α -Difluoromethylornithine-resistant cell lines obtained after one-step selection of *Leishmania mexicana* promastigote cultures

Cecilia P. SÁNCHEZ*‡, Juan MUCCI*, Nélica S. GONZÁLEZ*, Alberto OCHOA†, Mario M. ZAKIN† and Israel D. ALGRANATI*§

*Instituto de Investigaciones Bioquímicas 'Fundación Campomar', Facultad de Ciencias Exactas y Naturales, University of Buenos Aires, Patricias Argentinas 435, 1405 Buenos Aires, Argentina, and †Unité d'Expression des Gènes Eucaryotes, Institut Pasteur, Paris, France

Proliferation of *Leishmania mexicana* promastigotes in synthetic medium can be blocked by the depletion of intracellular polyamine pools induced by the presence of D,L- α -difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ornithine decarboxylase (ODC). Here we report that DFMO-resistant cell lines growing normally at DFMO levels of 10 mM have been obtained from non-proliferating cultures after a single-step selection in the presence of high concentrations of the drug. The DFMO-resistant promastigotes underwent a morphological transformation into an 'amastigote-like' form after incubation for several hours at gradually increasing temperatures up to 35 °C. The uptake of DFMO was not significantly altered in the drug-resistant cell lines but in both cases (promastigote and 'amastigote-like' forms) the ODC specific activity

was increased approx. 15-fold over the normal enzymic levels found in the wild-type *Leishmania*. The enzyme affinities for its substrate and for DFMO gave very similar values in the drug-resistant promastigotes and the wild-type parasites. In contrast, ODC from the 'amastigote-like' *Leishmania* showed a higher affinity for ornithine and a decreased capacity for the binding of DFMO. An 80-fold amplification of the ODC gene and a corresponding increase in its transcripts have been detected in both DFMO-resistant *Leishmania* cell lines. The drug-resistant phenotypes with their characteristic morphologies, the increased levels of ODC activity and the amplification of the ODC gene have been stable for at least 6 months in the absence of selective pressure.

INTRODUCTION

Leishmania mexicana is one of the aetiological agents of human cutaneous and mucocutaneous leishmaniasis. This parasite has a digenetic life cycle involving flagellated motile promastigotes present in the alimentary tract of sandflies and intracellular non-motile rounded amastigote forms that proliferate in the phagolysosomal system of mammalian macrophages.

To survive and complete its life cycle, *Leishmania* goes through several differentiation steps that involve the conversion of the promastigote into the amastigote form after infection of the mammalian host macrophages, followed by the opposite process occurring in the midgut of the insect vector [1]. Therefore promastigotes and amastigotes are adapted to the living conditions prevailing in their respective hosts; these conditions differ widely in biochemical composition, temperature and pH [2,3].

Because *Leishmania* promastigotes can grow *in vitro* on axenic rich or synthetic media [4–6], different studies have been able to show that the parasite proliferation could be controlled by polyamine intracellular levels [7,8].

After repeated passages of *L. mexicana* promastigote cultures in a polyamine-free defined medium containing D,L- α -difluoromethylornithine (DFMO), the polyamine internal pool of the parasite was markedly decreased by this irreversible inhibitor of ornithine decarboxylase (ODC; L-ornithine carboxylase, EC 4.1.1.17); at the same time *Leishmania* proliferation was arrested. The addition of exogenous putrescine or spermidine to the culture medium evoked a rapid increase in polyamine intracellular levels and concomitantly the reinitiation of parasite multiplication [8].

We have recently observed that cultures containing non-proliferating *L. mexicana* promastigotes inhibited by high concentrations of DFMO were able to resume growth after variable periods of time in the presence of the drug, even without supplementing the medium with exogenous polyamines. This apparent spontaneous reversion from growth inhibition was presumably due to the selection of DFMO-resistant parasites. This was indeed true; here we describe and characterize two different *Leishmania* cell lines that proliferate normally in the presence of high levels of the antipolyamine drug.

The development of DFMO-resistance in our *Leishmania* cultures seems to be quite different from the stepwise selection process described previously for DFMO and other drugs in several parasites and mammalian cells [9–16]. Here we report our studies on the mechanisms involved in the one-step selection of DFMO resistance in cultures of *L. mexicana* wild-type strains.

MATERIALS AND METHODS

Materials

Brain–heart infusion, tryptose and yeast extract were obtained from Difco Laboratories (Detroit, MI, U.S.A.). Minimal essential medium (SMEM) and amino acids were from Gibco; haemin, bases, vitamins, polyamines, cycloheximide, pyridoxal 5'-phosphate, Hepes buffer and antibiotics were purchased from Sigma. L-[1-¹⁴C]ornithine (55.9 Ci/mol), [3,4-³H]DFMO (27.1 Ci/mmol) and deoxycytidine 5'-[³²P]triphosphate (3000 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). [5-¹⁴C]DFMO (60 Ci/mol) was obtained from Amersham (Little Chalfont, Bucks., U.K.). DFMO was kindly provided by Marion Merrell Dow.

