THE USE OF ACETO-ORCEIN-STAINED SQUASH PREPARATIONS FOR ENUMERATION OF NUCLEI IN MICROFILARIAE OF VARIOUS FILARIAL PARASITES*

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Abstract. The nuclear numbers situated between the cephalic space and the nerve ring (CS-NR) in the microfilariae of six filarial species were examined. For the counting of nuclei, aceto-orcein squashing preparations were used. This procedure revealed nuclei clearly visible under the phase-contrast microscope. The mean number of nuclei in the CS-NR region was very similar between *Brugia pahangi* and *B. malayi*, but was statistically different between *Onchocerca volvulus* and *O. gutturosa*. In the microfilariae of *O. volvulus*, two types of nuclei were clearly shown in this region. The procedure described is useful in the anatomical study of microfilariae.

Intraspecific differences in nuclear numbers in *Wuchereria bancrofti* microfilariae of different demes were demonstrated by Schacher et al.,^{1,2} based on the certainty of nuclear constancy (eutely). However, ordinary staining procedures usually do not readily reveal nuclei of microfilariae for counting, especially in *Onchocerca* spp. Recently, we found that an aceto-orcein squashing preparation was valuable in the enumeration of nuclei in microfilariae. By using this procedure, and based on the above premise, we have compared the number of nuclei of various microfilariae inter- and intra-generically.

MATERIALS AND METHODS

Preparation of microfilariae

Microfilariae of *Onchocerca volvulus* were obtained from onchocercal nodules extirpated from man in Guatemala, and those of *O. gutturosa* from the umbilical skin of bovines in Guatemala. The individual microfilariae were recovered in saline after an overnight incubation of excised tissue

from man and cattle. The identification of the species involved was made by the morphology of Giemsa-stained microfilariae.

Microfilariae of *Brugia malayi* were obtained from hemolyzed blood of cats experimentally infected in Nagasaki University through *Aedes togoi* with filariae of human origin (Che-ju Is., Korea). Microfilariae of *B. pahangi* were recovered from the peritoneal cavity of jirds infected with the Malaysian strain by transmission through *A. aegypti* in the same laboratory.

Microfilariae of *Dirofilaria immitis* were recovered from hemolyzed blood of dogs naturally infected in Beppu, Oita Prefecture.

Microfilariae of *Setaria digitata* were obtained by incubation of adult females which were collected from the abdominal cavity of bovines in Seoul, Korea.

The suspensions of microfilariae thus obtained were centrifuged at 1,000 rpm for 5 min and the sediment was fixed in glacial acetic acid-99.9% ethanol solution (1:3) for 24 hours. Thereafter, the sediment was washed several times with 70% ethanol and finally was stored in this solution until being stained.

Preparation of aceto-orcein

Aceto-orcein stain was made by adding 2 g of orcein powder (E. Merck) to 100 g of 60% acetic acid and boiling with stirring for 2 min. The so-

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TABLE 1

Number of microfilarial nuclei between the cephalic space and the nerve ring in six species of microfilariae (mf)

Microfilaria of	No. of examined	Mean no. nuclei	Standard deviation	Standard error	CV value*	Range of nuclear number (min.-max.)
<i>D. immitis</i>	100	99.12	5.31	0.53	5.36	89-110
<i>B. pahangi</i>	100	94.74	5.05	0.51	5.33	85-110
<i>B. malayi</i>	100	92.76	6.47	0.65	6.97	77-107
<i>S. digitata</i>	100	84.95	6.46	0.65	7.60	73-106
<i>O. volvulus</i>	100	87.73	6.87	0.69	7.83	73-111
<i>O. gutturosa</i>	100	79.23	6.87	0.69	8.67	64-92

* Coefficient of variation: CV = (SD × 100)/mean number of nuclei.

lution was cooled, filtered, and stored in a brown-colored bottle for later use. This staining solution has long been used in the field of chromosome studies.

Observation of nuclei

One drop of suspension of fixed microfilariae was placed on a glass slide to which was added 1 drop of aceto-orcein. The mixture was stirred gently for 1 min and then was covered with a glass coverslip (24 × 24 mm). The slide was then placed between two pieces of filter paper and was tightly squashed with a thumb bulb in order to increase microfilarial body width and to demonstrate arrangement better. When the microfilarial preparations were flattened adequately, the rim of the coverslip was sealed with melted paraffin-balsam (1:1), using the edge of a heated spatula. This process was effective in preventing the re-constriction of the cuticle. Unless the squashing was adequate, the nuclei would not be clearly enumerated. A phase-contrast microscope was used at a magnification of 1,000× since this procedure gave a clear outline of individual nuclei.

In this study, only those nuclei located between the cephalic space and nerve ring (CS-NR) were counted in 100 microfilariae of each species. The nuclei were stained a dark-brown color by the aceto-orcein.

RESULTS

As shown in Figures 3, 5, 6, and 7, the procedure described above clearly demonstrated individual nuclei and their arrangement in the CS-NR of microfilariae, and easily enabled us to enumerate the precise number of nuclei in individual filarial species. Results are presented in Table 1 and Figure 1.

Among all the microfilarial species observed, the number of nuclei in *D. immitis* microfilariae was the highest (99.12), followed by the two *Brugia* spp. (94.74 in *B. pahangi*, 92.76 in *B. malayi*). There was no statistically significant difference in the nuclear number between the two species ($t = 2.41$; $P > 0.01$). The number of CS-NR nuclei in *S. digitata* (84.95) fell between those of the two species of *Onchocerca*. The lowest number of nuclei (79.23) was seen in the microfilariae of *O. gutturosa*.

