Alicia Ponte-Sucre* · Alexis Mendoza-León · Heidrun Moll

Experimental leishmaniasis: Synergistic effect of ion channel blockers and interferon-γ on the clearance of *Leishmania major* by macrophages

Ion channel blockers and the clearance of *Leishmania major* by macrophages

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A.Ponte-Sucre (✉)
Laboratory of Molecular Physiology
Instituto de Medicina Experimental
Facultad de Medicina
Universidad Central de Venezuela
Apdo 68256, Caracas 1062-A
Venezuela
e-mail: aiponte@reacciun.ve

A. Mendoza-León
Laboratory of Biochemical and Molecular Biology of Parasites
Instituto de Biología Experimental
Facultad de Ciencias
Universidad Central de Venezuela
e-mail: amendoza-leon@hotmail.com

H. Moll
Institute for Molecular Biology of Infectious Diseases
University of Wuerzburg
Germany.
e-mail: heidrun.moll@mail.uni-wuerzburg.de

**Present address*:
Institute for Molecular Biology of Infectious Diseases, University of Wuerzburg, Roentgenring 11, Wuerzburg, D-97070, Germany.
Tel.: (0049)(0931)(312132)
Fax: (0049)(0931(312578)
e-mail: alicia.ponte-sucre@mail.uni-wuerzburg.de

Abstract We have previously shown that cultured *Leishmania* promastigotes are sensitive to drugs blocking K^+ and Na^+ channels and Na^+/H^+ transport systems and that the percentage of parasite-infected macrophages decreases significantly in the presence of the drugs. In the present work, we analyzed whether this drug susceptibility of intracellular amastigotes is associated with the activation of macrophage microbicidal mechanisms. Pre-treatment of the cells with glibenclamide (GLIB) increased their resistance to infection with *Leishmania*, an effect that may be mediated by calcium fluxes since it was reversed by EGTA. Notably, in infected macrophages post-treated with the drugs, the clearance of parasites was strongly enhanced when the cells were treated simultaneously with GLIB and interferon-γ; this effect correlated with an increased production of reactive nitrogen intermediates. In conclusion, the data suggest that GLIB treatment increases the resistance of macrophages to infection with *Leishmania* and potentiate the interferon-γ–stimulated clearance of parasites via the induction of nitric-oxide.

Abbreviations *AMIL* amiloride, *4-AP* 4-aminopyridine, *GLIB* glibenclamide, *IFN-γ* interferon γ, *EGTA* ethylene glycol bis (β–aminoethyl ether) N,N,N´,N´tetraacetic acid, *IL-1β* interleukin-1β, NO_2^- nitrite, *NO* nitric oxide, O_2^- superoxide, *PEC* peritoneal exudate cells, *THAP* thapsigargine, *TNF-α* tumor necrosis factor-α.

Introduction

Infection of humans with *Leishmania* parasites gives rise to a variety of clinical manifestations classically designated as visceral, cutaneous and mucocutaneous leishmaniasis which have been recognized as major causes of morbidity and mortality (Nakashima 1996). The complex life cycle of *Leishmania*, comprising the promastigote stage in the sand fly and the obligatory intracellular amastigote in the mammalian host, correlates with its ability to adapt to widely differing environments where the parasite is

frequently exposed to extreme pH changes (Zilberstein and Shapira 1994). Under these extreme conditions, ion transport systems are likely to be fundamental for the maintenance of the parasite´s intracellular homeostasis. Therefore, we used ion channel blockers to further our understanding of the associated mechanisms and to define potential targets for the development of new drugs.

