Cell differentiation and infectivity of *Leishmania mexicana* are inhibited in a strain resistant to an ABC-transporter blocker

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SUMMARY

We analysed whether markers of cell differentiation and infectivity differed when compared to the parental sensitive strain [NR(Gs)] in an *in vitro* selected *Leishmania* strain [NR(Gr)] resistant to Glibenclamide®, an ***A***TP-***b***inding-***c***assette (ABC)-transporter blocker. The data show that the cell body area was larger in NR(Gr) compared to NR(Gs) and that functional characters associated with an infective metacyclic phenotype, such as resistance to the lytic effect of the alternative complement pathway and expression of the Meta-1 protein, were reduced. The infectivity of NR(Gr) to J774.1 macrophages was also significantly reduced. These results suggest that resistance in *Leishmania* against Glibenclamide®, a general blocker of P-glycoproteins, could produce functional modifications that may be relevant for *Leishmania* differentiation, infectivity and survival.

Key words: cell differentiation, cell survival, drug resistance, infectivity, *Leishmania mexicana*.

INTRODUCTION

In *Leishmania*, *in vitro* drug resistance is often correlated with the increased expression of a P-glycoprotein (P-gp) that belongs to the ***A***TP-***b***inding-***c***assette (ABC) transporter family (Légaré, Hettema & Ouellette, 1994). Besides the specific membrane permeability changes, chemoresistance in *Leishmania* can involve functional modifications which are relevant for the parasite–host cell interaction; for example, alterations in membrane fluidity and in saturated fatty acid levels (Mbongo *et al*. 1998), or in mitochondrial enzyme activities and in lipophosphoglycan (LPG) expression (Basselin & Robert-Gero, 1998). There is a widely held belief that understanding such functional changes involved in drug resistance will eventually improve treatment outcome.

While in the sandfly, *Leishmania* parasites go through sequential differential stages from actively dividing non-infective parasites to non-dividing infective metacyclic forms (Sacks & Perkins, 1984) and morphological distinctions between non-infective and infective parasites are now clear (Rogers, Chance & Bates, 2002). Studies in *Leishmania* (*L*.) *major* show that (a) promastigotes obtained *in vivo* from infected sandflies shortly after parasite ingestion from mammals are, like their *in vitro* logarithmic phase counterparts, essentially avirulent and (b) in stationary-phase cultures, infective *L. major* promastigotes are routinely separated from non-infective parasites by taking advantage of their failure to be agglutinated by the *Arachis hypogaea* lectin peanut agglutinin (PNA). This change in lectin binding accurately reflects the development of metacyclic (infective) promastigotes (Sacks & da Silva, 1987). Differential PNA binding has not proved to be applicable to *L. mexicana*, and the definition and purification of metacyclic (infective) parasites have required the use of alternative methods (Bates & Tetley, 1993; Zakai, Chance & Bates, 1997), including the expression of the product of the *meta-1* gene, originally described in *L. major* (Nourbakhsh, Uliana & Smith, 1996), highly conserved in Old and New World parasites and predominantly expressed in metacyclic promastigotes of *Leishmania* (Berberich *et al*. 1998; Uliana *et al*. 1999).

The sulfonylurea (Glibenclamide® (GLIB))-sensitive receptor is a member of the ABC transporter family (Inagaki *et al*. 1995), to which the *Leishmania* P-gpA belongs; of note, *Leishmania* shows a moderate sensitivity to this inhibitor (Ponte-Sucre *et al*. 1997, 1998). In cancer cells with an increased expression of P-gp GLIB and two GLIB derivatives which are substrates for P-gp, can acutely increase [^{3}H]-colchicine accumulation and invert the multidrug resistance phenotype in a similar way to verapamil (Golstein *et al*. 1999). These data suggest that GLIB could be considered a general ABC transporter blocker that binds to a conserved motif. *Leishmania* parasites are extremely flexible and easily develop resistance against a great variety of

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compounds. To further our insight into the physiological basis of *Leishmania* drug resistance, we have selected *in vitro* a *L. mexicana* strain (NR(Gr)) resistant to GLIB, by continuous drug pressure. Herein we have analysed whether metacyclogenesis and infectivity differed between NR(Gr) and their parental *L. mexicana* (NR(Gs)) strain which are sensitive to GLIB (Ponte-Sucre *et al*. 1997; García *et al*. 2000). Our results show that resistance could influence important biological functions involved in the regulation of *Leishmania* differentiation and survival that may decrease the occurrence of putative metacyclics and parasite infectivity.

