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Comparison of the effect of various stimuli on the leishmaniacidal capacity of human monocytes *in vitro*

J. PASSWELL, R. SHOR, G. KEREN, G. MESSER & J. EL-ON *Division of Pediatrics, Chaim Sheba Medical Center and Department of Pathology, Ichilov Hospital and Sackler School of Medicine, Tel-Aviv University and Kuvin Center for Parasitic Diseases, Hebrew University-Hadassah Medical School, Jerusalem, Israel*

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SUMMARY

Leishmania organisms are obligate intracellular parasites of mammalian mononuclear phagocytes *in vivo*. In order to study the interactions of these parasites and mononuclear phagocytes, we have used a model of infection of *Leishmania major* in human monocytes *in vitro*. The presence of intracellular parasites did not alter the normal secretion of lysozyme or result in increased secretion of prostaglandin E_2 (PGE_2) or superoxide anion by the monocytes. Addition of concanavalin A (Con A), which binds to a specific membrane receptor, zymosan particles or endotoxin to infected monocyte monolayers, resulted in the expected increase in PGE_2 secretion. In addition, the production of superoxide by infected monocytes treated with phorbol myristate acetate was not different from control uninfected cultures. Despite this evidence of biochemical activation, neither endotoxin, zymosan nor Con A had any parasiticidal effect on the intracellular parasites. In contrast, Con A-induced lymphokines from human mononuclear cells resulted in an increased killing of the intracellular amastigotes. These studies have shown that the induction of leishmaniacidal capacity of human monocytes is dependent on the type of stimulus used to induce activation.

Keywords leishmaniasis lymphokines human monocytes

INTRODUCTION

Human monocyte derived macrophages may be infected *in vitro* with *Leishmania* organisms (Berman, Dwyer & Wyler, 1979). In this study we have examined whether infection of these macrophages with *Leishmania major* results in activation of these cells and whether the presence of the intracellular parasites interferes with the normal process of activation. In addition the effect of various exogenous stimuli including endotoxin, Concanavalin A (Con A) and Con A-induced lymphokines from mononuclear cells on the leishmaniacidal capacity of these monocyte derived macrophages was determined.

MATERIALS AND METHODS

Monocyte cultures. Monocyte monolayers were prepared as described previously (Passwell, Dayer & Merler, 1979). In brief, heparinized peripheral blood from non-immune volunteer donors

Correspondence: Dr J. Passwell, Division of Pediatrics, The Chaim Sheba Medical Center, Tel Hashomer 52621, Israel.

was first centrifuged at 400*g* for 5 min at 4°C and the plasma and buffy coat layer were removed. The white cell suspension was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifuged at 400*g* for 20 min at room temperature and the mononuclear cell layer was removed from the interface. These cells were washed three times in Hank's balanced salt solution (HBSS), resuspended in medium RPMI 1640 (Microbiological Associates, Bethesda, Maryland, USA) supplemented with 10% heat-inactivated millipore filtered fetal calf serum; penicillin (100 units/ml) and streptomycin (100 μg/ml) (GIBCO, Grand Island, New York, USA). The number of monocytes and lymphocytes in each preparation was determined by morphology of Giemsa stained cytocentrifuge preparations and positive staining for non-specific esterase. The cell concentration was adjusted to 5×10^5 monocytes/ml and aliquots of 0·2 ml (1×10^5 monocytes) were pipetted into wells of flat bottom tissue culture trays (8 mm diameter, Linbro Chemical Co. New Haven, Connecticut, USA). Adherence of monocytes was facilitated by gentle rocking at 37°C for 45 min, after which the non-adherent cells were removed by washing vigorously three times. These cell cultures were maintained in complete medium (0·2 ml/well) at 37°C in a humidified atmosphere of 5% CO_2. Leishmania major promastigotes were added to the monocyte monolayers in desired concentrations and left in culture overnight. The monolayers were washed the following morning and complete medium with the various test stimuli were added to the cell cultures. At the end of the experiments supernatants were collected for lysozyme and prostaglandin E_2 (PGE_2) assays. The cells were washed, fixed and stained with Giemsa stain within the tissue culture tray. The plastic well bottoms were carefully removed with a diamond tip cutter and mounted on slides. The percentage of infected cells and the numbers of parasites within each cell were determined with oil immersion light microscopy (× 1,000) after counting 200 cells per well.

Electronmicroscopy was performed on these monolayers after fixing and embedding the cells in epon (Passwell, Schneeberger & Merler, 1978).

Leishmania organisms. L. major LRC L-137 amastigotes (WHO new nomenclature, formerly known as *L. tropica major*) were isolated from cutaneous sores of an Israeli patient and were maintained *in vivo* in mice as previously described (Schnur, Zuckerman & Greenblatt, 1972). These amastigotes converted to promastigotes in parasite growth medium (NNN + 10% rabbit blood + RPMI 1640 + antibiotics) after culture for 48–72 h at room temperature. Aliquots were then kept at −20°C and thawed for subsequent use. Repeated passage of parasites resulted in a decreased rate of infectivity of the monocytes, therefore no more than three passages of the organisms from the infected animal were used in the cultures.

