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Resistance to the Drug Propamidine in *Leishmania donovani*[*]

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SUMMARY

1. Clones of *Leishmania donovani* were obtained and maintained by *in vitro* culture.
2. Propamidine was found to kill the organisms *in vitro* in concentrations of 4 μg/ml or higher. At 1 - 2 μg/ml the population size remained more or less stationary over a three week period. At 0.5 μg/ml growth occurred but it was significantly less than at 0.25 μg/ml which was similar to untreated controls.
3. Organisms able to survive and grow in the presence of 0.5 μg/ml of the drug appear with a frequency of about 1/160 normal cells.
4. This frequency of origin is too high to be accounted for by a spontaneous mutation rate, hence it is concluded that resistance is an induced change in response to contact with the drug.

INTRODUCTION

Explanations of the appearance of variants in populations of microorganisms have usually depended on one of two basis mechanisms. Either the variant is the result of mutation followed by selection or it is the result of changes induced by the environment (Luria and Delbrück 1943, Bryson and Szybalski, 1955). The frequency of origin of the variant can be of key importance in choosing between these alternative explanations. For the mutation selection hypothesis depends on spontaneous mutation as the source of variation and hence the frequency of origin cannot exceed the mutation rate-a value of the general order of 10^{-5} or 10^{-6}. Rates of origin significantly below this suggest the mechanism of induced changes.

The experimental work which has been used to develop the foregoing ideas has been derived largely from the study of bacterial populations. Extension of

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these studies to other cell systems is needed both to examine the general validity of these explanations and to analyze variation in nonbacterial cell populations.

The aim of this paper is to present a study of the frequency of appearance of drug resistant forms in a parasitic, flagellated protozoan, when grown *in vitro*. As will be seen from the experimental data, the rate of appearance of resistance is too high to be accounted for by spontaneous mutation. We tentatively conclude that resistance is a response by the organism to an environment which contains the drug.

MATERIALS AND METHODS

The organism used was *Leishmania donovani*, strain JOEL, obtained by Professor Inoki from South America. The organisms were cultured in test tubes of Tanabe's medium (Inoki, Nakanishi, Nakabayashi, 1958).

A line initiated from a single cell isolation was used throughout this work. This clone was obtained by using a modification of Inoki's technique for isolating single cells of *Trypanosoma* (Inoki, 1960). The details of the technique are as follows.

1. Place a small drop of sterile Tanabe's medium into the depression of a shallow depression slide.
2. Introduce a loopful of *L. donovani* culture into the sterile medium.
3. With a sterile micropipette (internal diameter about 50 μ) place at least ten small drops of the diluted culture on a sterile coverslip and invert this coverslip over the depression of a fresh, sterile depression slide.
4. Count the number of organisms per microdrop with a low power objective; any drops which appear to have only one organism must be further checked under higher magnification, for possible agglomeration of more than one organism.
5. If the average number of organisms per microdrop is such that one drop is not unexpected, isolate the drop onto a sterile fragment of a coverslip which is stuck to the underside of a large sterile coverslip by Tanabe's medium, and invert again over a sterile depression.
6. Check the microdrop again for presence of a single flagellate. If present, remove the coverslip fragment with its microdrop by means of sterile forceps and introduce the fragment into sterile Tanabe's medium containing Chloramphenicol (about 10 μg/ml) to inhibit bacterial contamination.

In the case of the clone used here, out of the eleven drops examined (step 5), six had no flagellates, three contained one flagellate, one had two, and one had a small agglomeration of flagellates. The single cell came from one of three microdrops containing a single flagellate apiece.

Quantitative measurements of growth were made by Thomas hemocytometer cell counts. The cells were immobilized for this purpose by mixing equal volumes of the culture and a methylene blue-formalin solution (1:20,000 methylene blue in 2 per cent formalin).

The propamidine was obtained by Professor Inoki through the courtesy of Mr. K. Mizobuchi, Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, New York, U.S.A.

