Int. J. Exp. Path. (1994), **75**, 277–284

Infection of human monocytes by *Leishmania* results in a defective oxidative burst

J.H. PASSWELL*, R. SHOR*, J. SMOLEN* AND C.L. JAFFE†

**Samuel Jared Kushnick Pediatric Immunology Laboratory, Sheba Medical Center, Sackler School of Medicine and †Department of Biophysics, MacArthur Center for Tropical Diseases, Weizmann Institute, Israel*

Received for publication 16 September 1993
Accepted for publication 18 April 1994

Summary. The effect of infection by prototypes from the three major species of *Leishmania* on the oxidative burst of human mononuclear phagocytes in culture was examined. The presence of intracellular parasites of the three species, *L.major*, *L.donovani* and *L.mexicana* decreased hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) production. This was particularly apparent when infected cells were compared to control monocytes following treatment with IFN-γ. Nitroblue tetrazolium (NBT) reduction by monocytes was also decreased in infected cells. This morphological analysis of infected monolayers clearly showed that infected monocytes were incapable of reducing the dye as compared to uninfected cells. Decrease in NBT positive cells and production of H_2O_2 and O_2^- was related to the degree of infection of the monocyte monolayers. These results suggest that the presence of intracellular *Leishmania* amastigotes in mononuclear phagocytes decreases the oxidative burst and may contribute to parasite survival. Failure of phagocytes from patients with chronic granulomatous disease to kill these intracellular parasites also emphasized the importance of the oxidative burst for this function. Nevertheless, the consistent increase in leishmaniacidal effect attained after IFN-γ treatment of the monocyte monolayers indicates that other non-oxidative mechanisms induced by this cytokine are also important in the killing of these intracellular parasites.

Keywords: *Leishmania major*, *Leishmania donovani*, *Leishmania mexicana amazonensis*, superoxide, hydrogen peroxide

The diseases caused by all species of the *Leishmania* genus are dependent on the fact that these parasites multiply and survive in the microbicidal environment of the mononuclear phagocyte (Pearson *et al.* 1983). This survival has to be seen in the context that both promastigotes and amastigotes of *Leishmania* are destroyed *in vitro* by the metabolites of the oxidative burst, hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) (Haidaris & Bonaventura 1982; Murray 1981), with H_2O_2 being more effective than O_2^-. Several *in vitro* studies with macrophages and human monocytes have shown that killing of *Leishmania* amastigotes, (similar to other intracellular pathogens), occurs when phagocytes are stimulated with either mitogen or antigen-induced

Correspondence: Dr J.H. Passwell, Samuel Jared Kushnick Pediatric, Immunology Laboratory, Sheba Medical Center, Sackler School of Medicine, Tel Hashomer, Israel.

lymphokines (Buchmuller & Mauel 1979; Murray *et al.* 1982; Nacy *et al.* 1981; Passwell *et al.* 1984; 1986a,b). The most active component in these lymphokine preparations has been shown to be interferon-gamma (IFN-γ) although other molecular species have been implicated in the killing of the intracellular parasites (Passwell *et al.* 1986a; Murray *et al.* 1983; Nathan *et al.* 1983). These T-cell lymphokines and IFN-γ stimulate macrophages to generate the oxygen metabolites, O_2^- and H_2O_2, which are toxic for intra and extra-cellular targets (Passwell *et al.* 1986a; Nathan *et al.* 1983). However, increasing attention as to the importance of non-oxidative pathways, particularly nitric oxide, have been reported in studies using murine macrophages (Green *et al.* 1990; Murray *et al.* 1989). In this study, we have examined and compared the effects of intracellular *Leishmania* amastigotes from three major species of this genus in the same donor on the generation of the oxidative burst by human monocyte cultures. The relative importance of the oxidative burst in the killing of these amastigotes was examined by determining the leishmaniacidal activity of monocytes derived from patients with chronic granulomatous disease. In addition, in order to examine the innate leishmaniacidal capacity of monocytes treated with IFN-γ from a particular donor, we have compared their ability to kill the intracellular amastigotes of each of the three major species of *Leishmania* parasites.