The difference of mean nuclear number between *O. volvulus* and *O. gutturosa* (8.5) was statistically significant ($t = 8.70$; $P < 0.01$). Analysis of the frequency distribution of nuclear number of these two *Onchocerca* spp. showed that of the microfilariae with a nuclear number of 90 or more in the CS-NR, 82.9% were those of *O. volvulus*, while of those with 79 or fewer nuclei, 88.9% were those of *O. gutturosa*.

The coefficient of variation of the nuclear number observed in the six species of filariae is shown

FIGURE 1. Number of nuclei between the cephalic space and nerve ring of six species of microfilariae. The solid lines indicate the range, the crossbar indicates the mean, and the box encloses one standard deviation on either side of the mean.

FIGURES 2-7. Microfilariae processed by aceto-orcein staining with or without squashing. All photos were taken under phase contrast, $\times 845$. 2. Microfilaria of *Onchocerca volvulus*, stained but not squashed. N, nerve ring; C, cephalic space. 3. Microfilaria of *O. volvulus*, stained and squashed; 4. Microfilaria of *O. gutturosa*, stained but not squashed; 5. Microfilaria of *O. gutturosa*, stained and squashed; 6. Microfilaria of *Setaria digitata* with wrinkled sheath around anterior tip, stained and squashed; 7. Microfilaria of *Brugia pahangi*, stained and squashed. S, sheath.

in Table 1. The values obtained were considered to indicate the internal consistency of microfilarial populations.

There were apparently two types of nuclei in the CS-NR region of the microfilariae, particularly in those of *O. volvulus* (Fig. 3). The first type was located centrally and was small and densely stained, while the other, located peripherally, was swollen and sporadically stained. By ordinary staining methods they were indistinguishable from each other.

DISCUSSION

Concerning the nuclear counts of microfilariae, Schacher et al.¹ first reported results with *Wuchereria bancrofti* microfilariae of different geographic origins. The theoretical basis for this methodology rested on the assumption of constancy of nuclear counts in microfilariae of the same species. Using Delafield's hematoxylin for staining, they counted the nuclei which were located in the CS-NR region; their results indicated that

there were distinct varieties of *W. bancrofti*, and that they were dealing with a species complex or sibling species.¹ Later, they compared 15 demes of *W. bancrofti* microfilariae based on the nuclear counts of various portions, and concluded that this methodology was useful, within reasonable limits, to test differences among microfilarial populations.² However, at the same time they were unable to apply this procedure to the microfilariae of other genera such as *Brugia* and *Onchocerca*. This is probably due to the nature of microfilariae in the smeared state. For example, Eichler reported the nuclear number in the CS-NR of *O. gutturosa* microfilariae from British cattle as 65.2–67.6.³ This count was obtained by Giemsa staining and is lower than that obtained in the present study. Our preliminary counting of nuclei in *O. volvulus* microfilariae smeared and stained with Giemsa's solution also resulted in fewer nuclei in comparison with those observed by the present technic. However, a possible taxonomic difference of microfilariae should also be taken into consideration in comparing bovine filariae from England with those from Guatemala.

Schacher and Crans used a 3:1 mixture of glacial acetic acid and absolute methanol in their study of *Foleyella flexicauda* microfilariae.⁴ Although this treatment is similar to the one we used, they applied this fixative to presoaked air-dried smears of blood before staining and they did not count the nuclei. The present specimens were not dried, and for this reason the body wall of the microfilaria retained flexibility and intercellular connections were rather loose. These features are preferable for squashing.

As shown in Figure 6 (*S. digitata*) and 7 (*B. pahangi*), the aceto-orcein squashing method is applicable not only to sheathless microfilariae but also to those that have sheaths, enabling easy examination of the microfilariae of some widely distributed filariae, such as *B. malayi*, in order to speculate on the evolution and geographic migration of the parasite.

Gibson reported on the morphologic features of microfilariae of *O. volvulus*, *O. gutturosa*, and *O. reticulata* (= *O. cervicalis*);⁵ these were of importance for field studies of onchocerciasis. He mentioned differences in size, and especially the arrangement, of anterior and caudal nuclei of individual microfilariae. A method of easily differentiating the microfilariae of *O. volvulus* from those of other species of the same genus would have practical value when dissecting vector black

flies in endemic areas of onchocerciasis. From this point of view it may be concluded that the present study adds a new criterion to the morphological features of microfilariae. For this reason, in the identification of onchocercal microfilariae not only the size, caudal nuclei and landmarks, but also the nuclear count would provide important information.

On the other hand, the close correlation of nuclear counts between *B. malayi* and *B. pahangi* further reflects that the features useful in differentiating these two species, such as the character of the inner body or staining by acid phosphatase,⁷ are limited. The present result may show the proximity of these two *Brugia* species as judged by the nuclear count.

Martínez-Palomo and Martínez-Báez showed two types of cells by using transmission electron microscopy; peripherally located muscle cells with large and clear nuclei, and centrally located undifferentiated cells with dense irregular nuclei. These cells were clearly demonstrable by the present method. Although no further investigation was performed with regard to the distribution and count of these types of nuclei, it is of interest to compare the features of nuclei among various species of microfilariae, and also to compare microfilariae of various geographic strains. However, more work is needed to examine whether the various fixatives such as formalin and AFA are suitable instead of the acetic-ethanol used for treatment in the present study.

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