MATERIALS AND METHODS

Chemicals

Glibenclamide® (GLIB) was purchased from Aldrich International Co., Milwaukee, USA and prepared as previously described (Ponte-Sucre *et al*. 1997). The other biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Gibco Life Technologies, (Gaithersburg, MD, USA).

Cell lines and cultures

Leishmania NR(Gs), MHOM/VE/80/NR, was kindly provided by Dr Angel Hernández, Universidad Central de Venezuela. It was originally isolated in 1980 from a Venezuelan patient with tegumentary diffuse leishmaniasis and identified as a member of *Leishmania mexicana* (Luis *et al*. 1998). Promastigotes of *Leishmania* NR(Gs) were grown as described (Eresh *et al*. 1993). The resistant strain NR(Gr) was selected *in vitro* by successive passages of the parental sensitive strain grown in the presence of 16×10^{-6} M GLIB as previously described (Ponte-Sucre *et al*. 1997) and maintained under the pressure of the drug. The macrophage cell line J774.1 was grown as previously described (Ponte-Sucre *et al*. 1998).

Parasite morphology

The morphology of *Leishmania* promastigotes was determined by previously described methods (Penin *et al*. 1992; Bates & Tetley, 1993). Parasite aliquots of 0·5 ml were removed and concentrated by centrifugation, 10 μl aliquots were air-dried, fixed in methanol and stained with Giemsa stain diluted in phosphate-buffered saline (PBS), pH 7·2. Afterwards the slides were observed through a calibrated microscope at 400× total magnification. The body length (L), the width (W) and the flagellum length (fL) were determined for 100 parasites analysed for each experimental condition.

Lysis of parasites by human serum

The lytic assay was carried out as described elsewhere (Louaissini *et al*. 1994) at 37 °C. Fresh human blood from healthy volunteers was allowed to clot at room temperature for 2 h and overnight at 4 °C. Aliquots of the resulting serum were stored at −70 °C until needed. Promastigotes at different growth phases were washed and resuspended to a density of 1×10^7/ml in PBS. They were exposed for 1 h to several dilutions of serum and the lytic reaction was stopped by the addition of 20 μl of 5×10^{-2} M EDTA. A control vial was exposed to 100% heat-inactivated human serum. The parasite viability was estimated in each experiment by checking the motility of the flagellum in 100 promastigotes for each experimental condition.

Electrophoresis and Western blotting

Crude cell homogenates were separated by polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes by standard procedures. The membranes were assayed with a polyclonal antibody prepared against the *Leishmania* Meta-1 protein (Meta-1) (1 : 1000) (Berberich *et al*. 1998) and a monoclonal antibody against *L. mexicana* flagellar proteins (B7) (1 : 10 000) (Ismach *et al*. 1989). Diluted peroxidase-coupled goat anti-rabbit (1 : 5000) or anti-mouse (1 : 2500) were used as secondary antibodies.

Infectivity

J774.1 macrophages ($2{\cdot}5 \times 10^5$ cells/ml) were resuspended and incubated at 37 °C under 5% CO_2 in air, for 2 h in 0·5 ml of Click-RPMI 1640 culture medium supplemented with 2×10^{-3} M L-glutamine, 20 μg/ml gentamicin, 2×10^{-5} M 2-mercaptoethanol and 10% fetal bovine serum, pH 7·2. Immediately thereafter, the non-adherent macrophages were removed by thorough washing with fresh Click-RPMI, and the adherent macrophages were infected with stationary-phase promastigotes of NR(Gs) or NR(Gr) for 4 h at a parasite to macrophage ratio of 5 to 1. After removal of extracellular parasites by thorough rinsing with fresh Click-RPMI, the wells were incubated further for 24 h either in the absence of drugs or in the presence of 3×10^{-6} M GLIB. Intracellular parasites were quantified at the end of either the 4 h infection or 24 h incubation by staining with acridine orange and ethidium bromide and were analysed by fluorescence microscopy at 495 nm (Ponte-Sucre *et al*. 1997).