Materials and methods

Monocyte cultures

Monocyte monolayers were prepared as described. In brief, human donor heparinized blood was centrifuged at 400 ***g*** for 10 minutes. The plasma and buffy coat layer was removed. The white cell suspension was layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden), centrifuged at 400 ***g*** for 20 minutes at room temperature and the mononuclear cell layer removed from the interface. These cells were washed three times in Hanks balanced salt solution and resuspended in RPMI 1640 medium (Microbiological Associates, Bethesda, MD) supplemented with heat inactivated Millipore filtered fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, NY). The cell concentration was adjusted to 5×10^5 monocytes/ml and aliquots of 1×10^5 monocytes were pipetted into wells of flat-bottomed tissue culture trays (16-mm diameter, Linbro Chemical Company, New Haven, CT). Adherence of monocytes was facilitated by gentle rocking at 37°C for 45 minutes, after which time the non-adherent cells were removed by washing vigorously three times. These cell cultures were maintained in complete medium (0.5 ml/well) at 37°C in a humidified atmosphere of 5% CO_2.

Monocytes derived from three patients with chronic granulomatous disease proven by clinical findings and their inability to generate either H_2O_2 or O_2^- *in vitro* were used to examine directly the effect of innate deficiency of the oxidative burst on leishmaniacidal capacity of the monocyte. The mode of inheritance in the three patients was autosomal recessive; two were brother and sister and in the third (a male), cytochrome B activity of leucocyte membrane was shown to be normal (results not shown).

Parasites and infection of the monocyte culture

Leishmania major (LRC L-137) was isolated from cutaneous sores of an Israeli patient as described (Schnur *et al.* 1972). *L donovani* (Khartoum) was obtained from Dr Keithly, Cornell Medical School, NY. and *L. mexicana amazonensis* (LTB0016) from Dr P Marsden, Universidade de Brasilia, Brazil. Virulent stocks of all species were isolated from animals. Aliquots were frozen at –70°C and thawed for subsequent use. Repeated passage of the parasites resulted in a decreased rate of infectivity; therefore, the parasites used in these experiments were kept in culture for no more than 3 passages after thawing. Promastigotes, that were used to infect monocyte monolayer cultures, were added when they were in a logarithmic phase of growth. Recombinant IFN-γ was obtained from Inter Yeda Laboratories, Rehovot, Israel. Phorbol myristate acetate was obtained from Consolidated Midlands Corporation, Brooster, New York, and dissolved in dimethylsulphoxide to a concentration of 2 mM. The stock solution was aliquoted and kept frozen in the dark at –70°C. Monocyte monolayers were infected with 1–2 parasites per cell for 2 hours, washed, and fresh medium added to the cultures. At the end of the experiments the cells were washed, fixed and stained with Giemsa stain. The cover slips were removed from the wells and mounted on glass slides. The percentage of infected cells was determined with oil immersion light microscopy (× 1000) after counting 200 cells per well.

H_2O_2 assay

A microassay method was used as described (Pick & Keisari 1980). Monolayers cultured in 96-well, flat-bottomed tissue trays were incubated in the presence of a phenol red solution (100 μl) which contained 140 mM NaCl, 10 mM potassium phosphate buffer (pH 7.0), 5.5 mM

dextrose, 0.56 mM phenol red (0.2 g/l), and 19 U/ml horse-radish peroxidase. The cultures were incubated for 60 minutes and the reaction stopped by the addition of 1 M NaOH (10 μl per well). The optical density at 600 nm of each sample was determined by a micro-ELISA reader (Model MR 580 Dynatech Laboratories, Alexandria, VA). Standard curves were established using H_2O_2 solutions of known molarity. Protein concentration of the monocyte monolayer was determined in replicate wells by the Lowry method.

Superoxide assay

Superoxide anion (O_2^-) production was measured by the reduction of ferricytochrome C (Type III, Sigma) (Pick & Mizel 1981). Washed cells were incubated in a (80 μM) solution of ferricytochrome C in phenol red free HBSS. Stimulants were added to the wells and cultures were incubated at 37°C in 95% air and 5% CO_2. Controls included parallel wells containing superoxide dismutase which were used as the blank reference, and control cultures without added stimuli. At the end of the incubation period plates were read in a microELISA reader. The reduction of ferricytochrome C was measured at 550 nm using a reference filter at 492 nm. Results were expressed as the Δ OD550/μg cell protein between cultures incubated in the presence or absence of superoxide dismutase according to the formula described by Pick and Mizel (1981).

