

In Vitro Growth of *Leishmania amazonensis* Promastigotes Resistant to Pentamidine Is Dependent on Interactions among Strains

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The in vitro growth of promastigote cells of *Leishmania amazonensis* was found to strongly depend on interactions among strains that differed in their pentamidine resistance. In particular, the growth of resistant strains was reduced when they shared the same environment with a less-resistant strain.

The aim of this experiment was to test whether closely related strains of *Leishmania amazonensis* promastigote cells, which differed only in their resistance to pentamidine, had an influence upon each other's in vitro growth when they shared the same environment. This approach contrasts with that normally used, where different strains grow in total isolation of one another. Our design has the advantage of allowing interactions among strains to be tested on the basis of their different drug resistance. The existence of such interactions would indicate that mechanisms giving rise to resistance have repercussions in addition to those which can be deduced from comparing strains grown in isolation from one another. Such interactions among strains could occur during mixed infections and strongly influence the relative numbers of sensitive and resistant cells produced.

Resistance to pentamidine in promastigote cells of *L. amazonensis* is known to entail changes in their kinetoplasts (1), metabolic activity (2), and membrane structure (3). However, these studies do not report any evident differences in the in vitro growth of resistant and sensitive strains. The current experiment tested whether the in vitro growth of a wild-type strain (LaWTCL1) and that of descendent strains resistant to 5 μ M pentamidine (LaR5CL1) or 20 μ M pentamidine (LaR20CL1) were influenced by being in each other's presence or not. The three strains used were each derived from a single wild-type cell (WHO designation, MHOM/BR/76/LTB-012). Each strain was maintained in identical conditions, only differing in exposure to pentamidine (Pentamidine Isethionate; Sigma), and for an equivalent period. Consequently, we could directly attribute any differences among lines to differences stemming from their strength of resistance to pentamidine and could eliminate all possibility that differences may have been due to any preexisting genetic variation among the strains being compared.

Promastigote cultures were maintained at $25 \pm 1^\circ\text{C}$ by weekly subpassages in medium RPMI 1640 (Gibco-BRL), buffered with 25 mM NaHCO_3 (pH 7.2) and supplemented with 20% heat-inactivated fetal calf serum (FCS). Each subculture was initiated with 5×10^5 parasites/ml of medium, with cells for the next subculture being harvested 6 days later at the end of the

logarithmic growth phase. Strains LaR5CL1 and LaR20CL1 were first stabilized for six subcultures in medium containing 0.05 μ M pentamidine before the drug concentration was increased in a stepwise manner until cell lines were resistant to the desired concentrations. The resistance index for these strains (IR: 50% inhibitory concentration [IC_{50}] after drug pressure/ IC_{50} before drug pressure) were 35 and 59, respectively, and were determined as described previously (7). The IC_{50} before drug pressure was $0.48 \pm 0.13 \mu\text{M}$ pentamidine (6). Strains LaR5CL1 and LaR20CL1 were then cultured in RPMI 20% FCS medium containing 5 and 20 μ M pentamidine, respectively. During this process each strain, including LaWTCL1, experienced approximately 50 subcultures. Thus, differential exposure of the strains to laboratory conditions is unlikely to explain our results.

Before the experimental assay, the effects of drug contamination were minimized by washing the resistant strain twice in 0.01 M phosphate-buffered saline (PBS; pH 7.2) and passing the strains through two subcultures in the absence of pentamidine. Strain LaWTCL1 experienced the same treatment.

The experimental assay itself involved growing promastigote cells in a 1-ml multiwell plate (Nunc) that was vertically divided into two equal volumes of 0.5 ml by a porous insert (pore size, 0.22 μm). This membrane allowed the free passage of the growth medium but prevented cells on each side of the divide from coming into direct physical contact. Thus, different pairwise combinations of strains could be grown on either side of the membrane and a strain's growth could be compared to that when it grew on both sides of the membrane, i.e., it grew in its own presence.

At the beginning of each assay, both halves of the well were seeded with 5×10^5 cells of a particular strain. Each of the nine possible pairwise combinations of strains were replicated three times. The number of cells at stationary phase (day 7 of the experiment) in each half of a well was estimated with a counting chamber and light microscope. We report data based on the identity of the strain in the upper half of a growth chamber. Equivalent analyses based upon the strain in the lower half of a growth chamber are very similar and reveal that the growth of strains was not influenced by its physical position within a growth chamber.

