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ABC Proteins In Leishmania Mexicana: Modulation of Parasite-Host Cell Interaction

N Silva1 y A Ponte-Sucre1.

1. Laboratory of Molecular Physiology, Instituto de Medicina Experimental, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela.

RESUMEN

Previamente hemos demostrado la sensibilidad de cultivos de *Leishmania* a substratos de transportadores ABC y hemos desarrollado una línea de parásitos resistente a este tipo de compuestos, [NR(Gr)]. En el presente trabajo estudiamos la influencia del desarrollo de resistencia a substratos de transportadores ABC en la interacción entre el parásito y su célula hospedera, el macrófago. Los resultados demuestran que la incorporación de [NR(Gr)] está incrementada en macrófagos tratados con glibenclamida (GLIB) y que su sensibilidad intracelular está significativamente aumentada. La reversión en la sensibilidad de los parásitos resistentes a la GLIB puede ser el resultado de una inestabilidad de la resistencia en el ámbito intracelular o de la modificación de la expresión de proteínas de superficie indispensables para la supervivencia del parásito en el medio intracelular. En su conjunto los resultados enfatizan la importancia de la comprensión de la modulación de la quimio-resistencia a lo largo del ciclo de vida del parásito.

Palabras Clave: Transportador ABC, Quimioresistencia, Infectividad, *L. mexicana*.

Abstract

Drug-resistant cell lines are useful for the study of the molecular basis of chemo-resistance. Due to changes in the adenosine 5´triphosphate binding cassette (ABC) transporters expression, these cells express dramatical increases in drug efflux through their surface membrane. We have selected a*Leishmania* strain [NR(Gr)] resistant to glibenclamide (GLIB), a drug which interacts with ABC transporters and analyzed whether the interaction of the parasite with its host cell changes for the resistant parasite. The data show that NR(Gr) incorporation is significantly increased in GLIB treated macrophages and that the intracellular parasite sensitivity against GLIB is regained. This reversion in drug sensitivity may be the result of an instability of the resistance during the infection and propagation of the intracellular stage of the parasite and could be related to an alteration in the expression of surface molecules in the parasite which may challenge its intracellular survival. These results emphasize the fact

that understanding of the modulation of chemo-resistance could be relevant for the comprehension of the transmission of drug-resistant strains during the life cycle of *Leishmania.*

Key Words: ABC transporter, Chemo-resistance, Infectivity, *L. mexicana.*

Introduction

The life cycle of *Leishmania sp.*, obligatory intracellular parasites causative of a wide spectrum of diseases, includes two morphologically developmental stages; the extracellular promastigote and the intracellular amastigote. The chemo-therapeutical approach is mainly directed towards the parasite intracellular stage and is based on drugs like sodium stibogluconate, meglumine, pentamidine and amphotericin-B. Which physiological mechanisms impair the successful treatment of leishmaniasis and explain the low level of efficacy frequently reported are of interest and, as resistance of parasites to different drugs is recognized as one of the main problems, strains selected *in vitro* have been used as tools to dissect the molecular basis of this chemo-resistance (Ullman, 1995).

The main mechanism related in *Leishmania* to drug resistance is the result of an increased expression of a P-glycoprotein (Pgp) which belongs to the ***a***denosine ***b***inding ***c***assette (ABC) transporter family (Callahan and Beverly, 1991; Legaré, et al 1994), is related to specific changes in membrane permeability, and should involve additional functional and biochemical changes. For example, amphotericin and pentamidine resistant *Leishmania* show alterations in membrane fluidity and saturated fatty acids levels (Mbongo, et al 1997) and in mitochondrial enzyme activities and lipophosphoglycan (LPG) expression (Basselin and Robert-Gero, 1998).