Abbreviations used: AR₁₀, PR₁₀, *L. mexicana* 'amastigote-like' and promastigote cell lines respectively resistant to 10 mM DFMO; CHEF, contour-clamped homogeneous electric field; DFMO, D,L- α -difluoromethylornithine; ODC, ornithine decarboxylase.

‡ Present address: Zentrum für Infektionsforschung, Röntgenring 11, Universität Würzburg, D-97070 Würzburg, Federal Republic of Germany.

§ To whom correspondence should be addressed.

Parasite cultures

Promastigotes of *L. mexicana mexicana* originally isolated by Dr. R. Zeledón (University of Costa Rica) were obtained from stocks of the Instituto Fatale Chaben (Buenos Aires, Argentina) and grown with shaking at 24 °C in Warren's modified medium [5] supplemented with haemin (20 mg/l), 20 % (v/v) fetal calf serum and antibiotics (100 mg/ml streptomycin and 100 i.u./ml penicillin). To elicit polyamine starvation, parasites were cultivated in a completely defined medium free of polyamines (HOSMEM II) described by Berens and Marr [6]; DFMO was added as specified in each experiment. *Leishmania* growth was followed by cell counting. The doubling time for wild-type parasites grown in synthetic media was 18–22 h, and maximal cell density was approx. 5×10^7 /ml.

Light microscopy

Parasites grown in HOSMEM II medium were centrifuged, washed and resuspended in the same medium at 3×10^7 cells/ml. After fixation for 15 min at room temperature with an equal volume of 2 % (v/v) formaldehyde in PBS, parasites were examined by phase-contrast microscopy.

Preparation of cell extracts and enzyme assay

Parasites were collected at the exponential phase of growth by sedimentation for 5 min at 7000 g; after being washed twice with PBS they were resuspended at 2×10^9 cells/ml in 50 mM Hepes sodium buffer, pH 7.0, containing 1 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM pyridoxal 5'-phosphate, 1 mM PMSF, 0.1 mM leupeptin and 0.01 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). *Leishmania* cells were lysed by incubation for 30 min at 0 °C in the presence of 0.5 % (v/v) Nonidet P40 followed by sonication for 10 s. Cell extracts were centrifuged for 30 min at 20000 g and then for 60 min at 100000 g. Supernatant fluids were collected and stored at –70 °C. Under these conditions ODC activity was stable for several months.

The enzyme activity was measured by the release of $^{14}\text{CO}_2$ from radioactive ornithine as previously described [17].

The protein content of enzyme preparations was measured by the method of Bradford [18] with BSA as standard.

Kinetics of ODC inhibition by DFMO

Studies of time-dependent inactivation of the enzyme by DFMO were performed after preincubation at 30 °C in 0.05 ml of the standard reaction mixture without ornithine and containing different concentrations of the inhibitor. Aliquots of 0.01 ml were removed periodically and the residual activity was measured at 37 °C after dilution to 0.2 ml with a complete reaction mixture that included radioactive ornithine. Because the inhibitor was still present at 1/20 concentration during the measurement of the enzymic activity, the corresponding controls were performed with the appropriate concentrations of DFMO in complete mixtures containing enzyme without the preincubation treatment. The values of K_i for DFMO were calculated by the method of Kitz and Wilson [19] with the ODC activities remaining after partial inactivation of the enzyme.

Sensitivity of *Leishmania* cultures to DFMO

Wild-type or DFMO-resistant parasites at the initial concentration of $(2\text{--}4) \times 10^6$ cells/ml were cultivated in synthetic media (HOSMEM II) in the absence (control) and in the presence of different levels of DFMO. Every 3 days all cultures, which were

in exponential growth, were diluted to the initial cell density with fresh medium containing the same concentrations of the inhibitor, and cultivation was resumed. After four passages with or without the drug, cells were counted and the percentage of growth inhibition was calculated. One passage corresponds to three or four generations of growth.

Polyamine analysis

Exponentially growing parasites were harvested by centrifugation, washed once with PBS and resuspended at a density of 10^9 cells/ml in 0.2 M perchloric acid. After removal of the precipitate, different aliquots of the supernatant liquids were dansylated and analysed by TLC and subsequent fluorometry [20]. Alternatively the concentration of polyamines was determined with an amino acid analyser [21].

