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The effects of diethylcarbamazine on the ultrastructure of microfilariae of *Wuchereria bancrofti* in vivo and in vitro

Received: 7 October 2003 / Accepted: 16 December 2003 / Published online: 9 March 2004
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Abstract An ultrastructural study of microfilariae of *Wuchereria bancrofti* was performed after treatment in vitro and in vivo with diethylcarbamazine citrate (DEC). The morphological alterations produced by treatment in vitro with 5 µg/ml of DEC were the loss of microfilarial sheaths and lysis of the cytoplasm, with the destruction of all organelles and the formation of several vacuoles, the contents of which presented various degrees of electron-density, or showed an empty appearance. Some of these vacuoles seemed to be extruding from the cytoplasm as apoptotic bodies and others presented organelles inside. Similar alterations were observed after in vivo treatment. At 40 min after treatment of a microfilaremic individual with DEC, almost all microfilariae observed had lost their sheaths; and, in some of them, remains of the microfilarial sheath on the larval surface could be detected. Numerous vacuoles were observed, mainly in the hypodermis and somatic cells, showing organelles inside or an empty appearance. Condensed chromatin was also observed in some somatic cells. At 1 h after treatment of a microfilaremic individual with DEC, microfilariae presented drastic morphological alterations, with large vacuoles within

somatic cell cytoplasm and complete lysis of all cellular organelles. Therefore, both treatments with DEC in vitro and in vivo had a direct mechanism of action on the microfilariae of *W. bancrofti*, including organelle damage and apoptosis.

Introduction

The antifilarial drug diethylcarbamazine citrate (DEC) has been in clinical use for more than 50 years, yet its pharmacological mechanism of action remains a matter of controversy. Several proposals suggested that DEC does not have a direct effect on the surface of the microfilariae, since exposure of microfilariae to concentrations of drug that exceed the therapeutic dose leaves them unharmed (Hawking 1979; Johnson et al. 1988). One of the most frequent findings is that DEC increases the microfilarial adherence to endothelial cells and granulocytes (for a review, see Maizels and Denham 1992). These studies led to the suggestion that DEC stimulates the innate arm of the immune system. Other studies suggested that nitric oxide (NO) might be involved in host defense against filarial parasites (Rajan et al. 1996; Taylor et al. 1996). However, Rajan et al. (1998) showed that DEC does not induce NO synthesis in mouse macrophages and rat endothelial cells.

Conversely, accumulated evidence indicates that a microfilaricidal effect of DEC is not dependent on a specific humoral response. Weiner and Soulsby (1982) showed that DEC reduced microfilariae levels by 95.8%, even when microfilariae of *Litomosoides sigmodontis* released in vitro were transfused into a naïve animal, suggesting that an adaptive immune response was not a sufficient condition for DEC effectiveness.

Recent ultrastructural studies developed in our laboratory showed drastic morphological damage to microfilariae of *Wuchereria bancrofti* after in vitro treatment with DEC, indicating a direct mode of action of this major

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anti-filarial drug. One of the first morphological alterations produced by treatment with DEC was the loss of microfilarial sheaths. DEC also severely affected organelles, inducing extreme cellular disorganization with an abundance of electron-dense degenerating organelles, numerous large vacuoles and nuclear condensation (Florêncio and Peixoto 2003; Peixoto et al. 2003).

The present work uses ultrastructural analysis by transmission electron microscopy (TEM) to reveal morphological damage to microfilariae of *W. bancrofti* obtained after treatment of microfilaricidal individuals with a single dose of 6 mg/kg of DEC, confirming previous evidence of a direct mode of action of this major anti-filarial drug.

Materials and methods

Definition of cases

Asymptomatic microfilaricidal subjects were life-long residents of *Wuchereria bancrofti* in endemic communities in Recife, Brazil. They had no history of lymphangitis, erysipelas, limb-swelling or any other stigmata of lymphatic dysfunction. Informed consent was obtained from all participants before clinical and parasitological studies. The study protocol and procedures were reviewed and approved by the Ethical Committee of the Centro de Pesquisas Aggeu Magalhães, Recife.

Microfilariae of *W. bancrofti*

Initial screening for microfilaricidal was done by microscope examination of 60 µl of night blood smears. After detection of positive patients, subsequent collection of 5–10 ml of venous blood was performed in EDTA (final concentration 0.5 M) for filtration by Nucleopore (Costar, Waltman, Mass.). No volunteer had received any previous DEC treatment.