Glibenclamide (GLIB), originally described as a $K+_{ATP}$ transport blocker, has been shown to interact with members of the ABC-transporter super-family such as the sulfonylurea receptor (SUR) (Inagaki, et al 1995), the cystic fibrosis trans-membrane conductance regulator (CFTR) (Schultz, et al 1996) and Pgp from different drug resistant cell lines (Golstein, et al 1999). As various species of *Leishmania* are sensitive to this drug (Ponte-Sucre, et al 1998), and in view of the increasing importance that drug resistance has in the treatment of leishmaniasis, we have experimentally selected a GLIB resistant*Leishmania* strain [NR(Gr)] and initiated the characterization of the molecular, biochemical and functional mechanisms responsible for this phenotype. The amplification of specific DNA sequences, as well as multiple biochemical and functional changes in enzymes such as pyruvate kinase and acid phosphatase, were found to be associated with GLIB resistance (Ponte-Sucre, et al 1997; García, et al 2000).

To further our insight into the physiological meaning and stability of GLIB resistance, in the present work we analyzed whether the interaction of *Leishmania* with its host

cell, and its sensitivity to GLIB while inside the macrophage, are changed for NR(Gr). The data show that GLIB resistant *Leishmania* are efficiently incorporated by GLIB treated J774 macrophages, but not by naive J774 cells, and that the drug resistance expressed by promastigotes is not retained by the intracellular parasites.

Materials and methods

Glibenclamide was purchased from Research Biochemical International and prepared as described (Ponte-Sucre, et al 1998). *Leishmania* (*L.*) *major* (MHOM/IL/81/FE/BNI) was cultured as described by Solbach, et al (1986). The origin and culture of the GLIB sensitive *Leishmania* NR(Gs) is described elsewhere (Ponte-Sucre, et al 1997). The resistant strain, NR(Gr), was selected *in vitro* by successive replications of NR(Gs) under continuous drug pressure as described by Ponte-Sucre, et al (1997). The cell line J774 was grown as described (Ponte-Sucre, et al 1998).

J774 (2 x 105 cells ml-1) were pre-incubated for 36 h, in a final volume of 0.5 ml of RPMI 1640 culture medium supplemented with 2 x 10-3 M L-glutamine, 20 µg ml-1 gentamicin, 2 x 10-5 M 2-mercaptoethanol and 10% fetal bovine serum, alone or in the presence of 3 x 10-6 M GLIB or 20 U ml-1IFN-g. Immediately thereafter, the macrophages were infected in the absence of drugs for 4 h with stationary phase promastigotes of *L. major*, NR(Gs) or NR(Gr) at a parasite to macrophage ratio of 5 to 1. After removal of extracellular parasites by thorough rinsing with fresh RPMI, the wells were incubated further for 24 h in the absence of drugs or in the presence of 3 x 10-6 M GLIB or 20 U ml-1 IFN-. In order to evaluate the percentage of infected cells and the average number of parasites per 100 macrophages, intracellular parasites were quantified either at the end of the 4 h infection or the 24 h incubation by staining with acridine orange and ethidium bromide (Ponte-Sucre, et al 1998) and analyzed by fluorescence microscopy at 495 nm.

The macrophages (1.25 x 106 cells ml-1), seeded in flat-bottom microtiter wells, were pre-treated, infected and further treated with or with out the drugs for 24 and 48 h using the protocol described in the previous section. The concentration of nitrite (NO2-) released by the macrophages was determined by adding 100 µl of the culture supernatant to 100 µl of Griess reagent (1% sulfanylamide, 0.1% naphtylethylene diamide dihydrochloride, 2.5% phosphoric acid) for 10 min at room temperature (Granger, et al 1996). The absorbance of the reaction product at 540 nm was measured in an ELISA reader. The NO2- concentration was determined using sodium nitrite as a standard.

The percentage of infection is expressed as mean values $\pm$ SEM of three experiments where at least 500 macrophages were analyzed. An infection index was calculated as {[percentage of infection (number of parasites/100 infected cells)]/1000}. Differences between the percentage of infection of untreated and treated macrophages were tested for statistical significance by the minimum $\chi 2$ method (Schefler 1981a).Differences in infection index and NO2- concentration of untreated and treated macrophages were tested for statistical significance by the unpaired Student *t* test (Schefler 1981b).