Monocyte and macrophage stimuli. Zymosan (Sigma Chemical Co., St Louis, Missouri, USA) was washed three times in HBSS and sterilized under an u.v. light source. Endotoxin (lipopolysacharide B from *Escherichia coli* B6; DIFCO Laboratories, Detroit, Michigan, USA) and Con A (Calbiochem., San Diego, California, USA) were prepared in HBSS in appropriate stock solutions. Phorbol myristate acetate (PMA) was obtained from Consolidated Midland Corporation (Brewster, New York) and was dissolved in dimethylsulphoxide (DMSO) to a concentration of 2 mM. This stock solution was kept frozen in small aliquots in the dark at −70°C. Lymphokines were prepared by stimulating mononuclear cell suspensions (1×10^7 cells/ml) with Con A (10 μg/ml) and the supernatants were harvested after 48 h of incubation. Control lymphokines were prepared by adding the Con A at the end of the 48 h incubation period.

Biochemical assays. Culture fluids were assayed for their content of PGE_2 by a radioimmunoassay (Levine, Cernosek-Gutierrez, & van Vunakis, 1971) (Miles Yeda, Rehovot, Israel). Lysozyme was assayed by determining the initial rate of lysis of a suspension of micrococcus lysodekticus (Sigma) in 1 M acetate buffer pH 6·2 at room temperature with a Gilford spectrophotometer fitted with a recorder. Egg white lysozyme (Sigma) was used as a standard (Gordon, Todd & Cohn, 1974). Superoxide (O^-_2) production was measured by the reduction of ferricytochrome C (Pick & Keisari, 1981). Monocyte monolayers were prepared in 16 mm diameter wells of culture trays and were covered with 0·25 ml of an 80 μM solution of ferricytochrome C (Type III, Sigma) in phenol red free BSS. Stimulants were added directly to the dishes and these were incubated for 90 min at 37°C in 95% air and 5% CO_2. Controls included monolayers without the added stimuli and wells with the cytochrome C solution and stimuli without cells. The optical density of pooled supernatants from four wells was measured at 550 nm.

RESULTS

The monocyte monolayers were readily infected with *L. major* promastigotes. The promastigotes converted to amastigotes within the phagocyte and no morphological evidence of activation such as increased spreading, prominent endoplasmic reticulum or ruffling of the plasma membrane was observed. Increasing the parasite: monocyte ratio resulted in an increased percentage of infected cells in the monolayer. Multiplication of the intracellular amastigotes was observed over a 5 day period of culture and was best assessed at a ratio of 1 : 1 parasite to monocytes. In this instance both the increase in the percentage of infected cells and the increase in the number of intracellular amastigotes were easily identified.

The presence of the intracellular parasites did not result in any alteration of lysozyme secretion, or increase in PGE_2 or superoxide production by the monocytes (Tables 1 & 2). Neither did their presence alter the expected increase in PGE_2 secretion following addition of endotoxin, zymosan or Con A (Table 1). In addition no difference in the amount of superoxide produced following stimulation with PMA was detected in the infected monocytes compared to the control cultures (Table 2).

The addition of either endotoxin, Con A or Zymosan particles did not promote killing of the amastigotes within the monocytes. However, morphological evidence of activation, such as increased spreading in the case of endotoxin and an increase in endocytic vacuoles in Con A treated monolayers was observed in both infected and control cultures.

Table 1. Effect of infecting human monocytes with *L. major* on their secretory products with and without stimulation *in vitro*

	Infected with *L. Major*	PGE_2 (ng/ml/culture)	Lysozyme (μg/ml/culture)
Control	−	2·2	4·9
	+	3	3·9
+ Con A (50 μg/ml)	−	60	2·8
	+	25	2·8
+Endotoxin (20 μg/ml)	−	3·6	3·5
	+	5·6	3·9
+Zymosan (1×10^7 particles/culture)	−	15	4·5
	+	11	3·5

Each result is the average of at least duplicate cultures from a single representative experiment. Similar results were obtained in two separate experiments.

Table 2. Effect of infecting monocytes with *L. major* on ability to produce superoxide following *in vitro* activation

	Infected with Leishmania	nM O^-_2/culture
Control	−	0
	+	0
+PMA (20 nM)	−	65·6 + 4·1
	+	70·7 ± 6·3

Each result is the mean ± s.e. of quadruplicate cultures from two separate experiments.