DEC in vitro treatment

Microfilariae were resuspended in RPMI 1640. In flatbottomed wells, 1,000 microfilariae were placed in 1 ml of one of the following final concentrations of DEC: 0 µg/ml and 5 µg/ml (Ministério da Saúde, Fundação Oswaldo Cruz, Farmanguinhos, Rio de Janeiro). The plates were incubated in 5% CO₂ for 1 h at 37 °C. The microfilariae were subsequently processed for TEM.

DEC in vivo treatment

Two patients participated in the in vivo assays. Before treatment, they had their blood collected for filtration and collection of pre-treatment microfilariae. After that, they received a single dose of 6 mg/kg of DEC during the daytime (provocative test) and were clinically monitored. A venous collection were performed in one individual at 40 min after ingestion of the tablet and in other at 1 h post-treatment. The microfilariae were subsequently processed for TEM.

Transmission electron microscopy

The living parasites were washed twice with phosphate-buffered saline (PBS) and fixed for 1 h in a solution containing 3.0%

glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, microfilariae were washed twice in the same buffer and then they were post-fixed in a solution containing 1% osmium tetroxide, 2 mM CaCl₂ and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in acetone and embedded in Epon 812 resin (Sigma Co., St Louis, Mo.). Polymerization was done at 60 °C for 2 days. Ultrathin sections were collected on 300-mesh copper grids, counterstained with uranyl acetate and lead citrate and examined by TEM, using a Zeiss EM 109 transmission electron microscope.

Results

Examination of thin sections of control microfilariae of *Wuchereria bancrofti* showed the following structures, as described by McLaren (1972): a thick sheath, a homogeneous cuticle layer, a hypodermis which forms the middle layer of the body wall, elongated muscle cells and somatic cells (Fig. 1). The morphological alterations produced by treatment in vitro with 5 µg/ml of DEC were the loss of microfilarial sheaths, although small numbers of apparently healthy ensheathed microfilariae could still be found. Severely impaired organelles were also observed, including lysis of the cytoplasm with destruction of all organelles, condensation and lysis of chromatin, formation of several vacuoles, the contents of which presented various degrees of electron-density or even an empty appearance (Fig. 2). Some of these vacuoles seemed to be extruding from the cytoplasm as apoptotic bodies (Fig. 2) and others presented organelles inside, like mitochondria (Fig. 3), and myofibrils (Fig. 4). Some cells presented mitochondria with a very condensed matrix (Fig. 3) and others showed fragmentation of the nuclear envelope (Fig. 4).

Examination of thin sections of pre-treatment in vivo microfilariae of *W. bancrofti* showed the structures as above described (Fig. 5).

At 40 min after treatment of a microfilaricidal individual with DEC, almost all microfilariae observed had lost their sheaths and, in some of them, remains of the microfilarial sheath on the larval surface could be detected (Fig. 6). Condensed chromatin was also observed in somatic cells (Fig. 6). Several microfilariae presented numerous vacuoles, mainly in the hypodermis and in somatic cells (Fig. 7). Whereas some of these organelles showed a membranous content, others presented an empty appearance (Fig. 8). The somatic cells shrank, producing a rippled appearance in the plasma membrane (data not shown).

At 1 h after treatment of a microfilaricidal individual with DEC, the microfilariae presented drastic morphological alterations. The loss of the microfilarial sheath was observed, although some larvae presented it in an intact form (Figs. 9, 10). Some somatic cells presented large vacuoles within the cytoplasm (Fig. 9). And in others the cytoplasm was observed as a remarkable electron-luscent region, indicating complete lysis of all cellular organelles. Strikingly, although the cellular content in some cells had disappeared, the plasma membrane maintained its integrity (Fig. 10).