Results

To determine whether the uptake of *Leishmania* by macrophages changes when GLIB resistant parasites are used, untreated, and IFN-g and GLIB treated macrophages were infected for 4 h in the absence of drugs. Untreated macrophages, showed a similar percentage of infection of 17% either by NR(Gs) or *L. major* but showed a 30% decrease ($p<0.05$) when naive macrophages were infected with NR(Gr) (Figure 1). The incorporation of parasites into macrophages was always stimulated by pre-treatment with IFN-g, pre-treatment of macrophages with GLIB decreased the percentage of infection with *L. major* and NR(Gs) 35 and 20% respectively and enhanced significantly the incorporation of NR(Gr) into macrophages ($p<0.05$ for all changes) (Figure 1).

When the macrophage/parasite cultures were incubated for 24 h without drugs, the infection index increased 100% ($p<0.05$) for *L. major* and 35% ($p<0.05$) for NR(Gs) and did not changed significantly for NR(Gr). When already infected macrophages were further treated with GLIB for 24 h, the infection index decreased 70% for *L. major* and 40% for NR(Gs) and, contrary to what we expected, decreased 35% for NR(Gr) ($p<0.05$ for all changes) (Figure 2).

To asses whether the clearance of sensitive and resistant strains could be explained by an enhanced microbicidal mechanism, we evaluated the NO formation in infected macrophages after the treatment with the drugs. The release of nitrite detected at 24 h in macrophages infected either with NR(Gs) or NR(Gr) was similar to the levels released by non-infected macrophages (Figure 3, continuous line). This finding suggest that differences in the triggering of this microbicidal mechanism cannot explain the clearance of NR(Gs) and NR(Gr) by macrophages. Only after a 48 h drug treatment with IFN-γ were we able to observe an increase in the nitrite concentration.

Figure 1: Percentage of infection by L. major, NR(Gs) and NR(Gr) of macrophages untreated or treated prior to the infection for 36 h with 3 μM GLIB or 20 U ml–1 IFN-γ. *p<0.05.

