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Semiautomated Assessment of In Vitro Activity of Potential Antileishmanial Drugs

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We have compared the in vitro activity of six agents against macrophage-contained *Leishmania tropica* amastigotes determined by the conventional Giemsa staining procedure, with the activity determined by the semiautomated assessment of incorporation of radiolabeled uracil into the nucleic acid of the organisms. Although the mean 50% effective dose of Pentostam by Giemsa staining (4.1 μg/ml) was somewhat higher than that by uracil incorporation (2.8 μg/ml), the ED_{50}s for the other two clinical agents (pentamidine, 0.035 versus 0.037 μg/ml; amphotericin B, 0.67 versus 0.70 μg/ml) and for three promising experimental agents (ketoconazole, 11.3 versus 11.3 μg/ml; the 8-aminoquinoline WR 6026, 1.6 versus 1.5 μg/ml formycin B, 0.018 versus 0.017 μg/ml) were virtually identical. The radiolabeling technique has several advantages over the Giemsa staining procedure. These include the need for relatively few macrophages, rapid and objective data generation, and viability of the test organism being measured. The successful application of the radiolabeling technique to at least six different chemical classes of compounds suggests that it would be useful for the routine assessment of antileishmanial activity in vitro.

The in vitro activity of antileishmanial agents is typically assessed by exposing amastigote-infected macrophages to drugs and manually counting the surviving organisms in Giemsa-stained cultures. The problems with this method include the need for large numbers of macrophages, the time-consuming and potentially nonobjective features of manual counting procedures, and the fact that Giemsa-positive organisms are not necessarily viable.

Quantitating the numbers of organisms that remain after drug exposure by determining the incorporation of radiolabeled nucleic acid precursors into parasite nucleic acids can potentially obviate these problems. Such experiments typically are performed in microtiter wells, which have one-seventh the surface area and therefore require one-seventh the number of macrophages needed for the 16-mm wells in current use (2). Scintillation counting of the cultures is both rapid and objective, and formation of nucleic acids is a measure of viability. Indeed, a procedure based on the incorporation of radiolabeled hypoxanthine into malaria-infected erythrocytes has proved useful in assessing the activities of various antimalarial agents against *P. falciparim* in vitro (5). Since macrophages incorporate hypoxanthine into their own nucleic acid, this precursor cannot be used to enumerate amastigotes in macrophages. However, intramacrophage *Toxoplasma* (7, 11) and *Leishmania* (9) strains can be specifically labeled with radiolabeled uracil.

We report here the quantitation of amastigotes remaining within human monocyte-derived macrophages after exposure to clinical and experimental antileishmanial agents, by incorporation of radiolabeled uracil.

MATERIALS AND METHODS

Macrophages. Macrophage cultures were derived from peripheral blood monocytes of healthy volunteers (2). Mononuclear cells were obtained by Ficoll-Hypaque fractionation of heparinized blood, and washed cells were suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml), to which 10% heat-inactivated autologous plasma (clarified by centrifugation at 2,000 × *g* for 5 min) had been added. Volumes of suspension containing 2 × 10^5 cells were added to each of the 96 wells (8 rows by 12 columns) of a microtiter plate (model no. 3596; Costar, Cambridge, Mass.). All manipulations were carried out with a digital multichannel pipet (Titretex; Flow Laboratories, McLean, Va.); all cultures were maintained at 37°C in 95% air–5% CO_2. At 3 days, the medium was replaced with RPMI plus 10% autologous human plasma; after 6 days, the monocytes had matured into macrophages. After two washings with Hanks balanced salt solution (GIBCO) to remove nonadherent lymphocytes, the cultures contained approximately 20,000 adherent macrophages.

***Leishmania* sp.** The WR 401 (NIH 173) strain of *Leishmania tropica* was maintained by a serial transfer of 5 × 10^6 amastigotes every 6 weeks in the footpads of BALB/c mice. Footpads were excised 3 to 6 weeks after inoculation; infected tissue was minced in 10 ml of Hanks balanced salt solution and pressed through a stainless steel sieve (60 mesh) with a plastic syringe plunger to free infected macrophages, which were disrupted by homogenizing the cell suspension twice. Monodispersed amastigotes liberated in this manner remained in the supernatant during centrifugation at 150 × *g* for 10 min, whereas unbroken macrophages, heavy subcellular debris, and clumps of amastigotes remained in the pellet. By these procedures, approximately 3 × 10^7 *L. tropica* amastigotes could be obtained per mouse footpad.