Uptake of DFMO

Parasites at the exponential phase of growth were collected, washed with PBS and resuspended in the same buffer at 2×10^7 cells/ml. After incubation in the presence of [^3H]DFMO (20 μM , 4 $\mu\text{Ci}/\text{ml}$) or [^{14}C]DFMO (80 μM , 5 $\mu\text{Ci}/\text{ml}$), aliquots were taken at different times, diluted with 3 vol. of cold buffer containing 1 mM unlabelled DFMO and quickly filtered through nitrocellulose membranes. Filters were washed three times with the same buffer solution and dried; the retained radioactivity was measured by scintillation counting.

Southern blot hybridization

Genomic DNA from wild-type and DFMO-resistant *Leishmania* was prepared essentially with the basic protocol described for mammalian tissue by Ausubel et al. [22]. *Leishmania* DNA was digested with *Eco*RI or *Pst*I restriction enzymes; the fragments obtained were separated by electrophoresis on 0.8 % agarose gels. After alkaline transfer to nylon membranes (Hybond N⁺ from Amersham) by Southern's procedure [23], DNA hybridization was performed with a ^{32}P -labelled 178 bp probe obtained by PCR of *L. mexicana* DNA. For this amplification a pair of appropriate primers complementary to both strands of a segment of the ODC gene coding region was used (I. D. Algranati, unpublished work).

Gel electrophoresis of extrachromosomal DNA

To separate extrachromosomal DNA from chromosomes, parasite DNA species were prepared in agarose blocks and subjected to contour-clamped homogeneous electric field (CHEF) electrophoresis with 1.5 % agarose gels, $0.5 \times \text{TBE}$ buffer [40 mM Tris/45 mM boric acid/1 mM EDTA (pH 8.3)] and pulse frequencies of alternating 300–900 s times for 12 h and 900–300 s for 12 h at 5 V/cm [13].

RNA analysis

Total RNA from wild-type and DFMO-resistant parasites obtained by guanidinium thiocyanate extraction and subsequent sedimentation through a CsCl step gradient [22], as well as poly(A)⁺ RNA purified by oligo(dT) bound to magnetic beads (Dynal, Paris, France) were analysed by electrophoresis on 1 % (w/v) agarose/formaldehyde gels and transferred to nylon membranes. Northern hybridization was performed with the same ^{32}P -labelled probe used for DNA analysis.

RESULTS AND DISCUSSION

Selection of *L. mexicana* cell lines resistant to high levels of DFMO

Previous results from our laboratory have shown that the proliferation of *L. mexicana* promastigotes can be arrested after several passages in a defined culture medium containing high levels (5–10 mM) of DFMO. Although the addition of the drug quickly inhibited ODC, a normal growth was maintained for at least two passages (about six generations) before intracellular polyamine pools were sufficiently depleted to block proliferation. Parasites remained viable after this treatment and growth could be resumed after the addition of exogenous putrescine or spermidine [8]. We have recently found that even in the absence of external polyamines, proliferation could be reinitiated spontaneously after a variable period of time in the presence of high concentrations of the drug. The recovery of *Leishmania* multiplication was slow at the beginning but after a few weeks it reached the normal growth rate of parasites cultivated in the absence of DFMO (Figure 1). After *Leishmania* cultures resistant to 5 mM DFMO had been obtained, the concentration of the drug could be doubled without any alteration of parasite proliferation.

The described one-step selection of *Leishmania* phenotypes resistant to elevated doses of DFMO is rather unusual because in most cases parasites or mammalian cell lines resistant to different inhibitors are obtained only after long periods of a stepwise selection process in the presence of gradually increasing drug levels [9–16, 24–26]. In our case the one-step selection of DFMO-resistant *Leishmania* was completed in a few weeks, whereas in all other reported resistant cell lines the multistep selection procedure takes several months. It could be argued that a fraction of our initial cultures of *L. mexicana* already contained an amplified ODC gene and that this subpopulation of cells was quickly selected and expanded after the addition of high levels of DFMO. To discard this possibility we isolated different clones by serial dilutions of the original wild-type cultures. After growing separately 15 different clones, their specific activities of ODC were measured. All the clones showed a similar ODC activity

Figure 1 Effect of DFMO on *L. mexicana* growth

Parasites were cultivated in synthetic media in the absence (●) or presence (○) of 5 mM DFMO. At the times indicated by the arrows, cultures were diluted with fresh HOSMEM II medium without (●) or with (○) the addition of DFMO.

Figure 2 Microscopy of wild-type and DFMO-resistant *L. mexicana*

Parasites grown in HOSMEM II medium in the absence or presence of 10 mM DFMO were collected, washed and fixed as described in the Materials and methods section. (A) Promastigotes; (B) 'amastigote-like' (AR₁₀) *Leishmania*. Magnification $\times 1000$.