Statistical analysis

Resistance to complement lysis is expressed as the mean ± S.E.M. of the dilution of serum capable of immobilizing 50% of a population of 100 parasites in

Table 1. Body and flagellum size and resistance to the lytic effect of human serum in exponential and stationary phase NR(Gs) and NR(Gr)

	Leishmania strain			
	NR(Gs)		NR(Gr)	
Growth phase	Exponential	Stationary	Exponential	Stationary
Body†				
Width (W) μm	2·16* ± 0·07	2·08 ± 0·05	2·44* ± 0·09	2·06 ± 0·06
Length (L) μm	10·64 ± 0·42	11·60** ± 0·49	11·06 ± 0·30	14·08** ± 0·37
Flagellum†				
Length (fL) μm	10·96*** ± 0·62	15·16*** ± 0·805	17·38 ± 0·60	17·70 ± 0·60
Lysis by human serum				
EC_{50}§	0·182* ± 0·019	0·275* ± 0·02	0·159 ± 0·014	0·170 ± .012

† The measurements were done in 2 independent experiments. Compared means ± S.E.M. are identified by the same number of asterisks: * $P<0·05$, ** $P<0·001$, *** $P<0·0001$.
§ EC_{50} ml/ml. The data represent the mean values ± S.E.M. of 3 experiments. Compared means ± S.E.M. are identified by the same number of asterisks: *$P<0·05$.

3 independent experiments, for each experimental condition carried out in duplicate. Statistical significance was evaluated by the Student's *t*-test (Schefler, 1981). The size of the promastigotes is expressed as the mean ± S.E.M. of a population of 100 parasites for 2 independent experiments for each experimental condition carried out in duplicate. Statistical significance was evaluated by the Student's *t*-test (Schefler, 1981). An infection index was calculated as {[(number of parasites/100 infected cells)$_{24\ h}$/(number of parasites/100 infected cells)$_{4\ h}$] × 100} of 3 experiments done in duplicate. Statistical significance was evaluated by the Student's *t*-test (Schefler, 1981).

RESULTS

Parasite morphology

Intact promastigotes showed their usual appearance under light microscopy; they did not have membrane damage and possessed flagellum motility. To determine if some morphological properties of *L. mexicana* are changed in NR(Gr), we evaluated the parasite body length (L) and width (W), as well as the flagellum length (fL) (Table 1). The flagellum length (a) was always greater for NR(Gr) than for NR(Gs), (b) increased significantly between log phase and stationary phase in NR(Gs) ($P<0·0001$), and (c) did not change significantly between log phase and stationary phase in NR(Gr). Exponentially growing NR(Gs) cells were significantly narrower ($P<0·05$) than NR(Gr) cells but had a similar body length; by contrast, parasites from both strains had a similar width at their stationary phase. Of note, NR(Gr) cells became significantly longer at stationary phase ($P<0·001$) than NR(Gs) cells. A frequency distribution of cell body area for parasites at their exponential and stationary phase of growth was calculated with these data (Fig. 1A, B). The frequency distribution

