability of the means was not illustrated using confidence intervals, but by the range, and/or the standard deviation (S.D.). The first step was a univariate analysis, taking into account each of the five factors investigated. The mean numbers of worms of a given category were compared using the Kruskal–Wallis test. For the second step, when significant differences were found using the Kruskal–Wallis test, non-parametric multiple-comparison tests were performed but, as the number of nodules in the different categories were not identical, these tests followed the procedure developed by Noether (1976). In all cases, a P-value of <0.05 was considered significant.

RESULTS

General Findings on the Population Structure of *O. volvulus*

Overall, 3234 palpable nodules (or groups of

nodules) were found at the preliminary clinical examination of the 576 patients from whom true *O. volvulus* nodules were excised, giving a mean number of 5.61 nodule sites/patient.

The 576 nodules processed contained 1246 female worms and 669 males in total, giving mean numbers (S.D.) of 2.16 (1.40) female and 1.16 (0.91) male worms/nodule, and a female:male sex ratio of 1.86:1. If only the live worms are considered (1056 females and 651 males), the sex ratio becomes 1.62:1. The maximum numbers of female and male worms found in a nodule were nine and five, respectively. Amongst the female worms, 554 (44.5%) were fecund, 502 (40.3%) were non-fecund, and 190 (15.2%) were moribund or dead. Most (61.1%) of the M and D female worms were totally or partially calcified. The mean numbers (S.D.) of fecund, non-fecund and moribund or dead female worms/nodule were 0.96 (0.89), 0.87 (0.89), and 0.33 (0.55), respectively. Only one nodule did not contain any female worms (it contained one male). Most nodules (66.8%) contained at least one fecund female, and 9.5% of all the nodules contained only moribund or dead females. The vast majority (97.3%) of the male worms were considered to have been alive at nodulectomy, the rest being moribund or dead. The mean numbers (S.D.) of live and dead males/nodule were 1.13 (0.91) and 0.03 (0.17), respectively. Some 77.3% of the nodules contained at least one male, and 3.1% at least one dead male.

Relationships with the Macrofilarial Population

THE LEVEL OF ENDEMICITY IN THE COMMUNITY (TABLE 1)

The mean numbers of moribund or dead female worms/nodule were significantly different between the CMFL classes ($P = 0.04$). The highest values observed (0.36 and 0.40) were recorded in the two groups of villages with the lowest levels of endemicity. However, the value was particularly low (0.22) in those patients living in villages where the CMFL ranged between 61 and 70 mff/ss.

THE WEIGHT OF THE NODULE (TABLE 2)

As might be expected, the mean numbers of worms of any given category increased significantly as the weight of the nodule increased. The only values which did not differ significantly between the weight classes were the mean numbers of moribund or dead females; this might reflect the relatively low numbers of worms in this category.

THE SITE OF THE NODULE (TABLES 3 AND 4)

The mean numbers of live females and of fecund females differed significantly with the localization of the nodule ($P = 0.02$, and $P = 0.01$, respectively), but there was no significant difference in the figures relating to non-fecund females. When multiple-comparison tests were performed, it appeared that the mean numbers of fecund females in nodules from the thorax (0.91) and at the iliac crests (1.02) did not differ significantly. However, the mean number of fecund females in nodules located over the knees and lower legs (1.21) was significantly higher than the mean numbers of fecund females in nodules located over the trochanters, iliac crests or thorax ($P < 0.05$).

With regard to the male worms, the mean numbers of live males, and of males of all categories, differed significantly with the nodule localization ($P < 0.0001$ and $P < 0.001$, respectively). As with the fecund females, the highest and lowest values were recorded in the nodules located over the knees and greater trochanters, respectively.

As there was no reason *a priori* that the nodules located at the greater trochanters should contain significantly fewer fecund females and male worms than those located on the knees and legs, further analysis was performed to evaluate whether this observation could be related to the fact that the trochanteric nodules were generally smaller (geometric mean weight = 0.29 g) than those collected from the other anatomical sites. The mean numbers of fecund and live females, and of males recorded at different anatomical sites were therefore compared within nodule weight-classes. In order to get sufficient numbers of nodules for comparison, only three site

TABLE 1

The macrofilarial population in relation to the community microfilarial load (CMFL) in the patient's village of residence

CMFL (no. of microfilariae/skin-snip)	*Mean no. of females/nodule*					*Mean no. of males/nodule*		
	Fecund	*Non-fecund*	*Total live*	*Moribund/dead*	*Total*	*Total live*	*Moribund/dead*	*Total*
10–40	0.99	0.90	1.89	0.36	2.26	1.25	0.05	1.30
41–60	0.90	0.86	1.76	0.40	2.16	1.11	0.01	1.12
61–70	0.97	0.88	1.85	0.22	2.08	1.07	0.03	1.10
>70	0.99	0.83	1.83	0.35	2.17	1.07	0.04	1.11