The analysis of functional phenomena related to the different developmental stages of *Leishmania* is commonly based on experimental systems that include the promastigote model, reflecting the initial steps of the infection, or take advantage of the use of axenically grown amastigotes (Callahan et al. 1997). As there are a number of biochemical, molecular and functional differences between axenic amastigotes and the intracellular amastigote form (el Azzouni et al. 1998) and since it has not been established whether these differences are relevant for the definition of the pathogenic intracellular stage, we analyzed the sensitivity of *Leishmania* amastigotes in macrophages to K^+ channel blockers, such as 4-aminopyridine (4-AP) and glibenclamide (GLIB), and amiloride (AMIL), a Na^+ transport system blocker. Our previous results demonstrated that these drugs induce a decrease in the percentage of infection and the survival of intracellular parasites (Ponte-Sucre et al. 1998). In the present study, we analyzed whether pre-treatment of macrophages with the drugs affects the uptake of parasites and whether the drug-stimulated clearance of intracellular parasites correlates with the activation of macrophage microbicidal mechanisms. In addition, as interferon-γ (IFN-γ) is one of the most important macrophage-activating cytokines and inducer of nitric oxide (NO) production in the mouse model (MacMicking et al. 1997), we tested whether the drugs modulate the effect of IFN-γ on macrophages. The data suggest that GLIB reduces the incorporation of *Leishmania* and increases the efficiency of IFN-γ-treated

macrophages in clearing the parasite. The effect of GLIB plus IFN-γ correlated with increases in NO levels generated by macrophages, a feature that may be of particular interest for a potential use of GLIB in therapeutic protocols.

Materials and methods

Chemicals, cell lines and cultures

The drugs 4-AP, GLIB and AMIL were from Research Biochemical International (Natick, Mass. U.S.A.) and prepared as previously described (Ponte-Sucre et al. 1998). *Leishmania* (*L. major*) (MHOM/IL/81/FE/BNI) was cultured as described by Solbach et al. (1986). Peritoneal exudate cells (PEC), obtained from thioglycolate-treated BALB/c mice, were used as the source of macrophages (Ponte-Sucre et al. 1998).

Treatment of macrophages with the drugs

BALB/c PEC (2 5 10^5 cells ml^{-1}) were pre-incubated with the drugs for 36 h and then infected in the absence of drugs for 4 h with stationary-phase *L. major* promastigotes at a parasite to macrophage ratio of 5 to 1, in a final volume of 0.5 ml of RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 20 µg ml^{-1}gentamicin, 2 x 10^{-5} M 2-mercaptoetanol and 10 % fetal bovine serum. Similar cultures were incubated with the drugs for the same time period but in the additional presence of either 0.5 mM EGTA, 0.5 mM EGTA plus 10 nM thapsigargine (THAP) or IFN-γ (20 U ml^{-1}) and infected as described above. After removal of extracellular parasites by thorough rinsing with fresh RPMI, the wells were incubated for further 72 h in the presence or absence of the drugs and or IFN-γ. Subsequently, intracellular parasites were quantified by staining with acridine orange and ethidium bromide (Ponte-Sucre et al. 1998) and analyzed by fluorescence microscopy at 495 nm.

Nitric oxide production and oxidative burst activity

BALB/c PEC (1.25 5 10^6 cells ml^{-1}) were pre-incubated in flat-bottom microtiter wells as described above, and were subsequently infected with *L. major* and treated with the drugs for 72 h. The concentration of nitrites (NO_2^-) released by the macrophages was determined by adding 100 µl of the culture supernatant to 100 µl of Griess reagent (1% sulfanilamide, 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 NO_2^- concentration was determined using sodium nitrite as a standard. Data are expressed as nmol NO_2^-/ 2.5 5 10^5 cells. For the evaluation of the intracellular oxidative burst, the cytochrome C reduction assay was used (Jupin et al. 1989). After removal of the supernatant, the pre-treated PEC were resuspended in 0.1 ml of Hanks medium and incubated at 37 ºC for 1 h in the presence of 2 mg ml^{-1} of cytochrome C III. The change in optical density was measured at 550 nm in an ELISA reader. Data are expressed as nmol of O_2^- released/ 2.5 5 10^5 cells.

Statistical analysis of the data

The data are expressed as mean values $\pm$ SEM of five experiments in which at least 500 macrophages were analyzed. Differences between the percentage of infection of treated and control macrophages were tested for statistical significance by the minimum χ^2 method (Schefler 1981a). An infection index was calculated as {[percentage of infection (number of parasites/100 infected cells)]/1000}. Differences in infection index, NO_2^- concentration and O_2^- of treated or control macrophages were tested for statistical significance by the unpaired Student *t* test (Schefler 1981b).