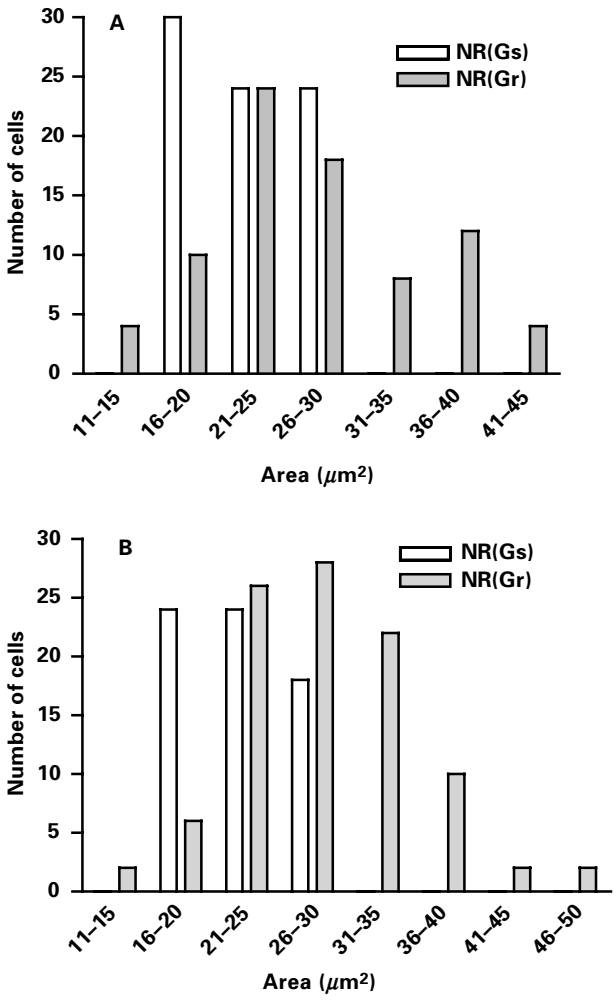

Fig. 1. (A) Frequency distribution of cell body area in exponential-phase wild-type [NR(Gs)] and GLIB-resistant [NR(Gr)] cultures. (B) Frequency distribution of cell body area in stationary-phase [NR(Gs)] and [NR(Gr)] cultures.

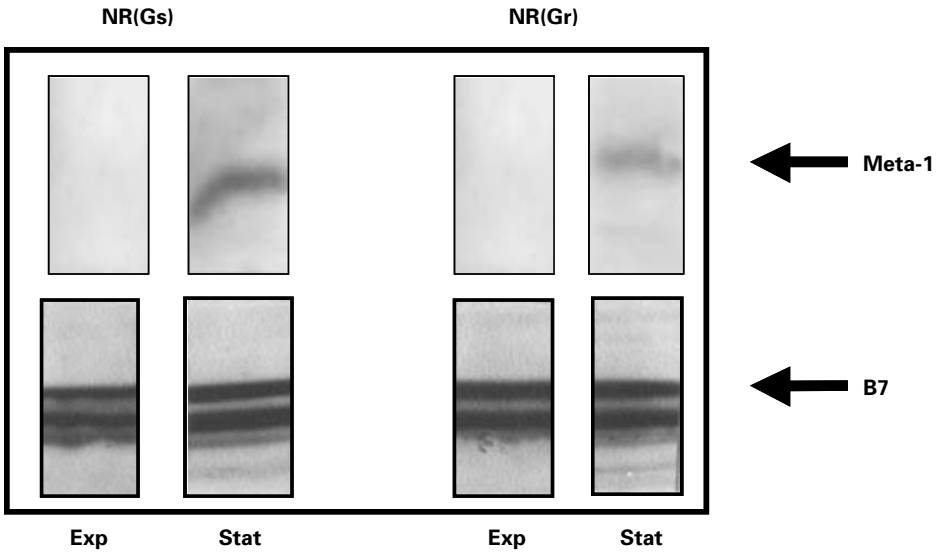

Fig. 2. Western blot analysis of exponential-phase (Exp) and stationary-phase (Stat) wild-type [NR(Gs)] and GLIB-resistant [NR(Gr)] strains. Homogenates were separated by PAGE, transferred into nitrocellulose membranes and exposed to B7 or Meta-1 antibodies.