The results of the experiment are reported in Table 1. The leading diagonal of the table shows the number of cells produced by each strain when it grew in its own presence. The

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TABLE 1. Number of stationary-phase cells produced by each strain as a function of the identity of the other strain in the environment

Other strain	No. of stationary-phase cells (SE) ^a produced by strain		
	LaWTCL1	LaR5CL1	LaR20CL1
LaWTCL1	23.96 (2.01)	3.96 (1.07)	2.28 (0.46)
LaR5CL1	10.18 (0.84)	20.06 (1.10)	3.43 (0.69)
LaR20CL1	14.19 (2.01)	6.72 (0.94)	16.97 (1.72)

^a Values are in millions of cells.

wild-type strain LaWTCL1 produced more cells than the strain resistant to 5 μ M pentamidine (LaR5CL1), which in turn produced more cells than the strain resistant to 20 μ M pentamidine (LaR20CL1). This trend for fewer cells to be produced as the strength of drug resistance increased, however, was not statistically significant (one-way analysis of variance, $F_{2,6} = 4.237$, $P = 0.071$).

When strains of differing resistance were paired in the same environment, there was a clear evidence that some form of interaction occurred among the strains that led to fewer cells being produced (comparison of mixed versus pure treatments, Welsh corrected one-way analysis of variance $F_{1,21,341} = 50.813$, $P < 0.001$). This effect was not simply due to being in the presence of another strain, since the relative decrease in cell production was greater as the resistance increased; the mean production values in a mixed versus pure environment were as follows: LaWTCL1, 50.0%; LaR5CL1, 24.3%; and LaR20CL1, 16.0%.

Interestingly, the number of cells produced in a mixed environment did not just depend on the resistance of the strain being measured but also upon the resistance of the other strain in its environment (two-way analysis of variance $F_{4,18} = 30.018$, $P < 0.001$). As a strain's strength of resistance increased, its ability to grow in the presence of a less-resistant strain was reduced. This effect increased as the difference in the strength of resistance among strains increased. Thus, the most-resistant strain, LaR20CL1, failed to grow well in the presence of either of the two less-resistant strains but did marginally better when paired with the resistant strain LaR5CL1. Equally, LaR5CL1 grew better in the presence of LaR20CL1 than the wild-type strain LaWTCL1. Finally, LaWTCL1 also produced more cells in the presence of the more resistant LaR20CL1 than when paired with the less-resistant LaR5CL1. Our ability to eliminate genetic variability among the strains of origin as a possible confounding factor means that we can be confident in attributing the source of these interactions to each strain's strength of pentamidine resistance.

There are two direct observations that can be made from our results. First, the growth of pure and mixed pairwise combinations of strains were markedly different. Second, the growth of strains in a mixed combination was determined by their relative strengths of drug resistance. This suggests that the mechanisms responsible for pentamidine resistance have an important role in determining how these strains influenced their environment or were influenced by it.

A possible interpretation of these results is that each strain secretes something into its environment which inhibits the growth of competitors (or non-self strains) but not itself. This would explain why each strain grew less well in mixed rather than pure treatments. The pattern of our results would additionally suggest

that the mechanisms responsible for pentamidine resistance either reduced the amount of product secreted or made the strain more sensitive to the secreted products of others. This type of effect is found in interactions among closely related strains of antibiotic-resistant *Escherichia coli* bacteria. These bacteria contain plasmids which produce antibiotics (colicins) that can be used to kill off rival strains in the local environment (5). Individual strains are resistant to the colicin they produce but differentially resistant to colicins secreted by other strains (4); an effect only evident when different strains share the same environment. Different mechanisms of resistance have different consequences for the cell and its ability to persist in the presence of other strains. For example, increased colicin resistance due to altered translocation pathways can disrupt the integrity of the cell membrane and make it more sensitive to changes in its environment (8). Pentamidine resistance is also known to alter the membrane structure of *L. amazonensis* promastigote cells (3). This may similarly make such cells more sensitive to changes in their environment due to the presence of different strains.

Promastigote cells are known to secrete and to be sensitive to a diverse range of products in their environment. This raises the possibility for interactions to exist among strains; our results indicate that pentamidine resistance is one such factor capable of creating such interactions. The situations in which different strains may come into contact and interact includes multiple infections, when sampled material contains strains of different origin, or when a mutation gives rise to sensitive cells within a resistant population. Our results suggest that interactions among sensitive and resistant strains would lead to the resistant strain being rapidly replaced by the sensitive strain. Such a mechanism would limit the establishment and persistence of resistant strains in the absence of the drug concerned. Further studies allowing for the possibility of such interactions among strains may help shed valuable light on how targets of drug action and mechanisms of resistance influence the biological properties of resistant cells.

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