EXTRACELLULAR KILLING OF *LEISHMANIA* 
PROMASTIGOTES AND AMASTIGOTES BY MACROPHAGE 
PRECURSORS DERIVED FROM BONE MARROW CULTURES

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Over the past few years our laboratory has been studying the functional activ-
ity of murine macrophage precursors. We have demonstrated that these cells are 
very potent natural effectors, exerting natural killer activity against YAC-1 
tumor cells and microbicidal activity against the yeast form of the fungus *Can-
dida albicans* (1, 2). Macrophage precursors can be isolated from bone marrow 
liquid culture or, in small amounts, from the peripheral organs (spleen and liver) 
of normal untreated mice (3). Under inflammatory or recruitment conditions, 
large numbers of macrophage precursors appear in the periphery (4, 5) in liver 
and spleen. Mature cells of the macrophage system appear to play a central role 
in the immune response against *Leishmania* parasites. More recently, evidence 
has been presented that adds support to the hypothesis of an involvement of 
cells with NK activity in the host recovery from visceral leishmaniasis (6).

Taking the central role of the macrophage compartment in leishmaniasis into 
account and the possible involvement of cells with NK activity in the recovery 
from the same infection, we were interested in examining in vitro the sponta-
neous leishmanicidal activity of cells in the early stage of macrophage differen-
tiation that have been shown to perform NK activity (1–5). Spontaneous cyto-
toxic activity of this effector cell type was tested against prelabeled promastigotes and 
amastigotes. The effector–parasite relationship has been investigated in terms of 
target binding, optimal effector cell to target ratio, and killing kinetics.

**Materials and Methods**

*Mice.* 6–8-week-old inbred C57Bl/6(H-2^b^ healer) or BALB/c(H-2^d^ nonhealer) mice 
of either sexes were obtained from the Zentrale Versuchstieranstalt, Hannover, FRG.

*In Vivo Treatment of Parasites.* *Leishmania enriettii* has been maintained by monthly 
subcutaneous passages in guinea pigs (Grand Island Biological Co., Grand Island, NY) 
and Dulbecco’s modified minimal essential medium (DMEM), supplemented with 5% 
FCS and 1% soluble hemoglobin (Difco Laboratories, Inc., Detroit, MI). *Leishmania major* 
(strain L38) has been maintained since then by subcutaneous passages in BALB/c mice.

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Promastigotes were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS.

*Leishmania donovani* has been maintained by intraperitoneal passages in hamster and *Sigmodon hispidus*. Promastigotes were grown in Steiger's medium (III) (7) supplemented with 10% FCS.

**Prelabeling of the Parasites.** The method has been recently described (7). Briefly, 10⁷ promastigotes in 10 ml of culture medium were incubated for 24 h with 50 μCi [³H]dThdR at 25°C in 5% CO₂, washed twice, resuspended in RPMI supplemented with 10% FCS at the concentration of 2 × 10⁵/ml and used as targets in the radiolabel-release assay. 2 × 10⁴ microorganism usually gave 2,000 ± 500 cpm.

**Preparation of *L. donovani* Amastigotes.** Amastigotes were prepared by culturing prelabeled promastigotes in J774 cells for 3 d. After mechanical destruction of J744 cells amastigotes were isolated by a two-step Percoll gradient.

**Isolation of Effector Cells.** Bone marrow (BM) cultures were established according to a slight modification of the technique described by Meerpohl et al. (2). BM cells were collected, incubated for 3 d in the presence of fibroblastic supernatant, and nonadherent cells were isolated by nylon wool treatment and Percoll gradient centrifugation (2).

**Results**

**Spontaneous Leishmanicidal Activity of BM-derived Macrophage Precursors Against [³H]dThdR Prelabeled *L. promastigotes*.** Different numbers of BM-derived macrophage precursors were cultivated *in vitro* with [³H]dThdR prelabeled *L. enriettii*, *L. major* and *L. donovani* promastigotes (2 × 10⁴/well) to assess their killing efficiency in a 12-h radio-label-release assay. As outlined in Fig. 1, a strong leishmanicidal activity was displayed by the effector cells already at very low effector/target ratios. The E/T ratio 0:1 indicates the [³H]dThdR release of the parasites incubated without effector cells (spontaneous release).

