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Cell structure and cytokinesis alterations in multidrug-resistant *Leishmania* (*Leishmania*) *amazonensis*

Abstract Multidrug-resistant *Leishmania* (*Leishmania*) *amazonensis* may be obtained by in vitro selection with vinblastine. In order to determine whether this phenotype is linked to structural alterations, we analyzed the cell architecture by electron microscopy. The vinblastine resistant CL2 clone of *L.* (*L.*) *amazonensis*, but not wild-type parasites, showed a cytokinesis dysfunction. The CL2 promastigotes had multiple nuclei, kinetoplasts and flagella, suggesting that vinblastine resistance may be associated with truncated cell division. The subpellicular microtubule plasma membrane connection was also affected. Wild-type parasites treated with vinblastine displayed similar alterations, presenting lobulated and multinucleated cells. Taken together, these data indicate that antimicrotubule drug-selected parasites may show evidence of the mutation of cytoskeleton proteins, impairing normal cell function.

Introduction

Leishmania spp. are parasitic protozoa that cause a wide spectrum of diseases affecting about 12 million individuals with 1.0–1.5 million new cases worldwide per year and at least 350 million people under risk of infection in 88 endemic countries (Desjeux 2004); however, since numerous cases remain asymptomatic for long periods, the incidence of the disease is presumably underestimated.

Chemotherapy still remains the major treatment and control method for most parasitic diseases, particularly because efficient vaccines are not available (Liu and Weller 1996). Nevertheless, drug-resistance mechanisms often reduce infection chemotherapy responsiveness (Sangster et al. 2002). The drugs of choice used for leishmaniasis include the antimonials, which have been in use for more than half a century. Besides important side effects, refractory cases are known to occur (Croft and Yardely 2002). Some degree of drug resistance may be observed in up to 70% of the cases in different forms of leishmaniasis (Grogl et al. 1991). Miltefosine (or hexadecylphosphocholine) is a rather promising compound recently incorporated into the therapeutic arsenal (Jha et al. 1999; Jacobs 2002), but miltefosine-resistant *Leishmania* have already been reported (Perez-Victoria et al. 2003a, 2003b). A detailed understanding of the mechanisms underlying drug resistance is required for the development of successful chemotherapy strategies (Cabral 2001; Ponte-Sucre 2003).

The expression of P-glycoprotein efflux pumps, such as the product of the *mdr1* gene, confers multidrug resistance (MDR) to protozoan cell lines and human tumors (McKeegan et al. 2003). The amplification of genes homologous to the mammalian *mdr* encoding P-glycoprotein-like molecules has been associated with the development of drug resistance in many *Leishmania* species (Perez-Victoria et al. 2002; Leandro and Campino 2003).

Tubulin binding agents, such as *Vinca* alkaloids and taxanes, target essential components of the mitotic and cell organization machinery and are frequently associated with mechanisms of efflux pump-mediated drug resistance (Dumontet 2000; Manetti et al. 2004). Tubulins are the major trypanosomatid proteins, and

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microtubules not only shape the parasites, but also regulate important cell biological events, such as cell morphogenesis and polarity, endocytosis, locomotion, nuclear and mitochondrial/kinetoplast division and cytokinesis (Ploubidou et al. 1999; Gull 2001; Kohl et al. 2003; Dantas et al. 2003). Cell biology approaches may shed light on antiprotozoal chemotherapy (De Souza 2002; Vannier-Santos et al. 2002).

Despite several studies on the molecular and biochemical aspects of MDR in protozoa, the direct effect of the drugs on parasite cell organization is poorly understood. In this study, we used promastigotes of the vinblastine-resistant CL2 clone of the RV100 line of *L.* (*L.*) *amazonensis* presenting a MDR phenotype and exhibiting cross resistance to the chemically unrelated drug adriamycin (Gueiros-Filho et al. 1995). Data presented here indicate that the use of cytoskeleton-perturbing compounds may be useful in the elucidation of *Leishmania* cell biology.