Results

Effect of macrophage treatment with ion channel blockers on the uptake of *Leishmania*

Phagocytosis is the functional event by which the clearance of microorganisms is initiated by the host. In the case of *Leishmania* promastigotes, this process involves the classical receptor-mediated endocytosis. To determine whether the incubation of naive host cells with the ion channel blockers results in a change of the uptake of *Leishmania*, macrophages were pre-treated with the drugs for 36 h and then infected with the parasites for 4 h in the absence of the drugs. Upon treatment of macrophages with GLIB, the subsequent uptake of *Leishmania* decreased by 40 % (Table I). This suggests that GLIB increases the cells´ resistance to become infected. AMIL and 4-AP did not have such an effect on macrophages (Table I). To explore whether the GLIB effect is related to an increase in cytoplasmic Ca^{2+}, due to either an increased flux from the extracellular media or the use of intracellular pools, we measured the uptake of *Leishmania* by macrophages pre-treated with EGTA alone or in combination with the Ca^{2+} pump antagonist THAP. Neither EGTA nor EGTA plus THAP by themselves affected the uptake of *Leishmania* (Table I). However, the resistance to infection observed after GLIB treatment was completely abrogated in macrophages treated with GLIB and EGTA, but not in cells treated with GLIB, EGTA and THAP. These results indicate that calcium fluxes through the plasma membrane might contribute to the resistance to infection induced by GLIB. Finally, in cells pre-treated simultaneously with the drugs and IFN-γ, 4-AP and AMIL reversed the enhanced parasite uptake observed in cells treated with IFN-γ, whereas this increased uptake remained high in cells treated simultaneously with GLIB and IFN−γ

(Table I). These results suggest that 4-AP and AMIL, but not GLIB, inhibit the increased avidity for parasites normally observed in IFN-γ-treated macrophages.

Effect of long-term treatment of macrophages with the drugs on parasite clearance

The leishmanicidal activities of macrophages involve the sustained induction of intracellular processes that finally result in the killing of parasites. When already infected macrophages were further treated with the drugs for 72 h, both GLIB and IFN-γ *per se* decreased the infection index 11 fold (from 0.95 in untreated cells to 0.10); this effect was strongly enhanced (73 fold decrease) in cells treated simultaneously with GLIB and IFN-γ (Fig. 1). Similar to what was found in cells treated with IFN-γ alone, the infection index remained 11 fold lower in cells simultaneously treated with 4-AP or AMIL and IFN-γ. These results support the idea of a synergistic action of GLIB and IFN-γ in the activation of macrophages, resulting in a potentiation of the clearance of *Leishmania* by these effector cells.

Effect of the drugs on the nitric oxide production and oxidative burst

The production of nitric oxide (NO) and superoxide (O_2^-) are cytotoxic functions exerted by macrophages to eliminate intracellular protozoan parasites and bacteria. To assess whether the drug-induced clearance of parasites is due to these macrophage microbicidal mechanisms, we evaluated the NO formation and respiratory burst levels in infected macrophages after treatment with ion channel blockers in the presence or absence of IFN-γ . A release of nitrite was not detected in infected macrophages further treated with the drugs alone (Table II). This finding suggests that the effect of the drugs *per se* does not correlate with NO production by the macrophages. On the other hand, the level of nitrite increased significantly in infected cells treated with IFN-γ and increased even further in cells treated with IFN-γ and the drugs. These results indicate that ion channel blockers,

especially GLIB (see Fig. 1), enhance the clearance of *Leishmania* as the result of a synergistic effect with IFN-γ on the induction of NO production by macrophages. The level of O_2^- release decreased in infected macrophages treated with the drugs in the absence and presence of IFN-γ (Table II). A correlation between the decrease of respiratory burst activity and the decrease in the infection index could not be established (Fig. 1, Table II). Together, these data show that the effect of the drugs correlates with NO production but not with superoxide production in these host cells.

Discussion

We previously demonstrated that *Leishmania* promastigotes are sensitive to the ion channel blockers 4-AP, GLIB and AMIL and that the rate of macrophage infection decreases in the presence of these drugs (Ponte-Sucre et al. 1998). The aim of the present study was to further our understanding of the mechanisms involved. Therefore, we used *Leishmania*-infected macrophages to analyze whether the drug sensitivity of intracellular amastigotes correlates with the activation of macrophage microbicidal mechanisms.