RESULTS

We shall first describe the growth of *L. donovani* in the presence of varying concentrations of propamidine and then describe the frequency of origin of resistant forms in a given concentration of the drug.

Figure 1 shows the results of growing *L. donovani*, strain JOEL, clone 6, in the presence of varying concentrations of propamidine. In this experiment an initial concentration of 4.16×10^4 organisms/ml was used, the sample being taken from a resting phase stock culture. The concentrations of drug were 16, 8, 4, 2, 1, 0.5, and 0.25 μg/ml, respectively, in each of seven tubes. In addition there was one control tube lacking the drug. The concentration of organisms was determined by hemocytometer counts over a period of 21 days.

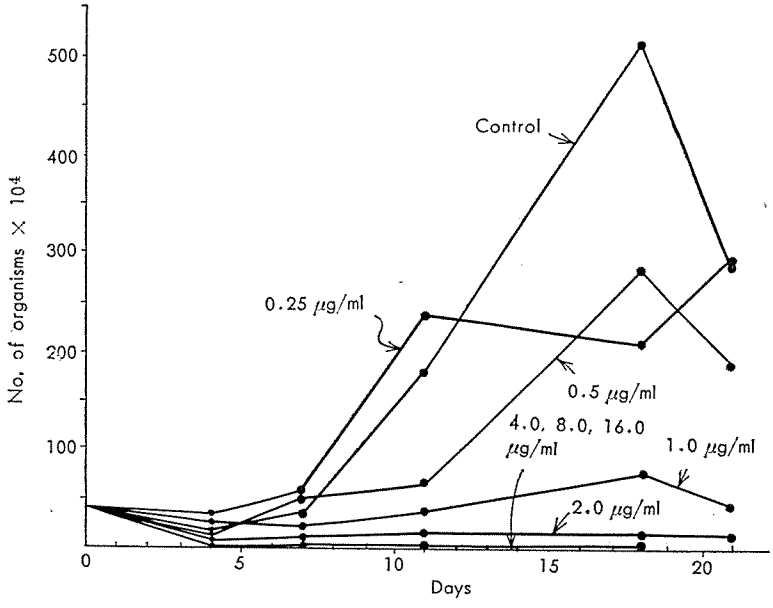

Fig. 1. The effects of Various Concentrations of Propamidine on the Growth of *L. donovani in vitro*

Certain general conclusions are illustrated by this figure. 1) The three highest concentrations of the drug resulted in disappearance of the flagellates. Even when the size (up to 1.1×10^5/ml) and physiological state (from log phase stock culture) of the inoculum were varied, this same result appeared. 2) In the next highest concentrations (2 and 1 μg/ml) motile flagellates persisted for the duration of the period of observation, but they showed no increase in number, suggesting that at these concentrations the drug may have some sort of leishmaniostatic effect, rather than the leishmaniocidal effects of the higher concentrations. These results were also seen in other runs of this same experiment. 3) Finally, at the lowest concentrations used, the drug had a small or no effect on the growth of the flagellate. At 0.5 μg/ml, growth in the presence of the drug was significantly below the control level. However, at 0.25 μg/ml, the effect, if any, is difficult to establish. The considerable divergence of the curve from the control on the 18th day is unex-

plainable and inconsistent with the otherwise similar growth of these two cultures. We tend to the view that this difference is not significant, and that 0.25 μg/ml of the drug is not effective against leishmanias grown under these conditions.

As a result of the foregoing it was decided to use a drug concentration of 0.5 μg/ml in the attempt to determine the frequency of origin of forms capable of growing in the presence of the propamidine.

The rationale of the experiment was the following. Assuming the origin of resistant forms has a finite probability, this can be determined by use of the first term of the Poisson distribution (Sager and Ryan, 1961) $P_0 = e^{-m}$ where P_0 is the probability of no growth (lack of resistance) in a set of samples, e is the base of natural logarithms, and m is the mean number of organisms per sample capable of growth under the conditions of the experiment. By observing two series of tubes, both inoculated with *L. donovani*, but one containing the drug (= experimentals) and the other lacking it (=controls), we can determine for each series the value of P_0 (the fraction of tubes showing no growth). From this we calculate m for each series and the ratio of m from the experimentals to m from the controls will be a measure of the frequency of resistant forms.