Exposure of infected macrophages to drugs. Macrophage cultures were inoculated with 1.5 × 10^5 amastigotes. After 4 h, the cultures were washed twice to remove nonphagocytized organisms, at which time serial dilutions of drug were added. For example, to provide twofold serial drug dilutions, 0.1 ml of medium (RPMI plus 10% heat-inactivated fetal calf serum) was added to each well of the columns that would subsequently contain drug. Then 0.1 ml of drug at 4 times the highest desired final concentration was added to the wells of the first column and gently mixed, followed by the transfer of

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0.1 ml of the solutions in the first column to the wells of the second column and gentle mixing. After completing the series of dilutions, 0.1 ml of the fluid from the wells in the last column was discarded, and all volumes in the microtiter plate were brought to 0.2 ml by the addition of media. Threefold and higher dilutions could be achieved by utilizing other proportions for the dilutions. One column was left without drug (infected positive controls), and one column was left without drug and without organisms (uninfected negative controls.) The macrophages were further cultured for 6 days with a change of medium, and drug where appropriate, on day 3. On day 6, culture medium was replaced with 0.2 ml of RPMI (without fetal calf serum) containing 2 μCi of [5-^{3}H]uracil (specific activity, 22 Ci/mmole; Moravek Biochemicals, La Brea, Calif.; final uracil concentration, 0.45 μM). Several rows of cultures (left unlabeled) were fixed, stained with Giemsa, cut out from the tray, and examined microscopically (2) for numbers of Giemsa-stained organisms. After 4 h, the radiolabeled cultures were harvested onto filter paper with an automated multiple sample harvester, the filter papers were dried, and scintillation was counted. Each drug was evaluated in triplicate.

Calculations. Net disintegrations per minute (dpm) in drug-treated cultures (dpm in treated cultures − dpm in uninfected controls) was expressed as a percentage of net dpm in infected controls. The mean number of Giemsa-stained organisms per 100 macrophages in drug-treated cultures was expressed as a percentage of the number in infected controls. For each experiment, a 50% effective dose (ED_{50}; dose of drug calculated to result in 50% of control net dpm or in 50% of control Giemsa-stained organisms) was calculated from the least-mean-squares plot of the percentages versus drug concentration. In addition, the mean ± standard error of the percentage surviving each dose of drug in all experiments was calculated and plotted against drug dosage.

Drugs. Antileishmanial drugs were obtained from the Walter Reed Army Institute of Research Drug Inventory. WR 6026 is an 8-aminoquinoline [6-methoxy-4-methyl-8-(6-diethylaminohexylamino)-quinoline].

RESULTS

Control cultures. On the day of inoculation, there were typically 300 to 400 Giemsa-stained amastigotes per 100 macrophages. After 6 days of in vitro cultivation, untreated control cultures contained a mean ± standard error of 1,283 ± 18 Giemsa-stained amastigotes per 100 macrophages (8 experiments). After exposure to [^{3}H]uracil for 4 h on day 6, there were 1,491 ± 373 dpm per control culture. Since uninfected (negative) controls exposed to uracil on day 6 contained 170 ± 51 dpm per culture, the uptake of radiolabeled uracil into the nucleic acid of infected macrophages was 8 to 9 times as extensive as that into uninfected macrophages. Comparison of the net incorporation of uracil per culture of 20,000 infected macrophages (1,321 dpm) to 256,600 Giemsa-stained organisms per culture revealed that there were 5.1 dpm of radiolabeled uracil incorporated into the nucleic acid of 1,000 non-drug-exposed amastigotes.

Drug-exposed cultures. The number of organisms apparently surviving drug exposure, as determined by the incorporation of radiolabeled uracil into the cultures or by manually counting the Giemsa-stained cultures, was expressed as a percentage of positive controls. Figure 1a to f shows the mean values of these percentages plotted against drug concentration for the six drugs. The computer-generated lines best fitting the uracil or Giemsa data are also depicted. Comparison of the lines for the uracil and Giemsa data suggests that the elimination of amastigotes determined by uracil incorporation in general closely approximated elimination as determined by Giemsa staining. This similarity was seen both for drugs for which the dose-response curves were steep (formycin B and amphotericin B) and for drugs for which the curves were more gradual (the other agents). The regression coefficient for the percent elimination determined by uracil incorporation versus the percent elimination determined by Geimsa staining was 0.83 to 0.97 (mean, 0.89) for the six drugs.