In contrast Con A lymphokine treated cultures resulted in a marked increase of destruction of the amastigotes. This was particularly apparent when the percentage of infected cells was observed, however, a decrease in the number of parasites per infected cell was also noted (Fig. 1). Addition of the lymphokine did not alter the phagocytosis of the parasite and by the second day of culture a decrease in the number of parasites per cell was noted (Table 3). Methyl mannoside (25 mM) did not inhibit the effect of the lymphokine. This leishmaniacidal effect could also be elicited by prior treatment of the monolayer with Con A lymphokine for 3 days and subsequent removal of the lymphokine resulted in a similar increase in destruction of the parasite (results not shown).

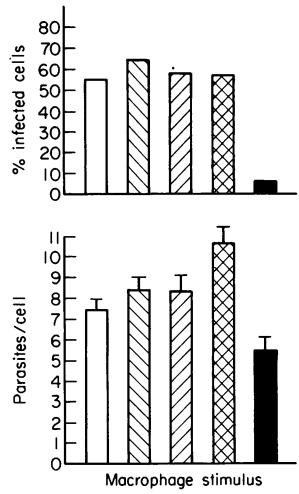

Fig. 1. Comparison of various stimuli on the leishmaniacidal capacity of human monocytes in culture. □ = control; ■ = Con A (50 μg/ml); ▨ = endotoxin (20 μg/ml); ■ = zymosan particles (1×10^7/ml); ■ = lymphokine (1:1, vol.:vol). The data presented in this figure were derived from quadruplicate cultures of a single experiment. The number of parasites in 200 cells was estimated in each culture. Similar results were obtained in four additional experiments.

Table 3. Kinetics of the effect of addition of the Con A lymphokine on the leishmaniacidal capacity of human monocytes *in vitro*

Experimental condition	Parasites/cell	% Infected cells
Control (24 h)	6·5±0·5	27·3±1·5
Lymphokine (24 h)	6·5±0·5	18·8±2·2
Control (48 h)	11·2±1	37·7±2·2
Lymphokine (48 h)	0	0
Lymphokine (48 h) & methyl mannoside (25 mM)	0	0

Results are the mean ± s.e. of quintuplicate cultures from a representative experiment. Similar results were obtained in three separate experiments.

DISCUSSION

We have further defined an *in vitro* system to study the effect of exogenous stimulation of human monocytes on their leishmaniacidal capacity (Berman *et al.*, 1979). The degree of infectivity of the monolayer was shown to be dependent on the parasite:monocyte ratio and intracellular multiplication of the amastigotes was observed in culture.

We have previously shown that various exogenous stimuli including zymosan, endotoxin, Con A and lymphokines induce the secretion of PGE_2 and other biochemical markers of activation. (Passwell *et al.*, 1979, 1980a.) In addition, we and others have shown that the qualitative and quantitative secretory response of activated human monocytes (Passwell *et al.*, 1980b) and also the production of superoxide and hydrogen peroxide is dependent on the stimulus used to elicit activation (Pick & Keisari, 1981). In this study this concept has been extended; while Con A, endotoxin and zymosan resulted in biochemical activation as evidenced by increased PGE_2 production, no increase in leishmaniacidal activity of these monocytes was noted. In contrast Con A-induced lymphokine promoted marked destruction of intracellular amastigotes. Clearly, then the leishmaniacidal capacity of human monocytes is dependent on the activation induced by lymphokine and is not related to the degree of biochemical activation elicited by other agents.

Mitogen- or antigen-induced lymphokines have been shown to restrict growth of intracellular micro-organisms and parasites and also result in several alterations of the biochemical function of the macrophage (Lazdins *et al.*, 1978; Murray & Cohn, 1980). Previous studies in mouse *in vitro* systems have shown that Con A-induced lymphokines is a potent inducer of macrophage leishmaniacidal capacity of several different Leishmania strains. (Buchmuller & Mauel, 1979; Murray, Masur & Keithly, 1982). Our studies corroborate those in animal macrophages, where intracellular replication of amastigotes was also inhibited by either prior pulse treatment with the lymphokine or treatment after infection (Nacy *et al.*, 1981).

In diffuse cutaneous leishmaniasis an adherent suppressor cell population has been shown to be responsible for the specific anergy to leishmania antigen. Indomethacin reversed this lack of response (Petersen *et al.*, 1982). We did not find any evidence of monocyte activation including an increase in PGE_2 production in monocytes infected with *L. major*, which suggests that this was not the usual cause for the chronic cutaneous inflammation. Despite considerable evidence that lymphokines enhance the leishmaniacidal capacity of macrophages *in vitro*, it is unclear why the natural history of the cutaneous lesion in normal individuals is prolonged for several months. It has not been established whether humans elaborate lymphokines *in vivo* following Leishmania infection. However there is now increasing evidence that inappropriate sensitisation of suppressor T cells occurs consequent on intracellular monocyte infection with atypical mycobacteria (Watson & Collins, 1981) or leprosy (van Voorhis *et al.*, 1982) and also in experimental visceral leishmaniasis (Howard, Hale & Liew, 1981). The role of these T cells or their products in the pathogenesis of Leishmaniasis could be further studied in this *in vitro* model and warrant further investigation in humans with the disease.

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