[$\pm 18.2\%$ (S.E.M.)] to that found in the starting wild-type cultures. This fact supports the conclusion that the appearance of DFMO-resistance was a process of 'one-step' amplification rather than the selection of an already present subpopulation containing the amplified ODC gene.

It is interesting that one culture of *L. mexicana* promastigotes resistant to DFMO and growing for approx. 1 month in the presence of the drug at a concentration of 10 mM showed a morphological transformation into 'amastigote-like' forms when the temperature of the culture was increased slowly to 34–35 °C over a period of several hours. This cell line has maintained its phenotype of drug resistance and spherical shape for a long time even in the absence of selective pressure and at a cultivation temperature of 24 °C (Figure 2). *L. mexicana* promastigote and 'amastigote-like' cell lines resistant to 10 mM DFMO were designated PR₁₀ and AR₁₀ respectively; both have been grown continuously for almost 1 year in the absence and in the presence of the drug.

Sensitivity of wild-type and resistant *L. mexicana* to DFMO

Wild-type and drug-resistant parasites were grown for 40 or more generations in the absence of DFMO and then different concentrations of the drug were added to several aliquots of these cultures. After four passages under these conditions by diluting each time to the same initial concentration of parasites as described in the Materials and methods section, the number of cells was counted 3 three days after the last passage and the percentages of proliferation relative to the control values in the

Figure 3 Effect of DFMO on the growth rate of wild-type and resistant cells

Drug-sensitive (●), PR₁₀ (○) or AR₁₀ (▲) *L. mexicana* were grown in the presence of increasing concentrations of DFMO. After four passages under the conditions described in the Materials and methods section, parasite growth at different concentrations of inhibitor was measured. The results were plotted as percentages of the growth in the absence of the inhibitor.

absence of inhibitor were calculated. Figure 3 indicates that growth of the wild-type strain was markedly decreased (by approx. 50%) at 100 μ M DFMO, whereas resistant parasites (promastigote and amastigote forms) did not change their growth rate even in the presence of a drug concentration 200-fold higher.

The resistance to the ODC inhibitor might be caused by one or more of the following mechanisms: (1) a decreased uptake of the drug [27–30]; (2) a mutation of the enzyme that decreases its affinity for the inhibitor [31] or increases either its catalytic efficiency or its stability [15]; (3) the amplification of the ODC coding gene causing overproduction of the enzyme [14,25,32,33]; and (4) the stimulation of ODC transcription or translation without gene amplification. We investigated all these possibilities; the results are analysed below.

DFMO uptake in drug-sensitive and drug-resistant *Leishmania*

The direct measurement of radioactive DFMO uptake into the drug-sensitive and resistant parasites gave comparable although very low values of approx. 1–3 pmol/h per 10^8 cells. To reach

different concentrations of the inhibitor we used ^3H - or ^{14}C -labelled DFMO for these experiments, obtaining very similar results in all cases. We also used an indirect approach to investigate the possibility of a decreased uptake of DFMO in *Leishmania* cells resistant to the drug. The inhibition of ODC activity by DFMO *in vivo* should depend on the intracellular accumulation of the drug resulting from its transport as well as on the sensitivity of the enzyme to the presence of the inhibitor. With the above-mentioned aim, parasite cultures corresponding to sensitive and resistant phenotypes were grown for 24 h in the presence of 10 mM DFMO, after which the residual activity of *Leishmania* ODC was measured on parasite samples taken at different times. After this treatment both types of *Leishmania* were viable and growing normally, as can be seen in Figure 1. Cells were carefully washed several times with PBS before the preparation of extracts, to eliminate completely any exogenous DFMO, which might inhibit the enzyme activity during the assay. The results indicated that the rates of ODC inhibition in DFMO-sensitive and resistant *Leishmania* were very similar and that after 24 h with the drug the enzyme activity in all cases was almost completely abolished, supporting the conclusion that the uptake of DFMO was not altered in the drug-resistant parasites (I. D. Algranati, unpublished work).

ODC activity and polyamine endogenous levels in wild-type and DFMO-resistant parasites

To measure the enzyme activity under different conditions in both *Leishmania* cell lines, DFMO was added for the indicated times and then removed from cultures for several days to avoid the inhibition of the newly synthesized ODC. Table 1 shows that the levels of enzyme activity in DFMO-resistant cells increased rapidly, reaching values 10–15 times higher than those found in extracts of wild-type *L. mexicana* treated in a similar way. Furthermore the recovery of enzyme activity after DFMO removal, which represents the *de novo* synthesis of active ODC, followed similar kinetics in both kinds of parasite.