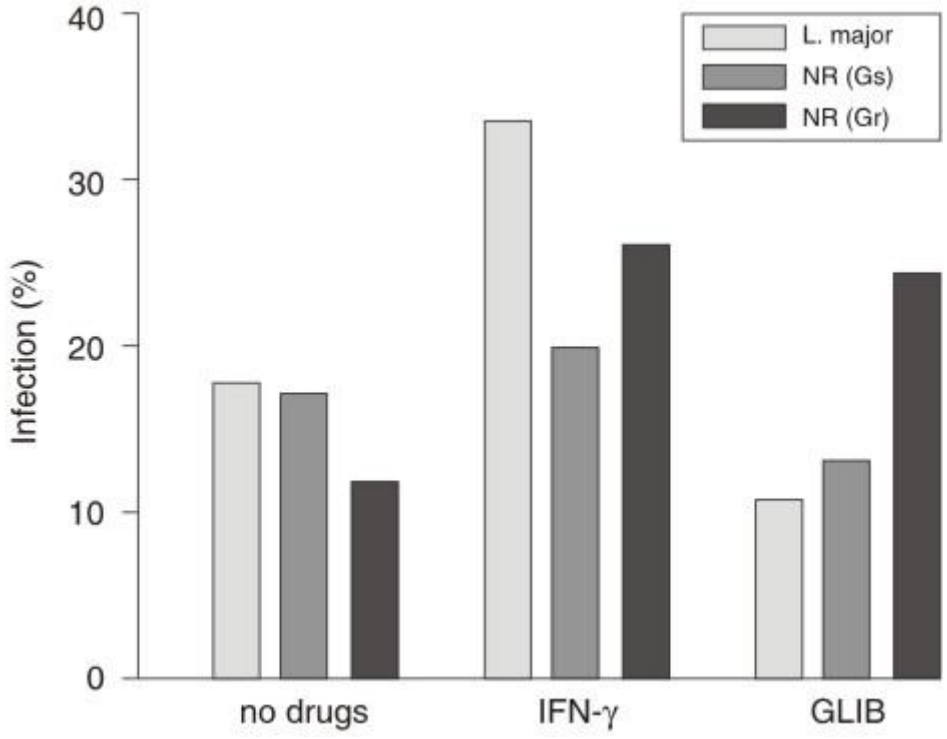


Figure 2: I**nfection index of macrophages untreated or treated post infection for 24 h with 3 μM GLIB or 20 U ml–1 IFN-γ. *p<0.05.**

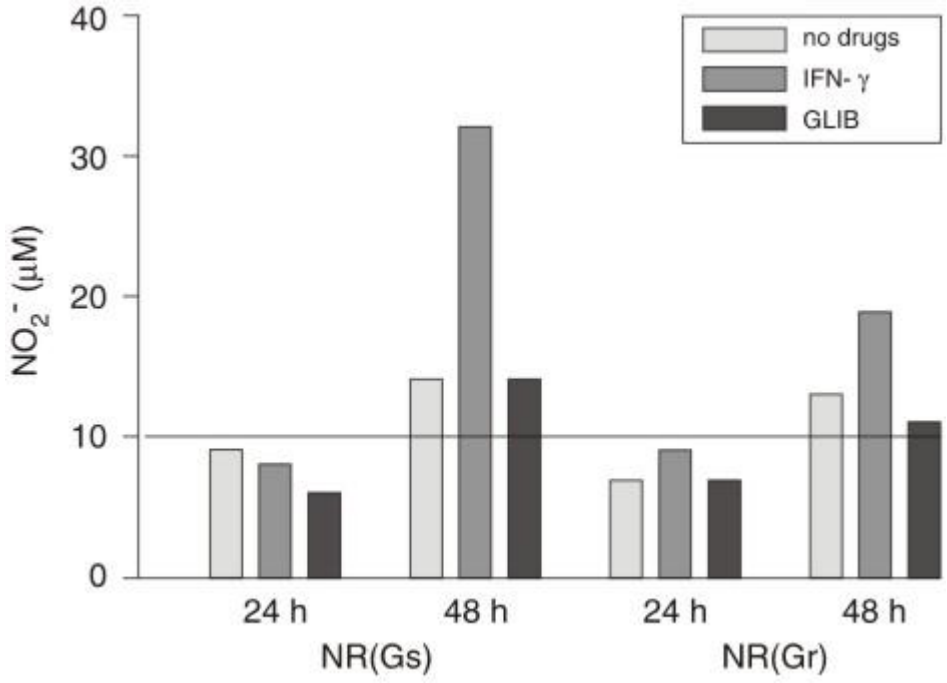
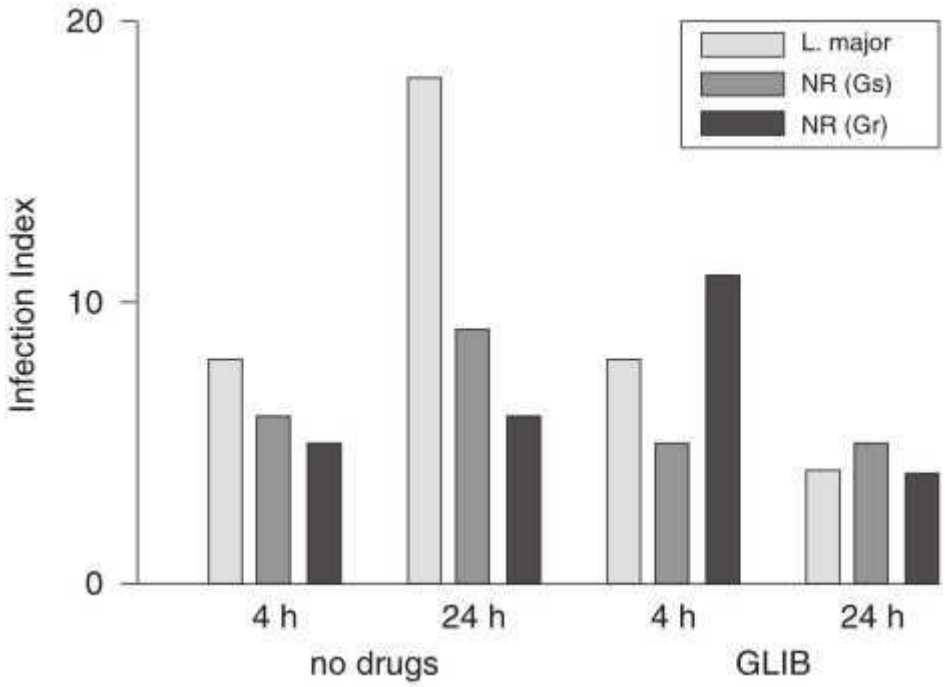