Materials and methods

Chemicals

Vinblastine (Velbam) was purchased from Eli Lilly do Brasil (São Paulo, SP, Brazil). Fetal calf serum, glutaraldehyde and osmium tetroxide were obtained from Sigma (St. Louis, Mo.). Reagents used in the culture medium were purchased from Difco Laboratories (Detroit, Mich.).

Parasite maintenance and growth conditions

Promastigotes of *L.* (*L.*) *amazonensis* (IFLA/BR/67/ PH8) were kindly provided by Dr. G. Grimaldi (Instituto Oswaldo Cruz, FIOCRUZ, Brazil) and maintained in brain and heart infusion (BHI) liquid medium supplemented with 10% fetal calf serum at 25°C (Vannier-Santos et al. 1995). The cells were subcultured weekly.

Selection of *Leishmania* drug-resistant cell lines

L. (*L.*) *amazonensis* promastigotes were selected by gradually increasing the vinblastine concentration as described previously (Coderre et al. 1983; Gueiros-Filho et al. 1995). Briefly, promastigotes were grown for 2 weeks in medium supplemented with 10 μM vinblastine. Then the cells were transferred to medium containing 20 μM vinblastine and allowed to grow for another 2-week period and so on until the promastigotes were able to grow in 100 μM vinblastine (RV100 line). We studied the CL2 clone of line RV100. Continuous drug pressure was maintained during all experiments. For short-term treatment, promastigotes were treated with 10 μM vinblastine for 1–72 h.

Electron microscopy

The promastigotes of *L.* (*L.*) *amazonensis* were rinsed in PBS, pH 7.4, fixed in 2.5% glutaraldehyde and 0.2% picric acid in 0.1 M cacodylate buffer, pH 7.4 for 60 min. They were then rinsed and postfixed in 1% OsO_4, 0.8% $K_3Fe (CN)_6$ and 5 mM $CaCl_2$ in the same buffer, for 1 h. For transmission electron microscopy (TEM), the samples were dehydrated in acetone and embedded in epoxy Polybed resin (Polyscience). Ultrathin sections were stained with uranyl acetate, lead citrate and were observed in a Zeiss 900 or Zeiss 109 electron microscope.

For scanning electron microscopy (SEM), fixed cells were adhered to coverslips covered with poly-L-lysine and post-fixed with osmium tetroxide as above. The specimens were then dehydrated in ethanol and critical point dried in a Balzers apparatus, mounted on metal stubs, covered with an approximately 20 nm-thick gold layer and observed in a Zeiss 940 digital scanning electron microscope.

Results

We compared the structural organization of the wild-type before and after vinblastine treatment and MDR^+ vinblastine-resistant *L.* (*L.*) *amazonensis* promastigotes by TEM and SEM.

Surface topography assessed by SEM showed that, contrary to wild-type parasites (Fig. 1a), the vinblastine-resistant promastigotes were abnormally shaped, presented short, twisted or absent flagella (Fig. 1b) and displayed numerous membrane invaginations (Fig. 2a, b) and occasionally multiple flagella (Fig. 2c), presumably indicating truncated or deregulated cytokinesis.

Ultrathin sections of the CL2 parasites revealed altered membrane-microtubule connections and microtubule-devoid membrane protrusions (Fig. 3a–c). The membrane-microtubule connecting filaments and the distance between them were irregular (Fig. 3a). It is noteworthy that endoplasmic reticulum (ER) cisternae were associated with, and even entered such extensions (Fig. 3b, c).

Promastigotes often presented surface invaginations forming long clefts associated with subpellicular microtubules and ER cisternae (Fig. 3d) which occasionally conferred the parasites a multilobulated appearance (Fig. 3e). We found CL2 parasites with remarkably enlarged kinetoplasts with several randomly distributed kDNA bars (Fig. 3f) and multinucleate cells (polykarions) were also seen, presenting nuclei with a normal ultrastructural appearance (Fig. 3g). Mitochondrial distribution also appeared atypical (not shown).