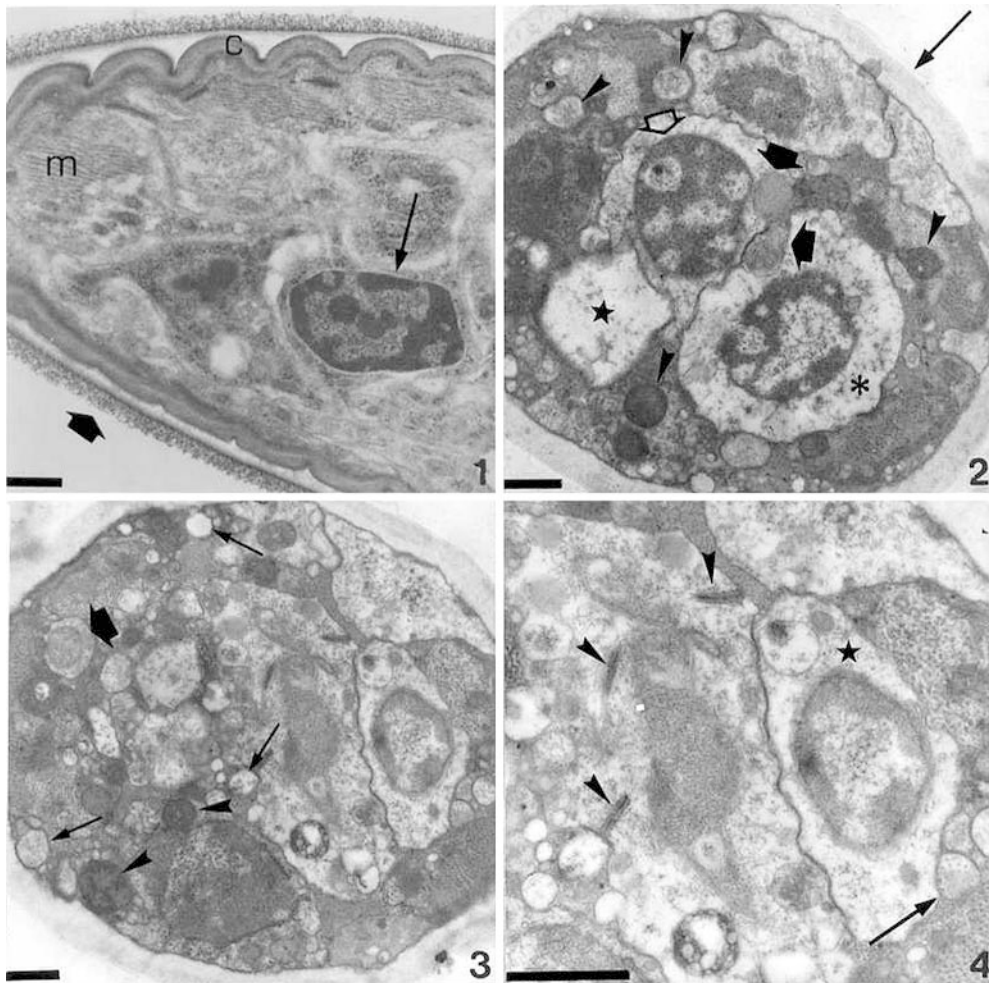


Fig. 1 Transmission electron micrograph of untreated control microfilariae of *Wuchereria bancrofti*. The following structures are observed: a sheath (short arrow), a homogeneous cuticle (c), muscle cells (m) and somatic cells (arrow). Bar 0.5 µm

Fig. 2–4 Transmission electron micrographs of microfilariae after treatment with 5 µg/ml of DEC. Bar 0.5 µm **Fig. 2** Observe the absence of sheath (thin arrow), formation of multiple vacuoles distributed throughout all microfilarial cells (arrowheads), some of them extruding from the plasma membrane (short black arrows), lysis of the cytoplasm of somatic cells (asterisk), an empty space probably related to a disappeared cell (star) and a condensed chromatin containing foci of lysis (short open arrow)

Fig. 3 Observe also an extensive vacuolization (thin arrows), condensed mitochondrial matrix (arrowheads) and a vacuole containing a mitochondrion (short arrows)

Fig. 4 Detail from Fig. 3. Observe the intense destruction of the somatic cell cytoplasm (star), fragmentation of the nuclear envelope (arrowheads) and a vacuole containing myofilaments (thin arrow)

Discussion

Previous studies showed that DEC has no effect on filarial parasites in vitro (Hawking et al. 1948; Hawking and Laurie, 1949; Johnson et al. 1988). These and many other observations led to the suggestion that DEC only stimulates host defense activity (for a review, see Maizels and Denham 1992). However, we recently

demonstrated by scanning electron microscopy and TEM analysis that treatment with DEC for 2 h in vitro stimulates the exsheathment of microfilariae of *W. bancrofti* and also promotes severe damage to microfilarial cells, including the presence of several large vacuoles and completely lysis of the cytoplasm, with the destruction of all organelles in some cells and the absence of nucleolus and condensed chromatin, features which resemble an apoptotic process (Florêncio and Peixoto 2003).