We also determined the intracellular concentration of putrescine and spermidine in drug-sensitive and resistant phenotypes cultivated with DFMO and after removal of the drug (Table 1). The results indicate that the ODC inhibitor caused a sharp decrease in putrescine levels in the wild-type as well as in AR₁₀ parasites, although in the latter case the endogenous concentration of putrescine remaining in the presence of the drug

Table 1 ODC activity and polyamine concentrations in wild-type and DFMO-resistant *Leishmania* cultures subjected to different treatments

Cultures of wild-type and AR₁₀ *Leishmania mexicana* in HOSMEM II medium were treated as shown in each case. Where indicated, parasites were collected, washed and resuspended in fresh medium lacking the inhibitor at 5×10^6 cells/ml and cultures were reinitiated. Aliquots were taken at intervals and the corresponding cell extracts used to measure ODC specific activity (means $\pm$ S.E.M. for three determinations) and polyamine levels (averages of duplicate experiments). Abbreviation: CC, Continuous culture with 10 mM DFMO (4 months).

<i>L. mexicana</i> cell line	Treatment of cultures	ODC specific activity (nmol of CO ₂ /h per mg of protein)	Polyamine concentration (nmol/ 10^8 parasites)	
			Putrescine	Spermidine
Wild-type	None	43.3 $\pm$ 3.5	142.0	12.3
	10 mM DFMO for 48 h	1.7 $\pm$ 0.2	2.9	10.3
	24 h after DFMO removal	4.5 $\pm$ 1.6		
	48 h after DFMO removal	29.4 $\pm$ 3.2	62.3	15.0
	72 h after DFMO removal	40.2 $\pm$ 3.8		
AR ₁₀	CC	18.9 $\pm$ 2.4	15.8	12.6
	24 h after DFMO removal	144.7 $\pm$ 13.8		
	48 h after DFMO removal	521.5 $\pm$ 45.6	131.4	9.0
	72 h after DFMO removal	557.4 $\pm$ 67.3		
	15 days after DFMO removal		189.7	10.2

Table 2 Kinetic properties of ODC obtained from wild-type and DFMO-resistant *L. mexicana*

Wild-type and drug-resistant parasites were cultivated in HOSMEM II medium in the absence and in the presence of 10 mM DFMO respectively. PR₁₀ and AR₁₀ cells were collected, washed, resuspended in fresh medium lacking the inhibitor and growth was resumed for 3 days before the preparation of cell extracts. Kinetic parameters were calculated as indicated in the Materials and methods section. All values are means $\pm$ S.E.M. for three determinations.

Cell line	K_m (ornithine) (mM)	K_i (DFMO) (mM)
Wild-type	0.79 ± 0.05	0.22 ± 0.02
PR ₁₀	0.63 ± 0.08	0.23 ± 0.02
AR ₁₀	0.38 ± 0.06	0.43 ± 0.05

was 5-fold higher than in the inhibited wild-type *Leishmania*. These levels seem to be sufficient to support the normal growth of the parasite, as shown in Figure 1. After cultivation in fresh medium lacking DFMO for 2 days, ODC activity had almost completely recovered, with a concomitant marked increase of putrescine levels. Drug-resistant parasites showed a further increase in the intracellular putrescine concentration after 15 days of the removal of DFMO. At the same time the amount of putrescine secreted into the medium by these cells was approx. 60 % higher than in wild-type *Leishmania*. We do not yet know whether the increased levels of intracellular and secreted putrescine could account for a fully active overexpressed ODC or for an enzymic activity partly down-regulated *in vivo*. In contrast, ornithine does not seem to be limiting in the resistant organisms because the addition of this amino acid to the cultures did not alter the polyamine levels significantly. Treatment of wild-type and resistant parasites with DFMO elicited only minor changes in spermidine concentration, as shown in Table 1.

Properties of ODC in DFMO-sensitive and DFMO-resistant *L. mexicana*

We compared the half-life *in vivo* of the enzyme from wild-type and resistant parasites. Cycloheximide (50 μ g/ml) was added to exponentially growing *Leishmania* cultures and the specific activity of ODC was measured after different periods. Our results indicate that both kinds of *Leishmania* cell lines contained a stable ODC (half-life longer than 12 h) and that in neither case could the enzyme activity be altered by incubation of the cultures for 24 h with 1 mM putrescine (N. S. González, unpublished work). To investigate whether the development of DFMO resistance resulted from some change in ODC kinetic parameters we measured the apparent constants K_m for ornithine and K_i for DFMO in enzymic preparations from wild-type and resistant strains. The results shown in Table 2 indicate that, whereas the ODC species from wild-type and PR₁₀ cells have very similar parameters, the values obtained for the enzyme from AR₁₀ cells suggest a structural or conformational alteration to ODC leading to a higher affinity for the substrate and a decreased capacity for the binding of the inhibitor, two changes consistent with an increased resistance to DFMO.