The mean ED_{50} for a drug was calculated from the mean of the ED_{50} of that agent in each of three experiments. The remaining data (Table 1) indicate that, for all agents except Pentostam, antileishmanial effects as determined by uracil incorporation were virtually identical to those determined by Giemsa staining. In the case of Pentostam, the ED_{50} by uptake of radiolabel was less, although not significantly so, than that determined by staining.

DISCUSSION

The activity of antileishmanial agents is best determined against the amastigote form of the organisms within macrophages. The extracellular, insect promastigote form is relatively insensitive to concentrations of clinically active agents attainable in serum (4). Although models in which nonadherent macrophages are used have been presented (8), adherent macrophage cultures with either human monocyte-derived macrophages (4) or mouse peritoneal macrophages (10) are generally employed. At least in the human macrophage model, control organisms mutliply once in 4 days (2), the same approximate time as for *Leishmania donovani* in hamsters (6). A 6- to 7-day period of drug exposure is used because concentrations of pentavalent antimony, the major clinical antileishmanial agent, comparable to those maintained in serum are only partially effective over 3 days (4). Amastigotes that are visually present after a 6-day period of drug exposure appear viable. When drug was removed after 6 days and the ability of intramacrophage amastigotes to transform into and multiply as promastigotes over the subsequent 3 to 4 days was assessed, the number of promastigotes from drug-exposed cultures (expressed as a percentage of non-drug-exposed controls) was similar to the number of Giemsa-stained amastigotes at the time of drug removal (expressed as a percentage of stained controls) (1).

In the present work, the activity of antileishmanial agents against amastigotes within human macrophages was determined by semiautomated quantitation of the incorporation of radiolabeled uracil into parasite nucleic acid and compared with the activity determined by the classical method of manually counting Giemsa-stained cultures. The ED_{50}s determined by the two methods were virtually identical for two of the three established clinical agents (pentamidine and amphotericin B) and for all three of the promising experimental agents (ketoconazole, WR 6026, and formycin B.) As for the third clinical agent (Pentostam), for which elimination as determined by uracil was somewhat greater than that determined by the Giemsa method, we have previously reported that, subsequent to Pentostam exposure, the loss of amastigote viability may precede the loss of nuclear staining by Giemsa (1). We note that the Giemsa ED_{50}s determined here in microtiter wells compare very well with those previously determined in larger chambers (3, 4), so that antileishmanial activity under these conditions is apparently valid.