measured in exponentially growing parasites (Fig. 1A) showed statistically significant differences; the mean body cell areas were $22{\cdot}6 \pm 0{\cdot}9\ \mu m^2$ and $27{\cdot}2 \pm 1{\cdot}27\ \mu m^2$ for NR(Gs) and NR(Gr) respectively ($P < 0{\cdot}001$). This difference was maintained in stationary phase parasites (Fig. 1B) and the cell body areas were $24{\cdot}40 \pm 0{\cdot}86\ \mu m^2$ in NR(Gs) and $29{\cdot}40 \pm 1{\cdot}18\ \mu m^2$ in NR(Gr) ($P < 0{\cdot}001$). Also, the shape of the frequency distributions differed in that the NR(Gs) cell body area had a narrower range than the cell body area of NR(Gr). These results suggest that in stationary phase cultures of NR(Gs) there is a higher proportion of cells with a morphology suggestive of metacyclic forms than in NR(Gr) cultures.

Functional signs of metacyclogenesis

To test if this difference in cell body area reflects changes in the proportion of differentiated cells in NR(Gr) compared to NR(Gs), functional signs of metacyclogenesis were quantified in *Leishmania* by the use of two techniques i.e. lysis by human serum (Table 1) and expression of the Meta-1 protein (Fig. 2). Resistance to the lytic effect of human serum was calculated by reference to a control experiment where the parasites were exposed to 100% heat-inactivated serum. Resistance to complement lysis was always greater in NR(Gs) than in NR(Gr) and increased significantly in the stationary phase of NR(Gs) promastigotes. In contrast, exponentially growing cells of NR(Gr) showed a resistance to the lytic effect of human serum that did not increase significantly in stationary phase. Regarding the expression of Meta-1, a protein band of a relative molecular mass of 12 kDa, similar to the subunit previously described for various *Leishmania*, was recognized exclusively in stationary phase parasites

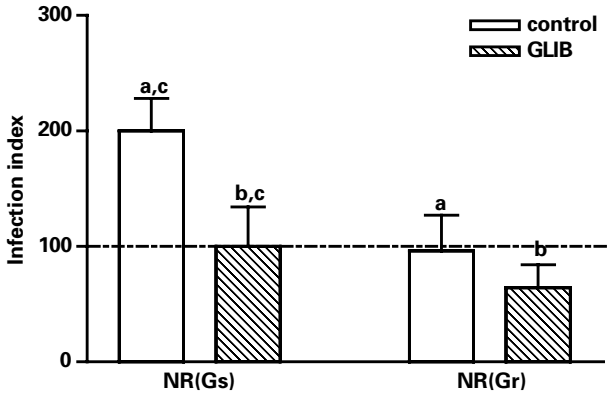

Fig. 3. Infection index of stationary-phase wild-type [NR(Gs)] and GLIB-resistant [NR(Gr)] within J774.1 macrophages either untreated or treated for 24 h with 3×10^{-6} M GLIB. Dashed line at 100 indicates infection index at 4 h. Compared means are identified by the same letter: $^{a}P < 0{\cdot}001$, $^{b}P < 0{\cdot}001$, $^{c}P < 0{\cdot}05$.

(Fig. 2). The amount of protein loaded for each sample was similar, as demonstrated by the intensity of the signal recognized by the B7 antibody against flagellar pocket proteins; nevertheless, in NR(Gs) the band recognized by the Meta-1 antibody was stronger than in NR(Gr). Altogether these results indicate again that a greater proportion of promastigotes expressing metacyclic properties were present in stationary phase cultures of NR(Gs) compared with NR(Gr).