Amplification of the ODC gene in DFMO-resistant *L. mexicana*

Genomic DNA was obtained from wild-type and DFMO-resistant parasites; after a complete digestion with *Eco*RI or *Pst*I restriction enzymes the resulting fragments were separated by electrophoresis on agarose gels and then transferred to nylon membranes. DNA hybridization was performed with a radioactive 178 bp probe specific for ODC that was prepared by PCR on the *L. mexicana* DNA template. The sequence of this probe showed a 93 % identity with a similar fragment described for *Leishmania donovani* ODC [12]. Figure 4(A) shows that only one

Figure 4 Southern blot analysis of DNA from wild-type and DFMO resistant *Leishmania*

(A) Genomic DNA from AR₁₀ (lanes a and b) and wild-type parasites (lanes c and d) were digested with *Pst*I (lanes a and c) or *Eco*RI (lanes b and d) and subjected to electrophoresis on a 0.8 % agarose gel. After transfer to a nylon membrane the separated products were probed with a radioactive 178 bp fragment encoding a segment of the *L. mexicana* ODC gene prepared as described in the Materials and methods section. In all cases the same amount of DNA (4 μ g) was used. The mobilities of molecular size markers are shown at the left. (B) Different amounts of DNA from wild-type (lanes 1, 2, 7 and 8) and AR₁₀ cells (lanes 3–6 and 9–12) digested with *Pst*I (lanes 1–6) or *Eco*RI (lanes 7–12) were analysed by electrophoresis on a 0.8 % agarose gel; after transfer to a nylon membrane the subsequent hybridization was performed with the same radioactive probe as described above. The amounts of DNA loaded in each case were: lanes 1, 3, 7 and 9, 5 μ g; lanes 2 and 8, 2.5 μ g; lanes 4 and 10, 1.66 μ g; lanes 5 and 11, 0.5 μ g; lanes 6 and 12, 0.16 μ g. The mobilities of molecular size markers are shown at the left.

Figure 5 Southern blot analysis of extrachromosomal DNA

CHEF electrophoresis of parasite DNA from wild-type (lane 1) or DFMO-resistant *Leishmania* (lane 2) was used to separate chromosomes from extrachromosomal elements. After transfer to a nylon membrane, Southern hybridization was performed as described in the Materials and methods section. The arrow indicates the position of chromosomes.

hybridizing fragment was obtained in each case with two different restriction enzymes. These fragments were of 14.7 and 5.3 kb for *EcoRI* and *PstI* digestion respectively. Furthermore the hybridization signal corresponding to DFMO-resistant *Leishmania* cell line AR₁₀ was markedly stronger than the band derived from the DNA of wild-type parasites. This result, which suggests a high level of ODC gene amplification in the drug-resistant cells, was confirmed by the titration of both kinds of DNA (Figure 4B). Densitometric scanning of the corresponding autoradiograms indicated that the ODC gene copy number in DFMO-resistant *L. mexicana* was approx. 80-fold higher than in wild-type parasites. Therefore a high level of ODC gene amplification has been detected in DFMO-resistant *Leishmania*. We obtained similar results with the cell line PR₁₀.

Southern analysis after partial digestion of DNA from amastigote resistant parasites with the restriction enzyme *PstI* did not show a ladder of hybridization bands with multiple sizes characteristic of a tandem of repeated gene units (I. D. Algranati, unpublished work). In addition, we performed CHEF gel electrophoresis on DNA from wild-type and resistant parasites followed by hybridization with the labelled probe specific for the ODC gene. The electrophoresis conditions we used permitted the concentration of all chromosomes in the position shown in Figure 5 with the extrachromosomal DNA remaining at the origin of the gel, where we observed a very intense hybridization signal only with DNA from drug-resistant parasites; the same band was almost undetectable with the wild-type DNA sample. In contrast, the hybridization bands corresponding to both kinds of DNA were not very different at the position of the chromosomes in the gel. These results strongly indicate that the multiple copies of ODC gene in DFMO-resistant *Leishmania* are not organized in a tandem of repeating units at the chromosomal level and seem to be located in extrachromosomal elements.

ODC mRNA in drug-resistant parasites

Total and poly(A)⁺ RNA obtained from wild-type and DFMO-resistant *L. mexicana* were subjected to Northern hybridization analysis by using the same 178 bp ODC-specific probe described above. Two transcripts of 5.8 and 6.9 kb were detected. After

Figure 6 Expression of the ODC gene in wild-type and AR₁₀ cells

Poly(A)⁺ RNA (0.5 µg) from DFMO-resistant (lane 1) or wild-type (lane 2) parasites was subjected to electrophoresis on a 1% (w/v) agarose/2.2 M formaldehyde gel, transferred to a nylon membrane and then hybridized with the same radioactive ODC specific probe as described in the legend to Figure 4. After hybridization, the same membrane was exposed to Kodak X-OMAT AR5 films for 10 days (A) or 3 h (B). The mobilities of RNA size markers were shown at the left.

normalization by hybridization with a probe corresponding to the actin I gene of *Plasmodium falciparum* [34], the 5.8 kb transcript was found to be 60–80-fold more abundant in the drug-resistant phenotype than in the wild-type strain (Figure 6). The level of amplification was even higher for the 6.9 kb RNA.

