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

By MANUELA BACCARINI, STEFAN HOCKERTZ, ALBRECHT F. KIDERLEN, AND MARIE-LUISE LOHMANN-MATTHES

From the Fraunhofer-Institute for Toxicology and Aerosol Research, Department of Immunology, 3000 Hannover 61, Federal Republic of Germany

Over the past few years our laboratory has been studying the functional activity 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 *Candida albicans* (1, 2). Marcrophage 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 spontaneous leishmanicidal activity of cells in the early stage of macrophage differentiation that have been shown to perform NK activity (1–5). Spontaneous cytotoxicity 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.

M. Baccarini's present address is Albert Einstein College of Medicine, Dept. of Microbiology and Immunology, Bronx, New York 10461. Address correspondence to Prof. M.-L. Lohmann-Matthes, Dept. of Immunology, Fraunhofer-Institute of Toxicology and Aerosol Research, Nikolai-Fuchs-Str. 1, 3000 Hannover 61, FRG.

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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, 10^7 promastigotes in 10 ml of culture medium were incubated for 24 h with 50 μCi [^{3}H]dTdR at 25°C in 5% CO_2, washed twice, resuspended in RPMI supplemented with 10% FCS at the concentration of 2×10^5/ml and used as targets in the radiolabel-release assay. 2×10^4 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 [^{3}H]dTdR Prelabeled L.promastigotes. Different numbers of BM-derived macrophage precursors were cultivated *in vitro* with [^{3}H]dTdR prelabeled *L.enriettii, L.major* and *L.donovani* promastigotes (2×10^4/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 [^{3}H]dTdR release of the parasites incubated without effector cells (spontaneous release).

L.major and *L.donovani* appeared to be killed more efficiently that *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. [^{3}H]dTdR-labeled amastigotes were prepared as described in Materials and

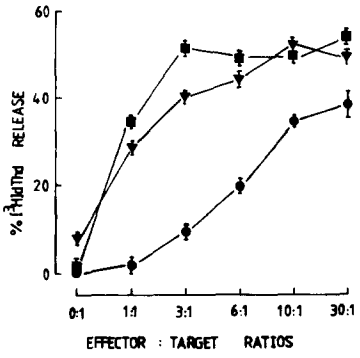

FIGURE 1. Spontaneous cytotoxic activity of BM-derived macrophage precursors obtained from C57/B16 mice against *L.enriettii, L.donovani* and L.*major,* prelabeled late log-phase promastigotes in a 12-h [^{3}H]dTdR-release assay. The effector/target ratio 0:1 represents the spontaneous release of the parasites incubated without effector cells. Vertical bars represent the standard errors of the mean. (—●—) *L.enrietti,* (—■—) *L.major,* (—▼—) *L.donovani.*

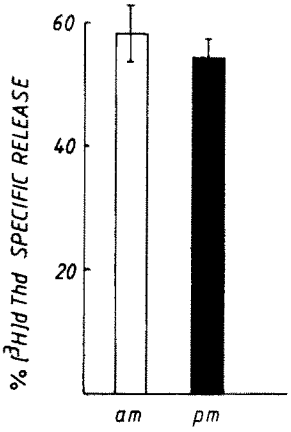

FIGURE 2. Spontaneous cytotoxicity of BM-derived macrophage precursors against *L.donovani* amastigotes (□) and *L.donovani* promastigotes (■). Effector/target ratio was 10:1. The graph shows the specific kill of prelabeled *L.donovani* promastigotes (*pm*) and amastigotes (*am*) in an 18-h release assay.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

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]dTdR 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.enriettii* 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.

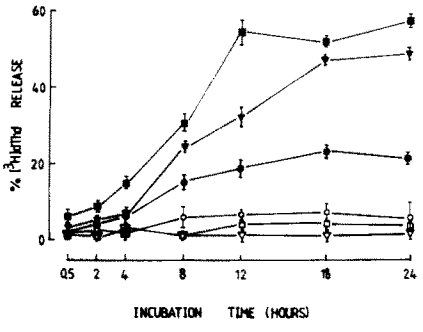

FIGURE 3. Kinetics of killing of *L.enriettii*, *L.donovani*, and *L.major* late log-phase promastigotes by BM-derived marcophage precursors at the effector/target ratio 10:1 (—●—) as compared with parasites spontaneous release (—○—). Vertical bars represent the standard errors of the mean. (—●—) *L.enrietti*, (—■—) *L.major*, (—▼—) *L.donovani*.

TABLE I
Target Binding Ability of Bone Marrow–derived Macrophage Precursors as Measured in 5-, 30-, and 120-min Assays

Bound Leishmaniae	Bound *L.enriettii* after incubuation time of:		
	5 min	30 min	120 min
		%	
Live	82 ± 6*	53.5 ± 8	19.5 ± 4
Damaged	3.5 ± 1.5	26 ± 10	60.5 ± 16
Dead	—	9 ± 7	8 ± 6

* Data represent the mean of three experiments ± SE of the mean. The assays were run at the temperature of 29°C. At least 200 cells/slide were counted; no intracellular parasite was ever observed.