Nitroblue tetrazolium (NBT) reduction test

Monocyte monolayers prepared on cover slips in 16-mm diameter wells were used for a semi-quantitative analysis of NBT reduction. After incubating the infected monocytes in the presence of IFN-γ (100 units/ml) or PMA (20 nM) the cells were washed with a balanced salt solution and 100 μl of a NBT solution (1 mg/ml in HBSS) containing the stimulus was added to the cell cultures. The cells were reincubated at 37°C and after 60 minutes the cover slips were removed and stained with Giemsa stain. The number of cells infected with parasites was counted using oil immersion microscopy (×1000) and the number of NBT positive cells recorded. This method permits a direct measurement of the oxidative burst in those cells infected with parasites.

Results

Monocyte cultures were readily infected by promastigotes of the three *Leishmania* species used (*L. major, L. m. amazonensis* or *L. donovani*). Using a ratio of 1:2 parasites/cell, approximately 60% of monocytes were infected with one of the three species. We previously reported that maximal effects of the rIFN-γ were attained after 72 hours incubation, and this was chosen as the time point to study the effects of IFN-γ on the three species. In any monocyte monolayer culture we consistently noted that some of the cells were apparently resistant to infection, while other monocytes contained phagocytosed parasites. After incubation of the monolayer in the presence of rIFN-γ significant killing of the intracellular amastigotes of all three species was noted. The degree of leishmaniacidal effect attained was dependent on the concentration of IFN-γ used and on the donor's monocytes rather than the species of *Leishmania*, although *L. donovani* appeared to be more resistant at low IFN-γ concentrations. Increasing the dose of IFN-γ above 100 units/ml did not markedly increase the killing of the amastigotes in most cases. In addition, we consistently noted that although enhanced killing by IFN-γ was observed with all the species, some cells were still infected with *Leishmania* after the three-day incubation period (Table 1).

The monocytes from each of the three patients with chronic granulomatous disease phagocytosed the parasites normally; however, they failed to kill intracellular

Table 1. Effect of IFN-γ on the leishmaniacidal capacity of human monocytes for three species of *Leishmania*

	Leishmaniacidal capacity (%)*		
	L. major	*L. donovani*	*L. mexicana*
Experiment 1			
IFN-γ Addition			
100 U/ml	81.2 ± 4.0	59.0 ± 1.8	79.7 ± 2.2
250 U/ml	86.8 ± 2.7	76.9 ± 7.3	85.9 ± 2.2
500 U/ml	85.9 ± 1.3	84.6 ± 1.8	90.7 ± 4.4
Experiment 2			
100 U/ml	38.4 ± 1.3	34.9 ± 2.1	34.9 ± 12.1
250 U/ml	63.4 ± 1.3	39.8 ± 10.2	28.0 ± 3.8
500 U/ml	73.2 ± 5.1	45.8 ± 15.3	44.0 ± 3.8

* Leishmaniacidal capacity (%) was calculated by the formula:

$$1 - \frac{\text{infected cells following IFN-gamma treatment (\%)}}{\text{infected control cells (\%)}} \times 100$$

Results are the mean ± s.d. ($n = 4$) of two representative experiments, where monocyte monolayers derived from two separate donors were infected with each of the three *Leishmania* species. Following infection of the monocyte layers with 1–2 promastigotes/monocyte, the monolayers were treated with a designated concentration of rIFN-γ for 72 hours. At the end of this incubation period, the percentage of infected monocytes was determined in each monolayer and the percentage leishmaniacidal capacity calculated. Similar results were obtained in three additional experiments.

Table 2. Effect of monocytes derived from patients with chronic granulomatous disease on the leishmaniacidal capacity for *L. major*

(a)	Control*	IFN-γ†
Normal	55.3 ± 6.8	29.6 ± 4.5
Patient MA	68.0 ± 5.0	76.1 ± 2.8
Patient MC	41.1 ± 7.1	49.8 ± 7.2
Patient CY	26.4 ± 6.9	32.5 ± 4.5

(b)	Control	IFN-γ	ConA LK	M-CSF
Normal	31.0 ± 3.3	10.5 ± 2.42	8.0 ± 3.8	15.1 ± 6.9
Patient CY	30.2 ± 2.1	25.0 ± 4.6	34.0 ± 3.0	36.5 ± 3.7

Results are expressed as the percentage infected monocytes observed after a 72-hour culture period without any treatment or following treatment with cytokine. Each results is the mean ± s.d. ($n - 4$).