Stability of the ODC-overproducing phenotype and of resistance to DFMO after removal of selective pressure

To ascertain whether the drug-resistant cell lines obtained by a single-step selection with high doses of DFMO were stable in the absence of this substance, parasites cultivated for 1 month in the presence of DFMO were collected and, after resuspension in fresh medium lacking the drug, grown continuously in the absence of DFMO. Samples from these cultures taken at different times over a period of several months were subjected to measurements of ODC activity. Figure 7 shows that the overproduction of ODC in drug-resistant cell lines was fairly stable in the absence of DFMO for at least 6 months. In fact the specific activity of the enzyme, after a sharp increase occurring during the first days without the drug, showed a further enhancement and finally reached a plateau. In addition neither the PR₁₀ nor the AR₁₀ cell line changed its high resistance to DFMO and the level of gene amplification during the same period of continuous culture in the absence of the drug. Furthermore AR₁₀ maintained its 'amastigote-like' morphology (Figure 2) and contained an ODC with improved catalytic properties (Table 2).

The development of drug resistance is a process widely exploited by different types of cells to overcome the toxicity of enzyme inhibitors that might be used in antiproliferative treatments. Phenotypes resistant to methotrexate, a specific inhibitor of dihydrofolate reductase, or to DFMO, the irreversible inhibitor of ODC, have been obtained in mammalian cell and protozoan parasite cultures after stepwise selection in the pres-

Figure 7 Stability of ODC amplification in AR₁₀ and PR₁₀ cell lines

AR₁₀ (●) and PR₁₀ (○) parasites cultivated with DFMO for 30 days were collected, washed and resuspended in fresh HOSMEM II medium in the absence of the inhibitor. Cultures were resumed and aliquots were taken at the indicated times to measure the ODC specific activity. Each point represents the mean $\pm$ S.D. for three determinations.

ence of gradually increasing concentrations of the drug [9–16]. Gene amplification is one of the mechanisms more frequently used by cells to become resistant to these selective agents. Hanson et al. [25] obtained DFMO-resistant *L. donovani* mutants after a stepwise selection in the presence of increasing concentrations of the drug and in this case the resulting parasites with increased levels of ODC showed a rapid decrease in enzyme activity and unstable gene amplification several weeks after the removal of DFMO from the culture medium. The present paper is the first report on the emergence of parasite cell lines resistant to DFMO after only a one-step selection in the presence of the drug at high concentrations. We speculate that with appropriate selected agents and conditions a similar one-step amplification of other genes might appear.

The described cell lines that overproduce ODC have been also generated by a mechanism of gene amplification, but the DFMO-resistant phenotypes remained stable even after continuous cultivation for long periods in the absence of selective pressure in spite of the fact that the multiple copies of ODC gene are most probably located not in the parasite chromosomes but in extrachromosomal DNA. In addition, the enzyme from AR₁₀ cells showed a lower K_m for the substrate and a higher K_i for DFMO than shown by the wild-type ODC (Table 2). These results could indicate that gene amplification and a mutation in ODC gene might operate as coexistent mechanisms contributing to the DFMO resistance of 'amastigote-like' *Leishmania*. To investigate this possibility we are currently cloning and sequencing the ODC genes of wild-type and DFMO-resistant parasite cell lines. The simultaneous occurrence of gene amplification and a mutated enzyme has also been recently reported in methotrexate-resistant *Leishmania* cells [31].

It is interesting to point out that our results indicate a higher amplification of DNA and RNA than the corresponding enhancement of enzyme activity. This lack of correlation between the enzyme levels and those of ODC mRNA even in the absence of DFMO strongly suggests the involvement of an additional post-transcriptional down-regulation not mediated by polyamines in the process of ODC gene expression in *L. mexicana*.

We thank Dr. Eugenia Lamas (Pasteur Institute) for her help with Northern hybridization experiments; Dr. Lo Persson (University of Lund) for some determinations of polyamine concentrations; Dr. José Mordoh for the parasite microscopy; Dr. Michael Lanzer (University of Würzburg, Germany) for his advice in the experiments of CHEF gel electrophoresis; and Dr. Sara H. Goldemberg for helpful discussions. The collaboration between our two laboratories was made possible by an INSERM (France)–CONICET (Argentina) agreement. This work was supported by grants from the Swedish Agency for Research Cooperation with Developing Countries (SAREC), United Nations Industrial Development Organization (UNIDO), Centre National de la Recherche Scientifique (URA 1129), France, and the University of Buenos Aires, Argentina.