In the present work, we confirm and extend these previous observations. At 1 h after treatment with DEC in vitro, we observed that almost all microfilariae had lost their sheaths and presented numerous large vacuoles, mainly in somatic cells, some containing organelles inside. Also, some electron-dense vacuoles were observed extruding from the plasma membrane, resembling apoptotic bodies.

Similarly, treatment with 6 mg/kg in a single dose promoted after just 40 min the exsheathment of *W. bancrofti* microfilariae; and, in some embryos, remains of the microfilarial sheath on the larvae surface could be detected, indicating possible digestion of the microfilarial sheath. Also, cellular injury was clearly detected, with the presence of numerous and large

Fig. 5 Pre-treatment microfilariae of *W. bancrofti*. The following structures are observed: a sheath (arrow), a homogeneous cuticle (c), muscle cells (m) and somatic cell (s). Bar 0.5 μ m

Figs. 6–8 Transmission electron micrograph of microfilariae of *W. bancrofti* obtained at 40 min after treatment in vivo with DEC. Bar 0.5 μ m

Fig. 6 Remains of lost microfilarial sheath (arrowheads), condensed chromatin (thin arrow)

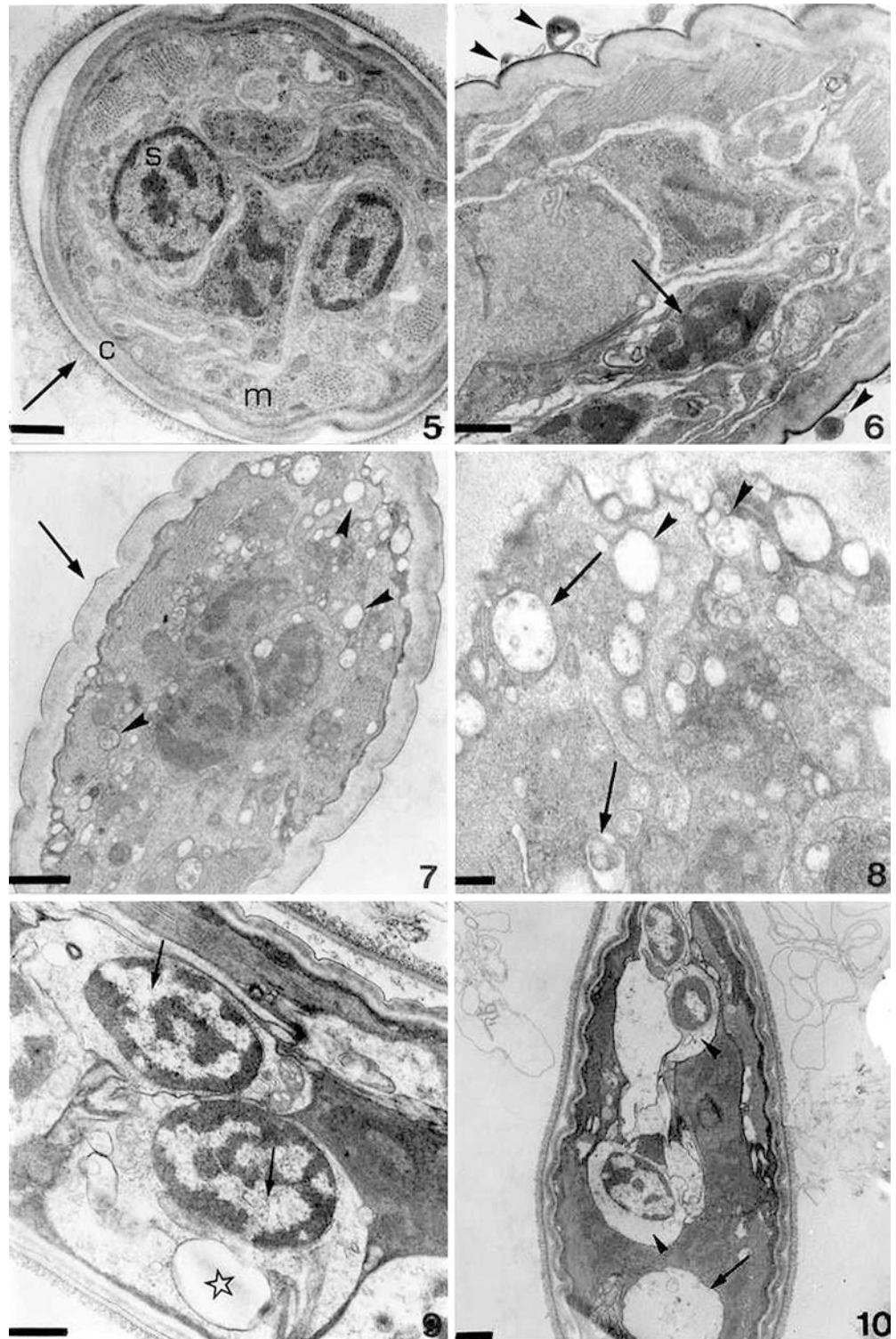
Fig. 7 Observe the loss of sheath (thin arrow), and the formation of numerous vacuoles dispersed throughout all microfilaria tissues (arrowheads)