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 [^{3}H]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

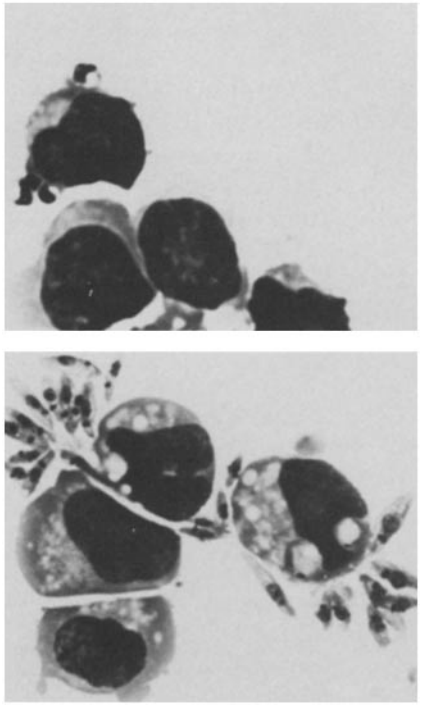

FIGURE 4. May-Grünwald Giemsa–stained cytocentrifuge preparations taken after 5 min of effector parasite cocultivation. After 5 min, the promastigote form of *L.donovani* is tightly bound to the effector cells. (*Top*) Stained by the same technique, *L.donovani* amastigotes firmly attached to the surface of the effector cells.

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 nonadherent, 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 logphase 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 [^{3}H]dTdR from prelabeled target organisms, allowing the quick, easy, and exact measurement of spontaneous extra- 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 [^{3}H]dTdR 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 marcophage 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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References

1. Lohmann-Matthes, M. L., W. Domzig, and J. Roder. 1979. Promonocytes have the functional characteristics of natural killer cells. *J. Immunol.* 123:1883.
2. Baccarini, M., F. Bistoni, and M. L. Lohmann-Matthes. 1985. *In vitro* natural cell-mediated cytotoxicity against *Candida albicans:* macrophage precursors as effector cells. *J. Immunol.* 134:2658.
3. Baccarini, M., F. Bistoni, and M. L. Lohmann-Matthes. 1986. Organ-associated macrophage precursor activity. Isolation of candidacidal and tumoricidal effectors from the spleen of cyclophosphamide-treated mice. *J. Immunol.* 136:837.
4. Decker, T., M. Baccarini, and M. L. Lohmann-Matthes. 1986. Liver-associated macrophage precursors as natural cytotoxic effectors against *Candida albicans* and Yac-1 cells. *Eur. J. Immunol.* 16:693.
5. Baccarini, M., A. F. Kiderlen, T. Decker, and M. L. Lohmann-Matthes. 1986. Functional heterogeneity of murine macrophage precursors from spleen and bone marrow. *Cell. Immunol.* 101:339.
6. Kirckpatrick, C. E., J. P. Farrell, J. F. Warner, and G. Dennert. 1985. Participation of NK cells in the recovery of mice from visceral Leishmaniasis. *Cell. Immunol.* 92:163.
7. Kiderlen, A. F., M. Baccarini, and M. L. Lohmann-Matthes. 1986. A fast and objective assay for cell-mediated intra- and extracellular killing of Leishmania promastigotes. *J. Immunol. Methods.* 86:199.
8. Herberman, R. B., editor. 1980. Natural Cell-mediated Immunity against Tumors. Academic Press, New York. 0–0.

9. Murphy, J. A., and O. McDaniel. 1982. *In vitro* reactivity of natural killer (NK) cells against *Cryptococcus neoformans*. *J. Immunol.* 128:1577.
10. Landolfo, S., M. G. Martinotti, P. Martinetto, and G. Forni. 1980. Natural cell-mediated cytotoxicity against *Trichomonas vaginalis* in the mouse. I. Tissue, strain, age distribution and some characteristics of effector cells. *J. Immunol.* 124:508.
11. Hatcher, F. M., and R. E. Kuhn. 1982. Destruction of *Trypanosoma cruzi* by natural killer cells. *Science (Wash. DC).* 218:295.
12. Kumagai, K., K. Itoh, R. Suzuki, S. Hinuma, and F. Saitoh. 1982. Studies on murine large granular lymphocytes. Identification as effector cells in NK and K. cytotoxicities. *J. Immunol.* 129:2788.
13. Luini, W., D. Boraschi, S. Alberti, A. Aleotti, and A. Tagliabue. 1981. Morphological characterization of a cell population responsible for natural killer activity. *Immunology.* 43:663.
14. Keller, R. 1978. Macrophage mediated natural cytotoxicity against various target cells. *In* Immunobiology of the Macrophage. D. Nelson, editor. Academic Press, New York. 487.
15. Domzig, W., and M. L. Lohmann-Matthes. 1979. Antibody-dependent cellular cytotoxicity against tumor cells. II. The promonocyte identified as effector cell. *Eur. J. Immunol.* 9:267.