In order to determine whether these alterations could directly result from the *Vinca* alkaloid, we incubated wild-type parasites with vinblastine for different periods. Promastigotes cultured with 10 μM vinblastine for 72 h
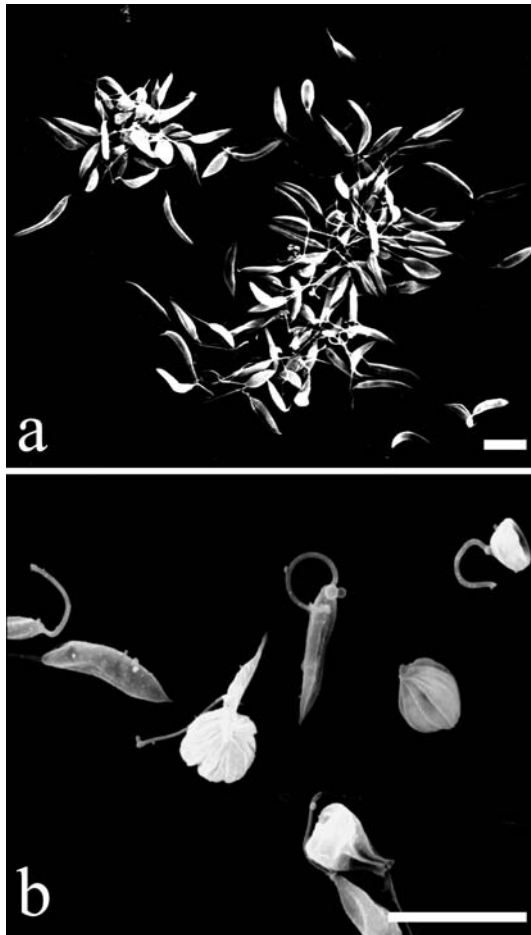
Fig. 1 a Scanning electron micrograph of control wild-type *Leishmania* (*L.*) *amazonensis* displaying the normal morphology of promastigotes. b SEM of CL2 vinblastine-resistant parasites showing irregularly shaped and often rounded forms with short, twisted and sometimes absent flagella. *Bars* represent 10 μm

presented multilobulated and multinucleated cells (Fig. 4), strongly indicating impaired cytokinesis.

Discussion

Antimicrotubule agents are useful tools in leishmaniasis chemotherapy studies both because they may be leishmanicidal (Chan and Fong 1990; Chan et al. 1995; Bhattacharya et al. 2002, 2004; Werbevetz 2002) and for their ability to select parasites with multidrug-resistant (MDR[+]) phenotypes (Gueiros-Filho et al. 1995).

Vinblastine-resistant parasites displayed abnormal shapes and loss of cell polarity, indicating an altered cytoskeletal function. Flagellar alterations suggest that the axoneme microtubules were affected in these cells. Deformed and twisted flagella were also reported in *Leishmania* (*L.*) *donovani* exposed to antimicrotubule agents (Havens et al. 2000).

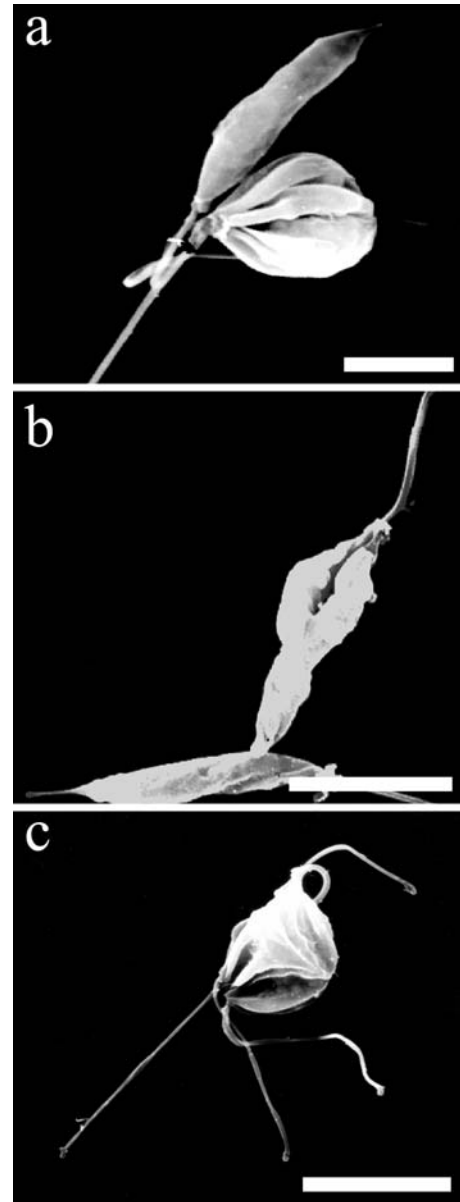Since the subpellicular microtubule membrane is rather stable in trypanosomatid parasites (De Souza