TABLE 2

The macrofilarial population in relation to the weight of the excised nodule

Weight of nodule (g)	*Mean no. of females/nodule*					*Mean no. of males/nodule*		
	Fecund	*Non-fecund*	*Total live*	*Moribund/dead*	*Total*	*Total live*	*Moribund/dead*	*Total*
<0.1	0.29	0.50	0.79	0.29	1.07	0.39	0.05	0.45
0.1–<0.2	0.42	0.70	1.12	0.22	1.36	0.68	0.03	0.71
0.2–<0.3	0.77	0.65	1.41	0.27	1.68	0.90	0.02	0.93
0.3–<0.5	0.96	0.73	1.69	0.33	2.02	1.15	0.02	1.17
0.5–<1.0	1.10	1.15	2.26	0.35	2.61	1.30	0.06	1.35
1.0–<1.5	1.71	1.14	2.86	0.57	3.43	1.98	0.02	2.00
≥1.5	2.00	1.30	3.30	0.43	3.73	1.95	0	1.95

TABLE 3
The macrofilarial population in relation to the site of the excised nodule

	Mean no. of females/nodule					*Mean no. of males/nodule*		
Site of the nodule	*Fecund*	*Non-fecund*	*Total live*	*Moribund/dead*	*Total*	*Total live*	*Moribund/dead*	*Total*
Head/arm	0.97	1.01	1.99	0.27	2.25	1.11	0	1.11
Thorax	0.91	0.86	1.77	0.31	2.08	0.98	0.03	1.01
Iliac crest	1.02	0.88	1.89	0.33	2.23	1.24	0.03	1.27
Trochanter	0.73	0.72	1.45	0.33	1.78	0.92	0.04	0.96
Knee/leg	1.21	0.93	2.14	0.41	2.55	1.43	0.06	1.49

TABLE 4
The macrofilarial population in relation to the weight and the site of the excised nodule

Weight of the nodule (g)	*Site of the nodule*	*No. of nodules*	*Mean no. of females/nodule*			*Mean no. of males/nodule*
			Fecund	*Total live*	*Total*	
<0.2	Trochanter	37				
	Knee/leg	24	NSD	NSD	NSD	NSD
	Other	102				
$0.2-<0.5$	Trochanter	29		1.21	1.62	
	Knee/leg	29	NSD	1.45	1.66	NSD
	Other	128		1.68	1.98	
$0.5-<1.0$	Trochanter	23	0.83	1.70	2.04	1.22
	Knees/leg	16	1.50	2.88	3.31	1.88
	Other	86	1.10	2.29	2.63	1.29
≥ 1.0	Trochanter	11	1.45	2.45	2.91	1.45
	Knee/leg	17	2.59	4.18	5.18	2.88
	Other	74	1.78	2.97	3.35	1.84

NSD, No significant difference between the three localizations ($P > 0.05$).

classes were used (trochanters, knees and legs, and other sites) and the nodules' weights were divided into only four classes (<0.2, $0.2-<0.5$, $0.5-<1.0$, and ≥ 1.0 g). The data presented in Table 4, where only the significant differences between the three localization sites are given, show that, at least for the larger nodules, the results obtained at the first analysis still persisted after stratification for weight. However, most of the differences in the multiple-comparison tests were found between the nodules located at the knees and legs and those located at the 'other sites', whereas there were no significant differences between the nodules located at the trochanters and those located at the 'other sites'. The latter result might be related to the relatively small numbers of nodules coming from the trochanters.

THE AGE OF THE PATIENTS, AND THE TOTAL NUMBER OF PALPABLE NODULES/PATIENT

No significant difference was found for any category of worm between the groups defined on the basis of the age of the patients or according to the number of sites where nodules were found (either at the pre-treatment examination or at the time of nodulectomy).

DISCUSSION

The only other detailed data available on the population structure of adult *O. volvulus* in patients living in areas where no control programme has been implemented appear to be those obtained from examinations of nodules from Liberia (Schulz-Key and Albiez, 1977; Albiez *et al.*, 1984, 1988; Duke *et al.*, 1990), Burkina Faso (Albiez *et al.*, 1984, 1988), Mali (Karam *et al.*, 1987; Duke *et al.*, 1990) and Guatemala (Duke *et al.*, 1990). The present study is therefore the first of this type to be conducted in Central Africa. In all the previous studies, the worms were collected after collagenase digestion of the nodules, whereas the worms in the present study were examined using histological techniques on sections of nodules. Comparisons of the two methods (Büttner *et al.*, 1988; Duke *et al.*, 1991) indicate that the main disadvantage of digestion is that it tends to fragment the dead worms (which are thus difficult to detect) whereas with sectioning it is often difficult, in multi-worm nodules, to be certain which of the cut coils belong to which worm. During the present study, the latter disadvantage was attenuated by the facts that all the slides were examined by two independent examiners who, whenever their original results were divergent, subsequently discussed them and reached a consensus, and that the larger nodules were sectioned at three levels, a procedure which facilitates significantly the counting of females, as well as the detection of the smaller male worms.