REFERENCES

- Sacks, D. L. and Perkins, P. V. (1984) *Science* **223**, 1417–1419
- Bard, E. (1989) *Biochem. Cell. Biol.* **67**, 516–524
- Glew, R. H., Saha, A. K., Das, S. and Remaley, A. T. (1988) *Microbiol. Rev.* **52**, 412–432
- Hendricks, L. D., Wood, D. E. and Hajduk, M. E. (1978) *Parasitology* **76**, 309–316
- Warren, L. (1960) *J. Parasitol.* **46**, 529–538
- Berens, R. and Marr, J. (1978) *J. Parasitol.* **64**, 160
- Kaur, K., Emmett, K., McCann, P. P., Sjoerdma, A. and Ullman, B. (1986) *J. Protozool.* **33**, 518–521
- González, N. S., Sánchez, C. P., Sferco, L. and Algranati, I. D. (1991) *Biochem. Biophys. Res. Commun.* **180**, 797–804
- Coderre, J. A., Beverley, S. M., Schimke, R. T. and Santi, D. V. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2132–2136
- Coons, R., Hanson, S., Bitonti, A. J., McCann, P. P. and Ullman, B. (1990) *Mol. Biochem. Parasitol.* **39**, 77–90
- Bellofatto, V., Fairlamb, A. H., Henderson, G. B. and Cross, G. A. M. (1987) *Mol. Biochem. Parasitol.* **25**, 227–238
- Hanson, S., Adelman, J. and Ullman, B. (1992) *J. Biol. Chem.* **267**, 2350–2359
- Chiquero, M. J., Olmo, A., Navarro, P., Ruiz-Pérez, L. M., Castanys, S., González-Pacanoska, D. and Gamarro, F. (1994) *Biochim. Biophys. Acta* **1227**, 188–194
- McConlogue, M. and Coffino, P. (1983) *J. Biol. Chem.* **258**, 12083–12086
- Mitchell, J. L. A., Hoff, J. A. and Bareyal-Leyser, A. (1991) *Arch. Biochem. Biophys.* **290**, 143–152
- Kameji, T., Hayashi, S., Hoshino, K., Kakinuma, Y. and Igarashi, K. (1993) *Biochem. J.* **289**, 581–586
- Sánchez, C. P., González, N. S. and Algranati, I. D. (1989) *Biochem. Biophys. Res. Commun.* **161**, 754–761
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Kitz, R. and Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Medrano, E. E., Goldemberg, S. H. and Algranati, I. D. (1983) *J. Cell. Physiol.* **117**, 141–147
- Holm, I., Persson, L., Heby, O. and Seiler, N. (1988) *Biochim. Biophys. Acta* **972**, 239–248
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York
- Southern, E. (1975) *J. Mol. Biol.* **98**, 503–517
- Wilson, K., Collart, F. R., Huberman, E., Stringer, J. R. and Ullman, B. (1991) *J. Biol. Chem.* **266**, 1665–1671
- Hanson, S., Beverley, S. M., Wagner, W. and Ullman, B. (1992) *Mol. Cell. Biol.* **12**, 5499–5507
- Papadopolou, B., Roy, G. and Ouellette, M. (1993) *Nucleic Acids Res.* **21**, 4305–4312
- Ellenberger, T. E. and Beverley, S. M. (1987) *J. Biol. Chem.* **262**, 13501–13506
- Dewes, H., Ostergaard, H. L. and Simpson, L. (1986) *Mol. Biochem. Parasitol.* **19**, 149–161
- Kaur, K., Coons, T., Emmett, K. and Ullman, B. (1988) *J. Biol. Chem.* **263**, 7020–7028
- Hyvönen, T., Seiler, N. and Persson, L. (1994) *Biochim. Biophys. Acta* **1221**, 279–285
- Arrebola, R., Olmo, A., Reche, P., Garvey, E. P., Santi, D. V., Ruiz-Pérez, L. M. and González-Pacanoska, D. (1994) *J. Biol. Chem.* **269**, 10590–10596
- Kahana, C. and Nathans, D. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3645–3649
- Alhonen-Hongisto, L., Kallio, A., Sinervirta, R., Seppänen, P., Kontula, K. K., Janne, O. A. and Janne, J. (1985) *Biochem. Biophys. Res. Commun.* **126**, 734–740
- Blum, H., Hannappel, M. and Arnold, G. J. (1990) *Nucleic Acids Res.* **18**, 7140