Infectivity

Finally, as metacyclogenesis is associated with increased infectivity and in order to further validate the results described above, we compared the infectivity to J774.1 macrophages and sensitivity to GLIB of

both NR(Gs) and NR(Gr). Previously (García *et al*. 2000) we had determined that at 4 h and in the absence of drugs, J774.1 macrophages are infected by NR(Gs) and *L. major* to a similar percentage (17%) and that this rate of infection is 30% smaller in J774.1 macrophages infected with NR(Gr). It should be noted that NR(Gs) and NR(Gr) had the same passage number. Herein we have measured the proliferation of parasites 24 h after a 4 h infection (Fig. 3). The degree of infection determined at the end of the 4 h is here referred to as 100%. In macrophage/parasite cultures incubated without GLIB for 24 h the infection index increased to 200% if infected with NR(Gs), but did not change for macrophages infected with NR(Gr) ($P<0{\cdot}0001$) (Fig. 3). The increase in the degree of infection observed in non-treated macrophages infected with NR(Gs) was impaired by treatment with GLIB for 24 h ($P<0{\cdot}001$). On the contrary, no further changes in the infection index were observed in macrophages infected with NR(Gr) and treated with GLIB. These results suggest that the proportion of parasites able to proliferate inside the macrophages was greater in NR(Gs) than in NR(Gr), that intracellular NR(Gs) parasites were sensitive to GLIB and that intracellular NR(Gr) parasites remained insensitive to GLIB.

DISCUSSION

We have investigated whether chemoresistance of *Leishmania* to GLIB, an ABC transporter blocker, was accompanied by changes in parasite markers of metacyclogenesis and infectivity, and found that, compared to NR(Gs), NR(Gr) parasites (a) have a longer cell body and a greater cell body area, (b) are more sensitive to the lytic effect of the activated human complement system, (c) show a decreased expression of the Meta-1 protein, and (d) show a decreased infectivity towards J774.1 macrophages.

Metacyclic parasites should possess short, narrow cell bodies (Bates & Tetley, 1993; Zakai *et al*. 1997). The present results indicate that stationary-phase NR(Gr) have greater length and area than NR(Gs), suggesting a reduction in metacyclogenesis. In stationary-phase NR(Gr), sensitivity to human serum was greater than in NR(Gs) suggesting that a modified expression of surface membrane carbohydrates, related to drug resistance, could also affect other important signals and functions, including the parasite resistance to human serum complement. The specific expression of Meta-1 protein in stationary-phase parasites has been associated with their development into metacyclic promastigotes (Nourbakhsh *et al*. 1996; Berberich *et al*. 1998). In NR(Gr) stationary-phase cultures, the level of expression of this protein was significantly lower than in NR(Gs), suggesting again that metacyclogenesis was reduced in NR(Gr) compared to NR(Gs).

Finally, metacyclogenesis has been associated with increase in infectivity. We previously showed (García *et al*. 2000) that infectivity, like metacyclogenesis shown in the present results was reduced in NR(Gr). It could be possible that the drug carried by the NR(Gr) on their way into the host cell during the initial 4 h infection may inhibit the activity of the macrophages to incorporate them. This possibility is ruled out, firstly since pre-treatment of macrophages with GLIB does not alter the J774.1 macrophage infection for *L. major* or NR(Gs) at 4 h (Silva & Ponte-Sucre, 2001) or the expression of surface markers such as the major histocompatibility complex II (MHC-II) and the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) (data not shown). Secondly, intracellular parasites hosted by naïve J774.1 macrophages were sensitive to GLIB even if the drug was added to the culture medium after an initial 18 h infection (Ponte-Sucre *et al*. 1998).

Alternatively, the mechanisms involved in the maintenance of resistance could interact *per se* with the parasite differentiation, survival and intra-macrophage sensitivity, as has been also suggested for antimony-resistant parasites (Sereno *et al*. 2001). Bates & Tetley (1993) have demonstrated that among the determinant factors for metacyclogenesis, the pH of the environment that surrounds the parasite is crucial; a change in the pH could induce physiological signals that may increase the probability of individual promastigotes differentiating into metacyclic forms (Bates & Tetley, 1993). We do not have direct evidence on how and when the decision to become a metacyclic form is made through the cell cycle, but the results presented herein are suggestive of differences between NR(Gs) and NR(Gr) and indicate that resistance to general blockers of P-gp such as GLIB is accompanied by a decreased ability to differentiate into metacyclic forms and indicate that important biological properties involved in the regulation of parasite proliferation and differentiation could be affected.

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