Fig. 2 a Many cells presented long, single or multiple surface invaginations which seemed to be formed from the anterior end (b). c Some parasites presented numerous flagella, indicating truncated cell division. *Bars* represent either 5 μm (a, b) or 10 μm (c)

1989), such microtubule-free protrusions as those observed in MDR[+] *Leishmania* are only found under particular conditions such as ligand-induced shedding (Saraiva et al. 1989; Vannier-Santos et al. 1992) and protein kinase C activation (Vannier-Santos et al. 1988). Protein kinase C is able to regulate P-glycoprotein activity (Fine et al. 1996).

Although the ER approaches the parasite plasma membrane (Pimenta and De Souza 1985), the biological role of this phenomenon remains to be elucidated. It was recently shown that P-glycoprotein is transported from the ER to the Golgi complex and then to the cell surface (Fu et al. 2004). Blockage of ER-Golgi trafficking by brefeldin A and monensin resulted in daunorubicin

Fig. 3 a–c CL2 clone cells often presented cytoplasm and microtubule-free membrane projections. **a** Microfilament connections between subpellicular microtubules and the plasma membrane were usually observed, as in controls (*arrow*), but irregular amorphous material was also associated with microtubules in drug-resistant parasites (*arrowhead*). **b**, **c** Endoplasmic reticulum cisternae were seen in association with and even entering such protrusions (*arrow* in **c**). **d** The surface invaginations were scaffolded by subpellicular microtubules, **e** and gave the parasites a lobulated appearance. **f** CL2 parasites showed an impaired kinetoplast, but not nuclear division (**g**). Observe the kinetoplast presenting multiple k-DNA bars (*K*), but the nuclei (*N*), sometimes multiple, kept a normal ultrastructure (**g**). *Bars* represent either 0.1 (**a–c**) or 0.5 μm (**d–g**)
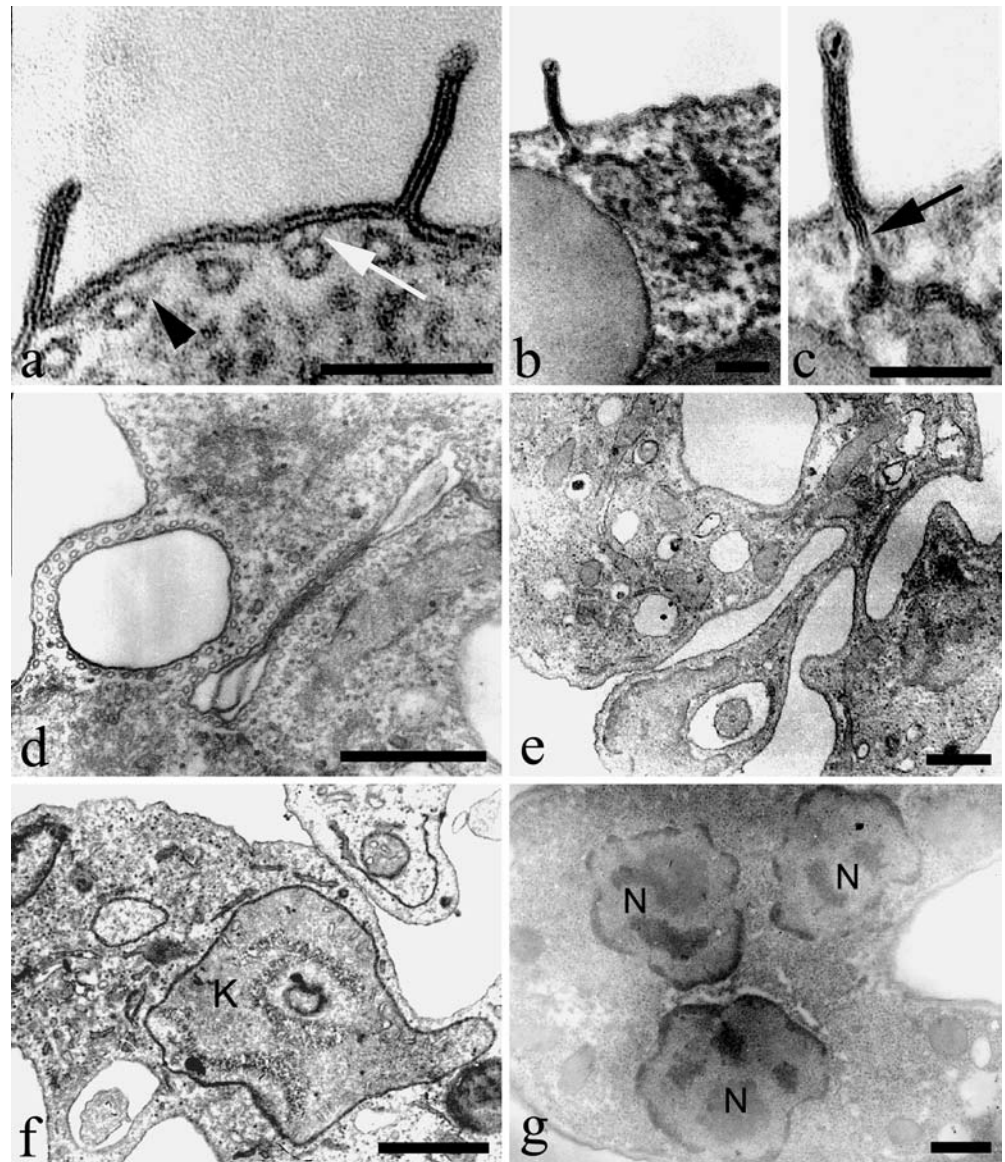
accumulation in HeLa cells. Nevertheless, the role of ER cisternae-membrane protrusions in the *Leishmania* MDR$^+$ phenotype is yet to be determined.