The design of the experiment was as follows. To 110 ml of Tanabe's medium, containing 12 μg chloramphenicol per ml, there were added log phase *Leishmania* sufficient to give a final concentration of 3/ml of medium. This inoculum size was determined after two preliminary trials with larger inocula, both of which have 100 per cent survival in all experimental tubes. One other trial using the inoculum size given here gave results similar to those reported below. From this work it was obvious that the resistant forms arose in a high frequency and a small inoculum was necessary. This material was dispersed as follows: 1.0 ml to each of 100 tubes (C tubes) and 3.0 ml to each of three tubes (C′ tubes). This is the control series. The experimental series of tubes was identical to the foregoing except for the added presence of 0.5 μg propamidine/ml of medium. Thus there were 100 E tubes, with 1.0 ml apiece, and three E′ tubes with 3.0 ml apiece. The growth of organisms in the C′ and E′ tubes was determined by hemocytometer counts, which are presented in the left half of Table 1. After growth had entered the log phase in the C′ cultures, fresh culture medium was added to all tubes. To the C and E tubes 1.0 ml of fresh medium was added, thus reducing the possible concentration of the drug to 0.25 μg/ml. To the C′ and E′ tubes fresh medium was added equal to the fluid remaining after the removal of samples for counting.

The reason for adding fresh medium was to encourage growth of any cells that could still do so after exposure to the drug.

Again counts were made on the C′ and E′ tubes (right half of Table 1) and when growth was indicated to be in the log phase, all C and E tubes were examined for the presence of viable flagellates. The results are that 4/100 C tubes showed no growth and 98/100 E tubes showed no growth.

Table 1. The Number of *L. donovani* per one ml of Culture before and after Adding Fresh Tanabe's Medium

(C' = tubes initially containing 3 ml of medium and no drug; E' = tubes initially containing 3 ml of medium plus 0.5 μg propamidine/ml)

Tube series	Tube no.	Inoculum	5th day	10th day	16th day	18th day	23rd day	28th day	33rd day
C'	1	3	0	0	100×10^4	Fresh culture medium added	84×10^4	80×10^4	90×10^4
	2	3	0	2×10^4	144×10^4		20×10^4	232×10^4	216×10^4
	3	3	0	20×10^4	364×10^4		12×10^4	118×10^4	48×10^4
E'	1	3	0	0	0		0	0	0
	2	3	0	0	0		0	0	2×10^4*
	3	3	0	0	0		0	0	2×10^4*

* Based on one organism/hemocytometer field. In both cases the observed entity might have been an artefact.

DISCUSSION

By use of the first term of the Poisson series, as applied to the C tubes, we see that $P_0=0.04$ and solving for m we find that $m=3.22$, which means, on the average there were 3.22 viable cells/tube in this series. This agrees very closely with the calculated inoculum of 3.0 cells/tube. On the other hand, solving m for the E tubes gives a value for m of 0.02, meaning that on the average there was 0.02 organisms capable of growth per tube containing 0.5 μg of propamidine. By comparing these two values for m we see that resistant forms are 160 times less frequent than the normal forms. Or, to state it another way, resistant forms are present on the average once in every 160 cells.

This frequency of resistant forms is too high to be accounted for on the basis of gene mutation, in contrast to the common situation among bacteria (for example, Demerec, 1948). We therefore must look for some alternative explanation. In the introduction to this paper, it was stated that the classical alternative is to propose a change induced by the environment, but before turning to this possibility we must examine certain others.

The high frequency of resistant forms could be the result of an abnormally high mutation rate, or, the result of mutation occurring early in the establishment of the clone and random sampling of the culture, as occurred during monthly serial transfer, may have resulted in a heterogeneous population containing roughly one resistant to every 160 sensitive cells at the time the clone was used for the experiment reported above.