Control * no addition; †IFN-γ used 250 U/ml; Con A LK, 1/10 dilution of extracellular medium of Con A stimulated mononuclears; MCSF, 250 U/ml.

L. major organisms after treatment with IFN-γ (Table 2a). In addition, neither Con A induced lymphokine nor M-CSF had an effect on CGD monocytes, but were effective in normal monocytes (Table 2b).

Effect of Leishmania *infection on monocyte H_2O_2 production*

IFN-γ resulted in a dose dependent increase in the H_2O_2 produced by monocyte monolayers in culture (Table 3). Similar effects were also observed on monocyte derived macrophages that had been in culture for periods of up to 8 days. Incubation for 72 hours with IFN-γ resulted in an optimal increase in H_2O_2 production (Passwell *et al.* 1986a). Monoclonal antibody to IFN-γ completely abrogated the H_2O_2 response to this cytokine (Passwell *et al.* 1986b and results not shown). PMA resulted in increased H_2O_2 production that was usually greater than for IFN-γ in each donor. Addition of PMA and IFN-γ was additive in these cultures (results not shown). The presence of intracellular amastigotes from *L. major* resulted in a decreased generation of H_2O_2 from monocytes in culture. This was more evident in infected cultures that had been treated with IFN-γ (Table 3). Viable parasites were required to show this effect as promastigotes that had been killed by two cycles of freezing and thawing and were phagocytosed by monocytes did not alter the amount of oxidative burst products generated. The difference in H_2O_2 produced between the control and IFN-γ treated cultures when using dead parasites was less than 5%. These effects were also seen in monocytes and monocyte derived macrophages and in monocytes treated with PMA alone. Similar findings were also observed using either *L.m. amazonensis* or *L. donovani* as the infecting parasite. The amounts of reduction observed with each species of parasite for any particular donor were comparable and no marked differences were observed between the different species of parasites (Table 4).

Table 3. Effect of IFN-γ on H_2O_2 production by monocyte monolayers infected with *L. major* promastigotes

Addition	Control	Infected	Killed parasites
	(nmol $H_2O_2/\mu g$ protein/60 min)		
	46.8 ± 10.8	29.4 ± 1.3	48.2 ± 4.5
IFN-γ (50 U/ml)	159.0 ± 14.7	17.4 ± 5.3*	166.9 ± 15.0
IFN-γ (100 U/ml)	192.4 ± 17.4	34.7 ± 1.3*	186.2 ± 16.2
IFN-γ (250 U/ml)	269.9 ± 8.0	85.5 ± 14.7*	252.8 ± 18.0

Monocytes were in culture for 72 hours in the presence of IFN-γ. H_2O_2 production was ascertained after a 60-minute incubation period. A similar decrease of H_2O_2 was obtained in both monocyte and monocyte derived macrophages from four different experiments. PMA (20 nM) added to 72-hour cultures just prior to incubation with the phenol red solution resulted in an increase of 370.1 ± 5.3 nmol $H_2O_2/\mu g$ protein production. Results are the mean ± s.e.m. ($n = 8$) of a representative experiment. Significant differences between control and infected cultures are indicated; *$P < 0.005$ (Student's *t*-test). Non-viable parasites were prepared by two cycles of freezing and thawing.

Effect of Leishmania *parasites on superoxide (O_2^-) production by monocytes*

A decrease in O_2^- production by monocyte monolayers infected with *Leishmania* was also observed (Figure 1). This effect was less obvious when PMA was used as a stimulus for O_2^- production. The differences were particularly marked following treatment of the monolayers with IFN-γ. The degree of reduction in O_2^- generated was related to the amount of infection of the monocyte monolayers (Figure 1).