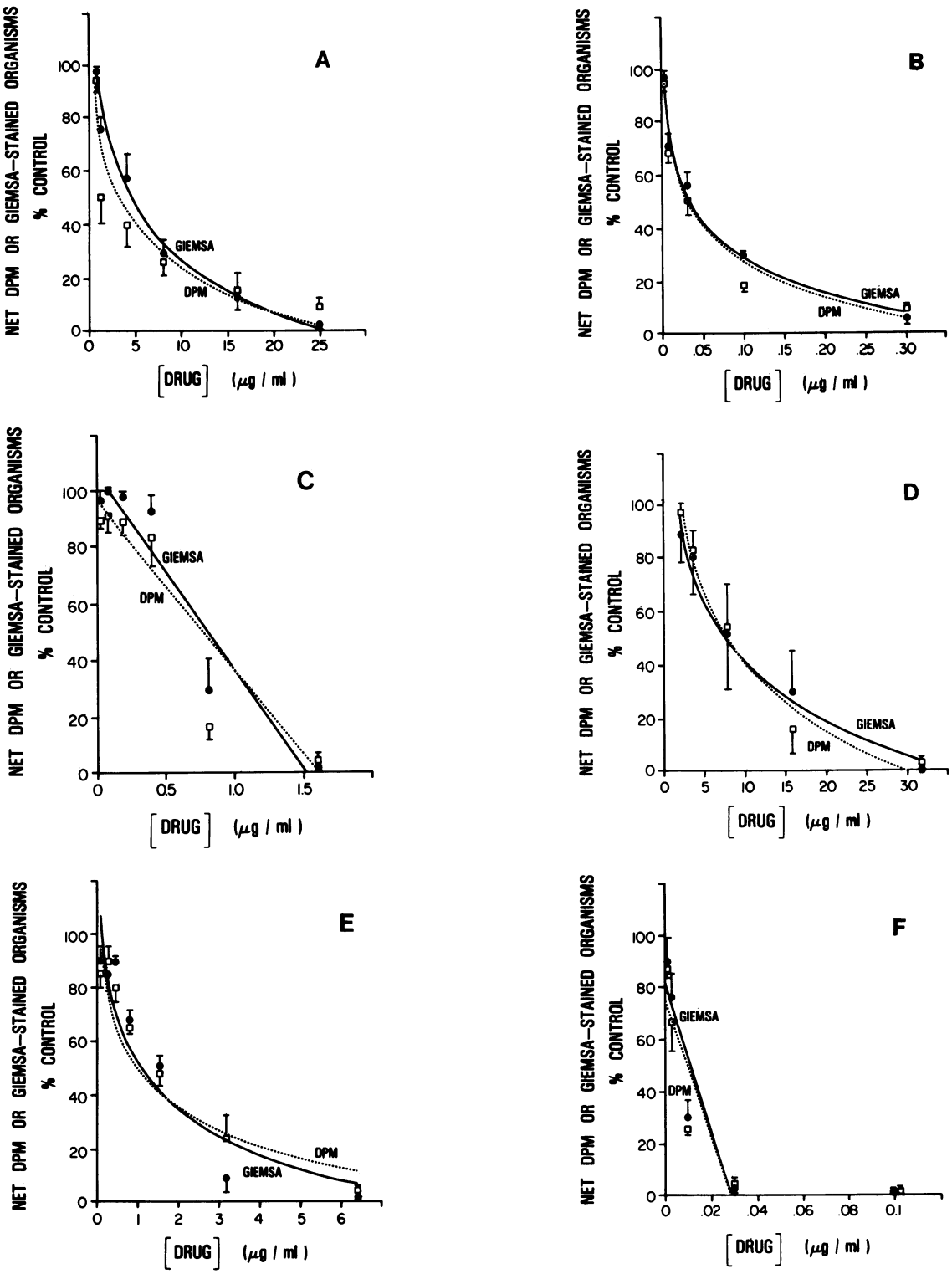

FIG. 1. Amastigotes within human macrophages were exposed to several concentrations of Pentostam (A), pentamidine (B), amphotericin B (C), ketoconazole (D), WR 6026 (E), or formycin B (F), and organism survival at several concentrations was determined by the incorporation of radiolabeled uracil into the cultures or by the number of Giemsa-stained organisms remaining in the cultures. Net dpm, or Giemsa-stained organisms, in drug-treated cultures was expressed as a percentage of the values in untreated controls. Each figure depicts the mean ± standard error of the mean of the percentages from three experiments for each drug plotted against drug concentration. Symbols: □, uracil incorporation data; ●, Giemsa staining data; - - - and ——, computer-generated least-mean-squares line for the uracil incorporation data and the Giemsa-staining data, respectively.

TABLE 1. Comparison of activity of antileishmanial agents as determined by uptake of radioactive uracil and by Giemsa staining[a]

Drug	ED_{50} (μg/ml) by:		P
	Uracil incorporation	Giemsa staining	
Pentostam	2.8 (1.6–3.6)	4.1 (2.2–5.3)	0.38
Pentamidine	0.037 (0.031–0.044)	0.035 (0.024–0.042)	0.77
Amphotericin B	0.70 (0.48–1.1)	0.67 (0.58–0.83)	0.92
Ketoconazole	11.3 (5.1–16)	11.3 (4.1–20)	0.99
WR 6026	1.5 (1.2–2.1)	1.6 (1.5–1.6)	0.92
Formycin B	0.017 (0.002–0.040)	0.018 (0.006–0.040)	0.97

[a] The ED_{50} (dose of drug calculated to result in 50% of control values) was calculated for each of the three experiments in which the activity of antileishmanial agents was determined by [^{3}H]uracil incorporation or by Giemsa staining. The mean (range) of the ED_{50}s for the three experiments is shown. *P* (statistical significance between ED_{50}s determined by the two methods) was calculated by the Student two-tailed *t* test.

The advantages of the radiolabeled uracil method—that relatively few macrophages are used per culture, that the uptake of radiolabel is a measure of viability, that antileishmanial activity is objectively assessed, and that the method works for all six compounds which represent six distinct chemical classes—together result in the more rapid and accurate determination of the ED_{50} of antileishmanial agents. The disadvantages of the method are that fungal or bacterial contamination results in falsely high values of radioactive incorporation and that toxicity of the drug to the macrophage hosts removes them from the monolayer and results in falsely low incorporation values. We address these considerations qualitatively by inspection of the cultures with an inverted microscope, before the addition of uracil, to ascertain contamination, loss of macrophages, or both. Notwithstanding these concerns, the advantages of this technique, for which the results are comparable to Giemsa staining, has led us to adopt it as our standard means to evaluate the activity of putative antileishmanial agents.

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