The multiple membrane infolds observed in CL2 parasites are interpreted as slowed or interrupted division furrows, which are observed particularly at the anterior end of cells where cell division begins in trypanosomatids (Molyneaux and Killick-Kendrick 1987). Cell surface microvilli form a diffusion barrier which may take part in MDR (Lange and Gartzke 2001), but it is not known whether the CL2 parasites numerous surface infolds, observed by electron microscopy, take part in drug resistance.

Multiflagellate and multinucleate cells appeared as a result of truncated cell division. Similarly, *Vinca* alkaloids produced a reversible blockage of cytokinesis in *T. cruzi* with the presence of multiple nuclei and kinetoplasts (Grellier et al. 1999). Other tubulin inhibitors impaired cytokinesis, producing aberrant cell types
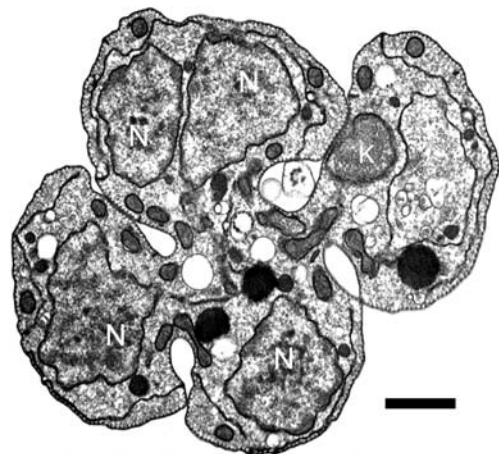