The female:male sex ratio of the live worms seen in the present study (1.62:1) was similar to those reported from Mali, Liberia and Burkina Faso (1.5:1 to 1.7:1; Schulz-Key and Albiez, 1977; Albiez *et al.*, 1984, 1988; Karam *et al.*, 1987).

The proportion of moribund or dead females among the total number of females was 15.2%, a value similar to those reported from hyper-endemic villages in Liberia (12.2%, 18.0% and 16.0%), but higher than those reported in Burkina Faso (9.9%), Mali (10.1%–12.0%) or Guatemala (4.7%) (Albiez *et al.*, 1984, 1988; Karam *et al.*, 1987; Duke *et al.*, 1990). Although it therefore seems possible that the adult females of *O. volvulus* in Liberia and Central Cameroon generally have shorter lifespans than those in West African savanna and Guatemala, this is difficult to ascertain because the only sites where this longevity has been accurately estimated are in the savanna area of Burkina Faso (Plaisier *et al.*, 1991).

The proportion of fecund worms among the total number of live females observed in the present study (52.5%) was similar to the values reported in Liberia (48.1%; Duke *et al.*, 1990) and Guatemala (57.9%; Duke *et al.*, 1990) and to that (53.0%) found in Mali by Karam *et al.* (1987), although markedly higher than that (41.1%) reported in Mali by Duke *et al.* (1990).

In Cameroon, 77.3% of the nodules contained at least one male (present study) and this value is within the range of those reported

from Liberia, Mali and Guatemala (82.2%, 61.0% and 62.0%, respectively) by Duke *et al.* (1990). Among the male worms recorded in the present study, 2.7% were moribund or dead. This proportion is similar to that reported from Mali (3.1%; Karam *et al.*, 1987) but slightly higher than that found in Liberia (1.9%; Albiez *et al.*, 1984) and Burkina Faso (2.0%; Albiez *et al.*, 1984). The relatively low proportions recorded in Liberia and Burkina Faso may reflect the difficulty in detecting these small and often fragmented worms using the collagenase-digestion technique.

The present analysis of the factors which might influence the population structure of the adult worms in the nodule demonstrated that the latter did not differ significantly with the age of the patient, or the total number of palpable nodules found at the examination of the patient. Although the design of the present study did not allow confirmation of this issue, it is known that these two factors, namely age and number of nodules, are closely related (Albiez, 1983). The results for patient age in Cameroon (present study) confirm those obtained in Burkina Faso, where the mean numbers of worms/nodule, and the sex ratios of the worms, were found to be similar in the different age-groups of patients (Albiez *et al.*, 1988). Thus, it seems that new infections and the appearance of new adult worms do not lead to an accumulation of worms in pre-existing nodules, but rather to the formation of new nodules, which are probably created around young females. This also explains why the mean weight of the nodules examined as part of the present study did not differ significantly with either the patient's age, or the number of his nodules (data not shown).

Regarding the level of endemicity, the mean numbers of moribund or dead female worms/nodule were relatively high in the villages with the lowest CMFL. This result confirms reports from Liberia, where the proportions of female worms that were old or dead were higher in the hypo-endemic villages (28% and 18%, respectively) than in the hyper-endemic (7% and 12%; Albiez *et al.*, 1988).

The present study indicates that the nodules located at the trochanters contain fewer worms, and those located at the knees contain more worms, than the nodules from other body areas. Similar observations concerning nodules around the knees have been reported previously from Liberia (Albiez *et al.*, 1988), but in that study the weight of the nodules was not taken into account. In addition, the relatively high total numbers of female worms in the nodules around the knees of the present subjects were essentially caused by high numbers of fecund females. This phenomenon poses a question: 'How can we explain that, for a given weight of nodule, there should be more fecund females in nodules present around the knees than in those from other sites?' Is it anything to do with the fact that, where *O. volvulus* is transmitted by *S. damnosum* s.l., species which bite mainly on the lower legs and feet, the first potentially good lodging point for the new, migrating, third- and fourth-stage larvae of *O. volvulus* (of both sexes) is probably in the region of the knees?

In conclusion, most of the present observations on the general population structure of adult worms of *O. volvulus* are very similar to those reported from the few studies from other areas dealing with the same subject. However, additional investigations might be performed to provide further details on the longevity of the worms in the forested areas of Central Africa, an issue which might influence decisions on the duration of APOC. The only factors which were found to influence the population structure of the worms in the nodules were the level of endemicity in the village of residence of the patient, and the localization of the nodule. These findings should be taken into account when selecting patients for trials of potential filaricidal drugs and when deciding which of their nodules should be excised.

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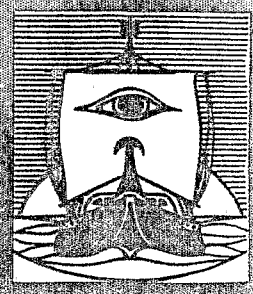

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