The uptake of parasites decreased significantly in macrophages treated with GLIB. Of note, this effect of GLIB was abrogated by the addition of EGTA. In the context of previous findings indicating that GLIB activity is related to an increase in membrane calcium fluxes (Boyd 1988), our results support the idea that the GLIB-induced increase in resistance to infection may be related to a calcium influx into the macrophage.

The infection index decreased 11 times in cells treated either with IFN-γ or GLIB and 73 times in cells treated with both GLIB and IFN-γ . These results imply a synergistic effect of GLIB and IFN-γ on the clearance of *Leishmania* by activated macrophages and support the idea of a close correlation between the ion currents and the functions of the macrophage. In fact, IFN-γ -induced expression of MHC class I and class II molecules on

the surface of macrophages is modulated by K^+ channel openers such as diazoxide or pinacidil (Zhu et al. 1995) and the secretion of interleukin-1β (IL-1β) by human monocytes and mouse macrophages is impaired by GLIB (Hamon et al. 1997). In this context, it should be noted that low levels of IL-1β have been associated with a mouse phenotype resistant to *Leishmania* infection (Cilliari et al. 1989).

The activation of macrophages *in vitro* results in the killing of intracellular parasites such as *Leishmania*. Reactive oxygen species have been implicated in the eradication of intracellular organisms (Chackraborty et al. 1996) but macrophage lines which do not produce reactive oxygen metabolites are also capable of efficiently eliminating *Leishmania* promastigotes and amastigotes via the formation of NO (Liew et al. 1990). To evaluate whether the GLIB-induced resistance to parasites and the drug-induced decrease of infection index is related to changes in the NO production and/or respiratory burst activity of macrophages, both parameters were analyzed. The reduction in infected cells correlated with changes in the nitrite levels only when IFN-γ was added to the cultures. These results suggest that the ion channel inhibitors may not be sufficient for the induction of NO production by macrophages but that triggering effect of IFN-γ might be enhanced in the presence of the GLIB. The drug action may thus imitate the effect of TNF-α which synergizes with IFN-γ in the activation of macrophages for the elimination of amastigotes (Bogdan et al. 1990). These conclusions are nevertheless, in line with the finding that a GLIB antagonist, Nicorandil inhibits the production of TNF-α (Pogrebniak et al. 1992),

In conclusion, our data suggest that the drugs, in particular GLIB, although do not promote the activation of microbicidal mechanisms of macrophages *per se*, enhance the

triggering effect of IFN-γ and therefore contribute to parasite clearance by macrophages via induction of NO formation.

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Figure Legends

Fig. 1. Infection index of macrophages treated with GLIB (3 μM), 4-AP (100 μM) and AMIL (10 μM) in the absence or presence of IFN-γ. Cultures of 2 5 10^5 BALB/c macrophages ml^{-1} were treated with the drugs for 36 h and then infected with *L. major*, for 4 h, at a parasite to macrophage ratio of 5 to 1, in a final volume of 0.5 ml of RPMI culture medium. After removal of extracellular parasites by exhaustive washing with RPMI culture medium, the drugs were added to the cultures which were then incubated for further 72 h.

