Multiplication of *Leishmania* in Human Macrophages In Vitro

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Received for publication 26 June 1979

To facilitate in vitro studies of the immunology of human leishmaniasis, we developed a method of growing pathogenic *Leishmania* in human monocyte-derived macrophages. After 6 days of incubation, adherent mononuclear cells were infected with *Leishmania donovani* amastigotes obtained from infected hamster spleen cells or with *L. tropica* amastigotes obtained from infected BALB/c tissue mouse footpad. Forty-eight percent of the macrophages were initially infected, with a mean of 3.0 amastigotes per infected macrophage. After 6 days of incubation, 59% of macrophages were infected and contained 8.8 amastigotes per infected macrophage, representing 2.9-fold multiplication. Electron microscopy revealed the presence of dividing parasites within phagolysosomes. These observations indicate that *Leishmania* survive and multiply within human monocyte-derived macrophages despite fusion of secondary lysosomes with the parasitophorous vacuole.

Leishmaniasis is a parasitic disease which poses a major public health problem in several developing nations. The disease is caused by a protozoan which invades and grows within host macrophages. Why some patients (e.g., those with cutaneous infections due to *Leishmania tropica*) can spontaneously resolve their infections and eliminate the intracellular parasite, whereas others (e.g., those with visceral disease due to *L. donovani*) experience chronic, disseminated, and even fatal infections, is unknown. One important aspect of virulence in leishmaniasis is the factor which permits the growth of *Leishmania* in macrophages, cells normally armed with efficient microbial capabilities. Studies of the interaction of host cells with *Leishmania* species pathogenic to humans have utilized in vitro cultures of tumor cells (6, 11, 12) or of macrophages derived from laboratory animals which are not the natural hosts of these species (1, 2, 4, 7, 14). The importance of investigating the interaction of parasites with macrophages derived from natural hosts is suggested by the possibility that the adaptation of *Leishmania* species with respect to their survival in macrophages may be highly specific. For example, Mauel et al. have observed that amastigotes of *L. enriettii* are not killed in vitro by macrophages of the natural host (guinea pig) under the same conditions which result in their elimination from macrophages of an artificial host (mouse) (13). To study mechanisms of host defense in human leishmaniasis, we therefore developed a method of cultivating *L. donovani* and *L. tropica* in human monocyte-derived macrophages.

**MATERIALS AND METHODS**

Macrophage cultures were derived from peripheral blood monocytes of healthy volunteer donors without previous leishmaniasis by a modification of the methods described by Johnson et al. (8). Briefly, 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 1-glutamine (4 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml) (referred to hereafter as supplemented medium), to which 10% heat-inactivated autologous plasma (clarified by centrifugation at 2,000 × g for 5 min) had been added. One milliliter of suspension containing 2 × 10⁶ mononuclear cells was added to each well (16 mm in diameter) of plastic culture trays (Costar, Cambridge, Mass.). After 4 h and again after 3 days of incubation at 37°C in 5% CO₂-95% air, medium was replaced with supplemented medium plus 10% autologous plasma. Nonadherent cells were removed after 6 days of incubation by washing cultures four times with warm (37°C) Hanks buffer (GIBCO). The medium was replaced with supplemented medium plus 10% heat-inactivated fetal calf serum (GIBCO). In preliminary studies, we determined that 6 days of incubation before removing nonadherent cells resulted in the largest number of macrophages which remained adherent after subsequent infection with *Leishmania*. The intracellular forms of *Leishmania* (amastigotes) were obtained either from spleen cells of hamsters infected 1 to 2 months previously by intravenous...
inoculation of 2.5 × 10^7 amastigotes of L. donovani (strain 1s, clone 2d [15]) or from footpad tissue of BALB/c mice infected 1 to 2 months previously by subcutaneous inoculation with 2 × 10^7 amastigotes of L. tropica NIH strain 173 (1a) (isolated from a patient in Iran with classical Oriental sore and kindly provided by A. Ebrahimzadeh, Shapur University School of Medicine, Ahwaz, Iran). Infected tissue was minced in 10 ml of Hanks buffer and pressed through a stainless-steel sieve (60 mesh) with a plastic syringe plunger. Infected cells were disrupted by rapidly passing the cell suspensions twice through a 25-gauge needle and then five times through a 30-gauge needle. Monodispersed amastigotes liberated in this manner were isolated into the supernatant during centrifugation (150 × g for 10 min), whereas unbroken cells, heavy subcellular debris, and clumps of amastigotes remained in the pellet. By using this procedure, approximately 6 × 10^7 L. donovani amastigotes could be obtained per hamster spleen and 3 × 10^7 L. tropica amastigotes could be obtained per mouse footpad.