There is no evidence in the protozoa to indicate a mutation rate of the order of 1/160. This, of course, does not disprove this possibility but it does render it highly unlikely.

The other possibility is not so unlikely for the following reason. During

serial transfers, calculations indicate, approximately 3.6×10^3 organisms are introduced into the fresh medium.* A mutant arising at the time of transfer would not give the necessary ratio of 1/160. One way to get this lower figure is to postulate that the necessary mutation occurred early in the establishment of the clone - for example, between the seventh or eighth fission when 128 to 256 cells were present - and this heterogeneity then persisted for about 12 transfers, since the final experiment was performed about one year after obtaining the clones and subculturing was done at least every month. This possibility cannot be disregarded since it would simply demand that on the average about 22 resistant cells be transferred at every subculture.

There remains, however, another consideration which opposes the mutational origin of the resistant forms by either high mutation rate or by origin of a mutant early in the establishment of the clone, and this is the graded response to different doses of the drug. (Fig. 1) The argument is as follows. A cell is resistant to a given degree as a result of mutation, or it remains sensitive. Hence, resistant cells do not show a growth rate that varies with each dosage of the drug to which they are exposed. They are resistant through several dosages up to a certain level and then above that a further mutation is often necessary (Demerec, 1948, and others) which confers another level of resistance. Our curves show neither a single phenotypic response to drug exposure nor evidence for a step-wise accumulation of resistance. The curves are best interpreted as showing an effect of propamidine on growth rate, which effect increases with concentration. And our data show further, that not all cells respond similarly to this effect, some are more resistant than others, *i.e.* at 0.5 μg/ml about 1/160 are resistant and able to show significant growth.

The final possibility remains then of an induced change, a physiologically adaptive response to the drug. If one could apply a fluctuation analysis (Luria and Delbrück, 1943) or one of its modifications (Newcombe, 1949; English and McCoy, 1951) a clear decision between this and the previous alternatives would be possible. However, the fluctuation analysis necessitates a plating technique, or its equivalent, which will detect the number of individually resistant cells in a given sample, and, unfortunately, such techniques are at present unavailable in the study of *Leishmania*. (The present authors made several unsuccessful attempts

* The basis for the calculation is the following: Cultures more than 20 days old have been found to have, on the average, 2.5×10^6 Leishmania/ml. However not all of these late resting stage forms are viable upon transfer. In one study of this problem the decrease in countable cells following addition of fresh culture fluid was by a factor of seven, *i.e.* one seventh of the cells originally present were found, by careful counting, to be present in the culture two days later. Hence, on the average about 3.6×10^5 cells/ml are viable on transfer. However the total number transferred depends on the volume of the loop used; in our work a loop whose internal diameter was about 3-4 mm was used and such a loop has a maximal capacity of 0.01 ml. Hence each transfer consisted of about 3.6×10^3 viable cells.

to develop such methods.)

Our conclusion, then, is that probably some induced change is responsible for the appearance of propamidine resistant *Leishmania*. There is general precedence for such a conclusion in the cases of induced heritable changes of antigenic types (Inoki, 1952; Inoki *et al.*, 1957, in the closely related form *Trypanosoma*; Sonneborn *et al.*, see review by Beale 1957, in *Paramecium*). More specifically, protozoan drug resistance (reviewed by Schnitzer and Grunberg, 1957) has been interpreted as an adaptive response, though this conclusion is not based on the quantitative analysis of the frequency of origin of resistant forms, such as reported here. Further work is needed to determine the nature of this postulated induced change.

We are therefore led to the conclusion that resistance is an induced change in the flagellates, presumably a response to the presence of the drug since this is the only difference between the C and E tubes.

This same conclusion has been reached in other studies on the protozoa (Schnitzer and Grunberg, 1957) but in none of these has there been a quantitative analysis of the frequency of origin of resistant forms, such as that described here, which can eliminate the possibility of gene mutation-selection as a possible explanation of this phenomenon.

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