*L. major* and *L. donovani* appeared to be killed more efficiently than *L. enriettii*. The killing of these both mouse pathogenic species reached a plateau at an E/T ratio as low as 3:1, whereas an E/T ratio of 10:1 was required for maximal killing of *L. enriettii*.

**Spontaneous Cytotoxicity of BM Macrophage Precursors against Prelabeled Leishmania Amastigotes.** We investigated whether a similar spontaneous cytotoxicity as shown for the promastigotes will also be active against the amastigote form. [³H]dThdR-labeled amastigotes were prepared as described in Materials and

![Figure 1](image_url). Figure 1. Spontaneous cytotoxic activity of BM-derived macrophage precursors obtained from C57/B16 mice against *L. enriettii*, *L. donovani* and *L. major*, prelabeled late log-phase promastigotes in a 12-h [³H]dThdR-release assay. The effector/target ratio 0:1 represents the spontaneous release of the parasites incubated without effector cells. Vertical bars represent the standard errors of the mean. (--●--) *L. enriettii*, (--■--) *L. major*, (--▽--) *L. donovani*. 
PARASITICIDAL ACTIVITY OF MACROPHAGE PRECURSORS

Methods. These amastigotes were then coincubated with BM macrophage precursor. As documented in Fig. 2, spontaneous cytotoxicity of BM macrophage precursor was comparable against promastigotes and amastigotes indicating that also the amastigote form is a suitable target for the described effector cells.

Kinetics of the Leishmanicidal Activity of BM-derived Macrophage Precursors. To determine the kinetics of the events leading to destruction of the promastigotes by the effector cells, we performed a series of experiments in which effector and target cells were cocultivated at a 6:1 ratio for different incubation periods. Fig. 3 shows that the $[^{3}H]d$TdT release from *L. donovani* and *L. major* target cells was already detectable after a 4-h cocultivation and increased over the next 8 h, reaching plateau values after 18 h. Killing of *L. enrietti* appeared to require a longer time, appearing after 8 h of incubation and reaching a plateau after 18 h.

Target Binding Studies. As shown in Table I, the binding of the target by macrophage precursors was a very quick event, 85% of the parasites being already tightly bound to the cells within the first 5 min of incubation. Fig. 4 gives examples of *Leishmania* promastigotes and amastigotes tightly associated with a BM macrophage precursor.

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**FIGURE 2.** Spontaneous cytotoxicity of BM-derived macrophage precursors against *L. donovani* amastigotes (□) and *L. donovani* promastigotes (■). Effector/target ratio was 10:1. The graph shows the specific kill of prelabeled *L. donovani* promastigotes (pm) and amastigotes (am) in an 18-h release assay. Vertical bars represent the standard error of the mean.

**FIGURE 3.** Kinetics of killing of *Lenrietti, L. donovani*, and *L. major* late log-phase promastigotes by BM-derived macrophage precursors at the effector/target ratio 10:1 (— — —) as compared with parasites spontaneous release (— —). Vertical bars represent the standard errors of the mean. (— — —) *Lenrietti*, (— ■ —) *L. major*, (— ◀ —) *L. donovani*. 
As outlined in Table I, target damage occurred much earlier (by at least 2 h) than it could be detected by the radiolabel-release assay. A complete lysis seemed therefore to be necessary for the [3H]dTdR label to be released. No intracellular protozoa were ever observed using the nonphagocytic precursors as effector cells throughout the assay time. The killing occurred, as in the case of YAC-1 and C.albicans, completely extracellularly.

Discussion

Spontaneous cell-mediated cytotoxicity plays a major role in innate resistances. Being present in nonimmunized individuals and being less restricted
PARASITICIDAL ACTIVITY OF MACROPHAGE PRECURSORS

than T cell–mediated immunity in its specificity, this natural cytotoxic activity represents the host’s first line of defense against pathogenic agents.