One-tenth milliliter of an amastigote suspension containing 7 × 10^8 amastigotes was added per well containing 6-day-old macrophage cultures (amastigote-macrophage ratio, 5:1). After 4 h of incubation at 37°C (5% CO₂–95% air), macrophage cultures were washed thrice with warm Hanks buffer and replenished with supplemented medium containing 10% heat-inactivated fetal calf serum. Cultivation at 37°C in 5% CO₂–95% air continued for up to an additional 2 weeks, during which time supplemented medium containing 10% heat-inactivated fetal calf serum was changed every 3 days. Macrophage cultures were fixed with absolute methanol, stained with Wright stain for 30 s and then stained with 5% Giemsa stain for 2.5 min, and examined by light microscopy (×1,000). The percentage of macrophages that contained amastigotes and the number of amastigotes present per infected macrophage were determined in replicate cultures by counting 200 cells per well.

To determine whether lysosomal fusion with the parasitophorous vacuoles occurred in Leishmania-infected cells, macrophages were labeled with thorium dioxide colloid (Thorotrast; Fellow Testagar, Sanurce, Puerto Rico) by the method of Jones and Hirsch (9), without modification.

**RESULTS**

After removal of nonadherent cells after 6 days of incubation, 145,000 ± 32,000 adherent cells per well (mean ± standard deviation of 7 experiments) were enumerated by visual inspection. These cells had the morphological and phagocytic properties of macrophages: they were large (typically between 20 and 60 μm in diameter), they had extensively ruffled plasma membranes, they had a large cytoplasmic-to-nuclear ratio, and 95% ingested latex beads during a 1-h incubation. Six days after infection with L. tropica or L. donovani, 120,000 ± 25,000 (seven experiments), macrophages remained adherent per well. The 17% loss of adherent cells was primarily due to cellular disintegration, since less than 2,000 intact macrophages per well could be recovered by cytocentrifugation of culture supernatants.

After infection of 6-day-old adherent cell cul-

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**Fig. 1.** Leishmania-containing human macrophages. Macrophages were infected with amastigotes of L. tropica (NIH strain 173) for 4 h, after which nonendocytosed parasites were removed by washing. (top) Macrophages immediately after infection: infected macrophages contain 1 to 7 amastigotes, with a mean of 3 amastigotes per infected macrophage (Wright-Giemsa stain; × 1,000). (bottom) Macrophages 6 days after infection: the macrophage in lower right contains approximately 40 amastigotes and illustrates the high degree of multiplication sometimes seen; other macrophages contain 7 to 13 amastigotes, with a mean of 9 amastigotes per macrophage (Wright-Giemsa stain; × 1,000).
tures with either *L. donovani* or *L. tropica* amastigotes, 48 ± 17% of macrophages were infected and contained 3.0 ± 0.8 amastigotes per infected macrophage (mean ± standard deviation of 10 experiments) (Fig. 1 and 2). After 6 days of further incubation, 59 ± 19% of macrophages were infected and contained a mean of 8.8 ± 1.9 amastigotes per infected macrophage (10 experiments) (Fig. 1 and 2). This represented a mean increase of 2.9-fold, or 190%, in 6 days.

The numbers of amastigotes enumerated in individual macrophages varied considerably on days 6 through 11 of infection (Fig. 3). Nevertheless, comparison of this percent increase with the expected value of zero if amastigotes did not replicate demonstrated that substantial multiplication of *Leishmania* had occurred in 6 days.

The mean number of amastigotes per infected macrophage began to decrease by 1.5 to 2 weeks of infection; amastigotes characteristically disappeared from macrophage cultures after 2 to 3 weeks of infection.