Figure 3: NO production by L. major NR(Gs) and NR(Gr) infected macrophages untreated or treated post infection for 24 h or 48 h with 3 μM GLIB or 20 U ml–1 IFN-γ.



Discussion

The treatment, prevention and control of leishmaniasis is more difficult everyday, not only because of the extreme low efficacy and high toxicity of the drugs currently in use, but because of the everyday increase in drug resistance among the strains that affect humans and other mammals. In the present study we investigated whether drug-resistance of parasites to ABC-transporter substrates modulates the interaction of *Leishmania* with its host cell, as well as its drug sensitivity while in the macrophage. We have found that NR(Gr) is less infective to naive macrophages than NR(Gs). This result suggest that physiological changes developed during the adquisition of GLIB resistance could be affecting the parasite-host cell interaction and therefore the selective incorporation of the parasites into the macrophages. In fact, in a previous paper we had demonstrated that the activity of a housekeeping enzyme, pyruvate kinase, and of the virulence marker, acid phosphatase, are significantly decreased in the resistant strain (García, et al 2000). Nevertheless, pre-treatment of the macrophages with GLIB increased their resistance to infection with *L. major*, as we have previously shown (Ponte-Sucre, et al submitted) and NR(Gs), but not with NR(Gr), a result at present very difficult to explain.

Contrary to what we expected, the infection index of macrophages loaded with NR(Gr) ad left untreated for 24 h did not increased and furthermore, the clearance of the parasites decreased 40% for NR(Gs) and 35% for NR(Gr) in GLIB treated macrophages ($p<0.05$) (Figure 2) without a significant increase in nitrite release (Figure 3). These results support the idea that the mechanisms involved in the maintenance of GLIB resistance could interfere *per se* with the amastigote survival and proliferation and that once intracellular, resistant parasites express, again, sensitivity against GLIB. A similar result, i.e., the intracellular dissapearance of chemo-resistance, has been observed in some drug selected pentamidine resistant parasites (Sereno and Lemesre, 1997).

Alternatively, if drug resistance emergence is linked to a dys-regulation in ion exchange through the membrane, as has been suggested by Roepe and Martiney (1999) and references therein, raising a cell line resistant against an ABC-transport blocker, would have strong effects on pH gradients, cell volume homeostasis and cell membrane potential and therefore, could challenge the survival of the drug selected parasite inside the acidic phagolysosomal compartment of the macrophage. In this line of evidences, preliminary results from our laboratory suggest that intracellular resistant but not sensitive parasites are extremely sensitive to amiloride, a Na+/H+ exchanger blocker. Altogether, these results emphasize the multiple functional changes which may arise from the phenomena of drug resistance and the attention that should be given to understand the biochemical and functional mechanisms involved, as well as the relevance of the transmission of drug resistance during the life cycle of the parasite.

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