TABLE I. Effect of treatment with ion channel blockers

on the uptake of *Leishmania* by murine macrophages

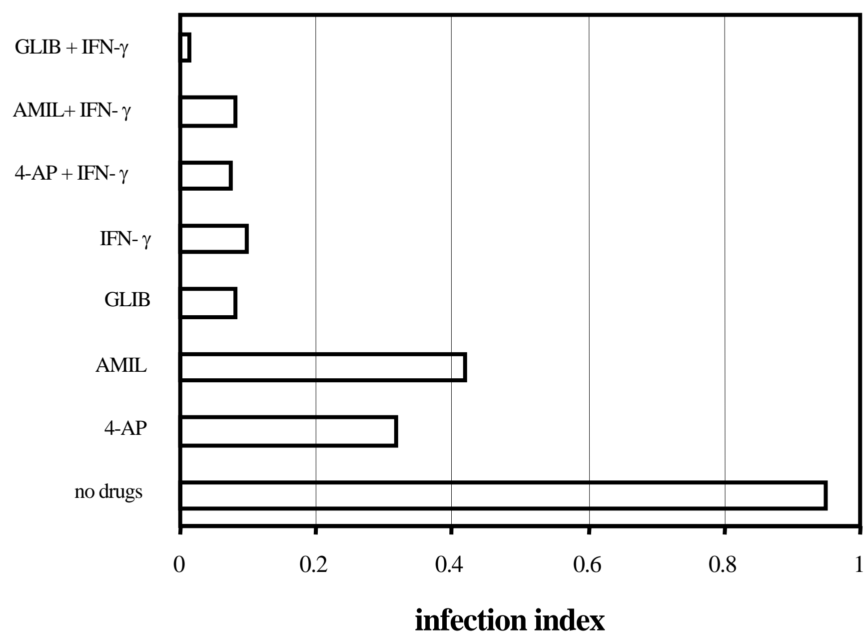
	percentage of infected macrophages			
	control	+ IFN γ [percent change of infection]	+EGTA	+EGTA/THAP
no drugs	17.84 ± 0.04[a,b]	33.60 ± 0.18[c,d,e] [+88]	17.90 ± 0.13	16.60 ± 0.02
4-AP	15.33 ± 0.02	20.00 ± 0.23[c] [+30]	ND	ND
GLIB	10.80 ± 0.05[a]	21.00 ± 0.17[d] [+94]	16.25 ± 0.09	13.00 ± 0.09[b]
AMIL	15.60 ± 0.14	19.60 ± 0.07[e] [+30]	ND	ND

Macrophages were pre-treated with GLIB (3 µM), 4-AP (100 µM) and AMIL (10 µM) in the absence or presence of IFN-γ (20 U ml^{-1}), EGTA (0.5 mM) or EGTA (0.5 mM) plus THAP (10 nM) for 36 h and infected with *L. major* for 4 h in the absence of drugs. Macrophages were counted immediately thereafter. The data represent 5 experiments where at least 500 macrophages were counted. [a,b,c,d,e] $p<0.0001$ for comparison between values identified with the same letter, other values without significant differences. ND= not determined. *[percent change of infection] expresses values in comparison to control cells .

TABLE II. Effect of treatment with the drugs on the NO_2^- production and respiratory burst of murine macrophages

	NO production (µM)		Respiratory burst (nmol 0_2^- / 2.5 x 10^5 cel)	
	control	+IFN-γ [change in NO_2^-] *	control [change in O_2^-]*	+IFN-γ [change in O_2^-] *
not infected				
No drugs	0.00	3.075 ± 0.005	1.45 ± 0.0002	1.31 ± 0.0002
infected				
No drugs	0.00	28.38 ± 0.018[a,b,c,]	1.28 ± 0.0002[d,e,f]	1.14 ± 0.0002[g,h,i]
4-AP	0.00	39.45 ± 0.013[a] [+11.07]	1.06 ± 0.002[d] [-0.22]	1.00 ± 0.003[g] [-0.14]
GLIB	0.00	38.51 ± 0.012[b] [+10.13]	0.99 ± 0.0003[e] [-0.29]	0.72 ± 0.0003[h] [-0.42]
AMIL	0.00	43.65 ± 0.012[c] [+15.27]	1.03 ± 0.002[f] [-0.25]	1.18 ± 0.002[i] [+0.13]

Macrophages were pre-treated with the drugs GLIB (3 µM), 4-AP (100 µM) or AMIL (10 µM) for 36 hours and infected with *L. major* for 4 h. Infected cells were treated for 72 h in the presence of the drugs and the NO production or respiratory burst were evaluated immediately thereafter. *[change] expresses values in comparison to control cells. The data represent 3-5 experiments with 2.5 x 10^5 cells per well. [a,b,c,d,e,f,g,h,i] $p< 0.0001$ for comparison between values identified with the same letter.



GLIB + IFN-γ
AMIL+ IFN- γ
4-AP + IFN- γ
IFN- γ
GLIB
AMIL
4-AP
no drugs
0
0.2
0.4
0.6
0.8
1
infection index