Fig. 4 A wild-type *L.* (*L.*) *amazonensis* promastigote treated with 10 μM vinblastine for 72 h. Four lobules, four nuclei (*N*) and one kinetoplast (*K*) can be seen, indicating impairment of cytokinesis. *Bar* represents 1 μm

including enucleated parasites or zoids in *L.* (*L.*) *donovani* (Havens et al. 2000) and *T. cruzi* (Grellier et al. 1999), as well as blockage of the promastigote cell cycle (Moulay et al. 1996). The interference with kinetoplast division in MDR$^+$ *Leishmania* may be explained by a microtubule-mediated mechanism of kDNA and kinetoplast repositioning as reported in *L.* (*L.*) *donovani* (Havens et al. 2000) and *Trypanosoma brucei* (Matthews et al. 1995), which may comprise a cell cycle checkpoint controlling cytokinesis (Ploubidou et al. 1999). Interestingly, nuclear division was not affected under the conditions employed here, suggesting the involvement of distinct tubulin isoforms or hampered access to the intranuclear spindle. Here, the cell vinblastine alterations are similar to those observed in the vinblastine-resistant *L.* (*L.*) *amazonensis* cell line.

Since the survival of drug-induced polykarions may be associated with an altered susceptibility to different compounds (Court et al. 1993), these aberrant forms must be considered in MDR studies. Although the early vinblastine effects on *Leishmania* may be reversed, the MDR phenotype and the ultrastructural alterations produced by long-term treatment were stable. This indicates that prolonged drug exposure may lead to the selection of populations bearing cytoskeleton protein mutations.

In this regard, different cell lines resistant to microtubule-targeting agents such as the *Vinca* alkaloids, colchicine, colcemid, griseofulvin and taxanes such as taxol, may rely on the rise of mutants displaying distinct β-tubulin isoforms with different biochemical properties. Drug resistance may be pgp expression-independent and not associated with the MDR$^+$ phenotype (Cabral et al. 1980; Jaffrezou et al. 1995; Kavallaris et al. 1997; Cabral 2001; Werbovetz 2002; Ponte-Sucre 2003). Similarly, drug resistance in *T. cruzi* infections may be unrelated to pgp gene expression (Murta et al. 2001), and multigene drug-resistance mechanisms have been reported in vinblastine-resistant *Leishmania* species (Henderson et al. 1992; Chow et al. 1993; Wong et al. 1994). Antimonial resistance in visceral leishmaniasis may rely on altered protein phosphorylation (Singh et al. 2003).

The alterations observed in resistant parasites may be epiphenomena rather than related to efflux pump activity (Ponte-Sucre 2003) or linked to the dysfunction of cellular volume control. In this regard, P-glycoprotein is a regulator of volume sensing (Okada 1997) and cell swelling may inhibit its activity (Sardini et al. 1994). Interestingly, P-glycoproteins play a role in phospholipid transport (Tannert et al. 2003). However, RV-100 promastigotes are sensitive to the pgp inhibitor verapamil, which leads to the accumulation of pgp substrates in the cells (Gueiros-Filho et al. 1995), indicating that although there are critical morphological alterations, cell survival is related to the MDR phenotype and overexpression of the MDR gene in RV100.

It is well known that microtubule function may influence actin filament architecture and the *T. brucei* subpellicular microtubules may be stabilized by low molecular weight cytoskeleton-associated proteins (Vedrenne et al.

2002). The MDR$^+$ phenotype is associated with actin microfilament organization and microfilament disruption causes loss of this phenotype (Tsuruo and Iida 1986; Takeshita et al. 1998). Interestingly, the actin network is only partially restored after colchicine removal (Erokhina et al. 1994). Furthermore, P-glycoprotein may be connected to actin through ezrin/radixin/moesin proteins (Luciani et al. 2002). Therefore, growth with vinblastine may lead to the selection of actin or microtubule-associated protein mutants. Thus, the effects of antimicrotubule agents in *T. cruzi* may be mediated by an altered interaction between microtubules and associated proteins (Grellier et al. 1999). In addition, a novel actin isotype has recently been observed associated with the *Leishmania* subpellicular microtubules (Sahasrabubbhe et al. 2004). It is noteworthy that the membrane-microtubule connection in the parasites was remarkably altered. Our data indicate that drug-induced selection of resistance may lead to an accumulation of mutants with anomalous cell functioning and the use of cytoskeleton-perturbing compounds may shed light on *Leishmania* cell and organelle architecture and division.

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