The first targets described to be susceptible to this reactivity have been tumor lines and virus-infected cells (8). More recently, spontaneous in vitro cytotoxicity has been reported to occur against fungal (2) and protozoan (10, 11) targets.

The effectors mediating spontaneous cytotoxicity are heterogeneous. Most of the attention in this field has been drawn to natural killer cells (large granular lymphocytes, LGLs; references 12, 13) and to mature macrophages (14). We have recently described the strong spontaneous cytotoxicity exerted by non-adherent, nonphagocytic cells in the early stages of macrophage differentiation against YAC-1 tumor cells (1–5) and against the yeast form of the dimorphic fungus Candida albicans (2–5). The present data extend the range of the natural cytotoxic activities of these effector cells to include the late log phase promastigote stadium and more important the amastigote stage of the protozoa of the genus *Leishmania*. The killing of the parasites was assessed by means of a recently developed technique, based on the release of [3H]TdR from prelabeled target organisms, allowing the quick, easy, and exact measurement of spontaneous extracellular and/or intracellular lysis of the parasites (7).

Even more important and of more direct relevance are the spontaneous killing data obtained using prelabeled *Leishmania* amastigotes as targets. The prelabeling technique of *Leishmania* promastigotes (7), which after in vitro phagocytosis by macrophages of the J774 cell line, are readily transformed to healthy dividing amastigotes, provides an ideal source for labeled amastigotes. Using these amastigotes we demonstrated that the nonphagocytic macrophage precursor cells have a strong potential to kill these amastigotes spontaneously. It is of particular importance, that early stages of the macrophage lineage display such a killing potential since at this stage of differentiation they are completely devoid of phagocytic ability and are therefore well suited to control the infection. We have shown previously that this cell type is present outside the BM in liver and spleen of normal animals and they are greatly enhanced in number in the organs of inflamed mice or of mice under recruitment conditions (3–5). Preliminary data obtained in our lab indicate, that a large proportion of the hepatosplenomegaly occurring during an *L. donovani* infection is caused by a strong increase in the macrophage precursor population (Hockertz, S., manuscript in preparation). It has been shown previously that macrophage precursors have in addition to their spontaneous cytotoxicity the ability, due to their Fc receptors, to perform antibody-dependent cell cytotoxicity (15).

Studies are in progress to clarify the role of organ-associated macrophage precursors during the course of a *L. donovani* infection and their possible cooperative effects with antileishmanial antibodies, which are usually present in the diseased animal in large amounts.

In conclusion, we have shown that macrophage precursor cells purified from BM culture possess, apart from their NK function against YAC-1 and *Candida albicans* (1, 2), a strong spontaneous activity to kill *Leishmania* promastigotes and, more important, also amastigotes. The prelabeling technique of both targets with [3H]TdR allowed a precise quantitative calculation of the activity of this spontaneous killer cell population. Further experiments will clarify the role
of spleen and liver associated macrophage precursors isolated during an ongoing infection and the possible contribution of this cellular differentiation stage to host recovery from parasite infection. Such a role may be of particular importance in the case of *L. donovani* infection, since this disease is mainly located in liver and spleen, both organs where the described effector cell is frequent and located in close association to parasitized macrophages. Since these macrophage precursors will, when matured to macrophages, become hosts for the parasite, balance mechanisms between the differentiation stages of this cell type may be important for the control of the disease.

**Summary**

Flagellates of the genus *Leishmania* are obligate intracellular parasites of vertebrates including man. The microorganisms reside and multiply inside the phagolysosomes of cells of the mononuclear phagocyte lineage. We here report on the spontaneous leishmanicidal activity exerted extracellularly by immature cells of the mononuclear phagocyte lineage. Highly purified, bone marrow–derived macrophage precursor cells displayed a strong spontaneous leishmanicidal activity already at very low effector/target rations (3:1, 6:1). This leishmanicidal activity was effective against both promastigotes and amastigotes as targets.

The cytotoxic effect was evident within 4 h and maximal after 12 h of effector–target organism cocultivation, as determined by a radiolabel-release assay. An intimate cell–cell contact seemed necessary for the parasites to be killed.

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