Electron microscopy of both *L. donovani* and *L. tropica*-infected macrophages treated with Thorotrast revealed amastigotes dividing within Thorotrast-containing vacuoles (Fig. 4).

**DISCUSSION**

The mean number of *Leishmania* amastigotes per infected macrophage increased by 190% over 6 days of infection. This indicated that amastigotes of *L. tropica* and *L. donovani* multiplied within human monocyte-derived macrophages. Since only 17% of the macrophages were lost from culture dishes during the *Leishmania* infection, it is unlikely that the observed increase in *Leishmania* amastigotes represented an artifact created by selective cell loss or by reinvasion of adherent cells by amastigotes released from

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**Fig. 2.** Growth curves of *L. donovani* and *L. tropica* in human monocyte-derived macrophages during 6 to 8 days of incubation. In each case, the mean number of amastigotes per infected macrophage and the percentage of infected macrophages were determined by counting 200 macrophages.

**Fig. 3.** Histograms illustrating frequency distributions of amastigotes of *L. donovani* within infected macrophages on days 1 and 11 of infection.
disintegrated cells. Electron micrographs of infected macrophages confirmed that amastigotes were indeed dividing in macrophage vacuoles (Fig. 4). The degree of intracellular *Leishmania* multiplication observed in this system contrasts with the low degree of multiplication (13 to 18%) seen in the *L. enriettii*-guinea pig macrophage system, the only previously described in vitro system that used a natural host-parasite combination (3, 13). The system we described therefore can serve as a useful model for investigating regulation of intracellular growth of pathogenic *Leishmania*. Furthermore, the appropriateness of using monocyte-derived macrophages is suggested by the fact that macrophages in many inflammatory lesions, including skin lesions, derive from blood monocytes (16, 17).

Localization of the parasite within the cellular ultrastructure was facilitated by the ability of lysosomes to concentrate Thorotrast endocytosed by the macrophage (9). The presence of Thorotrast in amastigote-containing vacuoles

![Fig. 4. Electron micrographs of macrophages exposed to Thorotrast and subsequently infected with *L. tropica*. (A) Dividing *Leishmania* amastigote (A) is located within a phagocytic vacuole. The presence of Thorotrast (arrows) within this vacuole suggests that phagolysosomal fusion has occurred. In addition to apparent cytokinesis, parasite viability is further indicated by the presence of Thorotrast in the amastigote flagellar pocket (F). A secondary lysosome (L) which has not fused with the parasitophorous vacuole is also seen (×26,000). (B) Amastigote (A) dividing within a parasitophorous vacuole. A cytoplasmic bridge joins the dividing *Leishmania*. The presence of Thorotrast (arrow) within a vacuole of the parasite indicates active parasite endocytosis of the marker (×24,000).]
and even within the parasite flagellar pocket (Fig. 4) indicates that lysosomal fusion with the parasitophorous vacuoles had occurred. The localization of *L. donovani* and *L. tropica* amastigotes to the phagolysosomes of human macrophages is similar to the observations previously described for *L. donovani* in hamster macrophages (4, 5) and for *L. mexicana* in mouse macrophages (1). Our observations extend these earlier ones by indicating that pathogenic *Leishmania* amastigotes also localize to this site in cells of the natural host. Collectively, these studies clearly indicate that *Leishmania* survives and multiplies intracellularly by some means of resisting digestion by lysosomal enzymes. This contrasts with observations in studies of *Toxoplasma gondii* and *Trypanosoma cruzi*, other intracellular protozoa whose behavior has been investigated in murine macrophages. *T. gondii* survives by preventing lysosomal fusion with the parasitophorous vacuoles and is destroyed if phagolysosomal fusion occurs (9). *T. cruzi* may evade lysosomal enzymes by escaping from the parasitophorous vacuole into the cytosol (10). The ability to culture *L. donovani* and *L. tropica* in human macrophages should facilitate immunological investigations in human leishmaniasis.

ACKNOWLEDGMENTS

We thank David Alling for assistance with statistical evaluation and M. Gue for typing the manuscript.

LITERATURE CITED