Fig. 8 Detail from Fig. 7. Note the extensive vacuolization of the hypodermis (arrowheads), some of these vacuoles presenting remains of organelles inside (thin arrow)

Figs. 9, 10 Transmission electron micrograph of microfilariae of *W. bancrofti* obtained at 1 h after treatment in vivo with DEC. Bar 0.5 μ m

Fig. 9 Note some somatic cells suffering an intense destruction of cytoplasmic organelles, with presence of large vacuoles (star), lysis of nuclear content and absence of nucleolus (thin arrow)

Fig. 10 Observe the complete lysis of all cellular contents (arrowheads) and the integrity of the plasma membrane of a cell that has disappeared (thin arrow)



vacuoles in the hypodermis and in somatic cells, some of them containing organelles.

At 1 h after treatment with DEC, the damage was more severe, with complete lysis of all cellular organelles, mainly in somatic cells, and the presence of an intact plasma membrane surrounding an electron-luscent space without any leakage of their

contents, suggesting an autolytic process. Possibly, DEC could stimulate proteases like procaspases, triggering an apoptotic process. Some microfilariae showed severe damage, even presenting their sheaths, indicating that the loss of this structure is not a condition for a subsequent cellular injury, as suggested in our previous work.

In vitro studies showed that some selective proteases (e.g. papain,) are effective in promoting exsheathment, whereas leucine aminopeptidase, collagenase, hyaluronidase and chitinase do not stimulate this process (Devaney and Howells 1979). For instance, the microfilarial exsheathment induced by DEC is possibly involved in altering specific biochemical pathways, such as the release of endoproteases.

Some ultrastructural changes in *Litomosoides sigmodontis* microfilariae following administration of DEC to gerbils have been described by Schardein et al. (1968). At 20 min after drug administration, exsheathed microfilariae were observed freely within sinusoids. And after 4 h, many of them underwent lysis, being the foci of inflammatory cells. In this context, it is not clear whether DEC causes the exsheathment by a direct or indirect mechanism of action.

Pharmacological studies have shown that DEC interferes with arachidonic acid metabolism, blocking a number of steps in both cyclooxygenase and lipoxygenase pathways. Prostaglandins and PGE₁/PGE₂ are potent vasodilators and highly active inhibitors of platelet aggregation and endothelial adhesion (Davies et al. 1984). Also, microfilariae secrete PGE₂ and PGI₂, which may facilitate their passage by increasing capillary network diameters. DEC blocks the PGE₂ and PGI₂ production from both MF and endothelial cells, causing a constriction of the capillaries and impeding the passage of larvae (Kanessa-thasan et al. 1991). Other studies have demonstrated that DEC increases the rate of microfilariae adherence to vascular endothelium and to platelets and granulocytes (Piessens and Beldekas 1979; for a review, see Maizels and Denham 1992). Together with vasoconstriction, the host cellular adhesion probably contributes to the antifilarial activity of DEC.

In view of the fact that the microfilarial damage produced by DEC is unequivocally identical to that observed in vitro, we concluded that DEC presents a direct mode of action for promoting severe damage of microfilarial cells, including the presence of large vacuoles, lysis of the cytoplasm and chromatin and bodies extruding from the plasma membrane, which are indicative features of an apoptotic process.

Acknowledgements This work was supported by the Fundação Oswaldo Cruz (FIOCRUZ), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq project 470038/2001) and FIOCRUZ/FIOTEC/FUNASA (project 268,sub-project 17).

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