Effect of intracellular Leishmania *parasites on NBT reduction by monocytes in culture*

The number of monocytes *in vitro* containing the characteristic blue staining of reduced NBT one hour after attachment ranged between 13 and 30%. Invariably, the number of NBT positive cells increased throughout culture *in vitro*, under basal conditions, increasing twofold after 48 hours in culture. Treatment of monocyte monolayers with IFN-γ resulted in a further twofold increase in NBT positive cells. This was apparent after even one day in culture. Treatment with PMA resulted in a consistent marked increase in NBT positive cells, usually four times higher than the control positive

Table 4. Comparison of the effect of infection by three species of *Leishmania* on H_2O_2 production by monocytes/ macrophages

	Addition	Percentage H_2O_2*		
		L. major	*L. donovani*	*L. mexicana*
Monocytes (Day 3)	–	18.0 ± 7.1	23.6 ± 10.5	13.7 ± 6.3
	PMA	13.2 ± 5.1	50.8 ± 6.2	77.8 ± 4.1
	IFN-γ	37.3 ± 2.8	37.3 ± 15.3	58.2 ± 4.5
Macrophages (Day 9)	–	29.5 ± 13.7	47.1 ± 25.1	21.2 ± 18.9
	PMA	77.6 ± 26.6	70.8 ± 23.4	60.5 ± 20.9
	IFN-γ	38.7 ± 16.2	49.6 ± 16.8	5.8 ± 15.0

Monocyte monolayers (3 days) or monocyte derived macrophage monolayers (9 days) from a single donor were infected with promastigotes from each of the three *Leishmania* species and then treated with rIFN-γ (100 U/ml) for 72 hours or PMA (20 nM) prior to determination of the amount of H_2O_2 in the subsequent 60 minutes.

*Results are expressed as the percentage of H_2O_2/μg protein produced by infected cells compared to that produced by non-infected monocyte monolayers; mean ± s.d. ($n = 8$ for each variable). Similar results were obtained in four separate experiments.

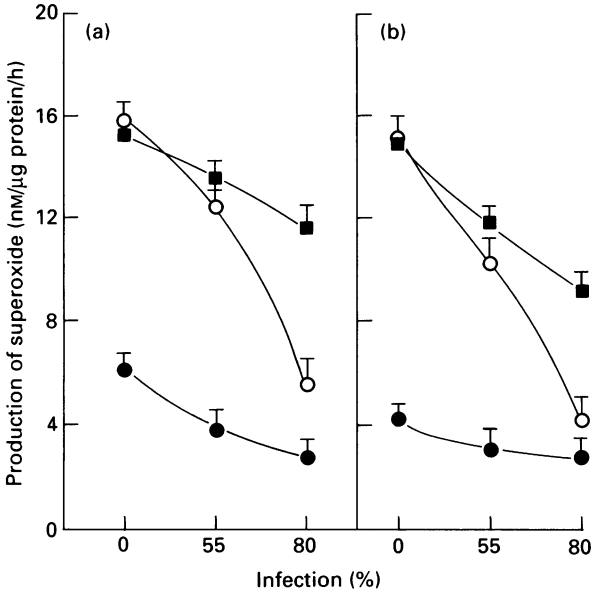

Figure 1. Effect of leishmanial infection on the production of O_2^- by human monocytes. Human monocyte monolayers infected with a, *L. major* or b, *L. donovani* at two different parasite/ monocyte ratios were treated with IFN-γ (250 μ/ml) for 48 hours or PMA (20 nM) for one hour and the amount of superoxide (O_2^-) generated from the monocyte monolayers was measured as described. The percentage of infected monocytes was determined morphologically in replicate plates at the end of the incubation period. Protein content of the monolayer was measured by the Lowry method in replicate wells. Results presented are for a representative experiment (mean ± s.d., $n = 8$ of each variable). Similar results were obtained in two additional experiments. ●, Control untreated monocytes; ○, IFN-γ treated monocytes; ■, PMA treated monocytes.

cells. The presence of *L. major* intracellularly resulted in a decrease in number of positive cells recorded (Figure 2). The reduction in number of NBT positive cells was a direct function of the percentage infected monocytes and was seen at all time points including one hour following infection. Reduction in NBT positive cells was more pronounced with the IFN-γ treated cells. The most striking finding was the consistent absence of dye granules in cells infected with the parasites, while none of the NBT positive cells contained parasites within the cytoplasm. Dye reduction was seen under both basal conditions and when the monocytes were treated with either recombinant IFN-γ or PMA. Similar results were also recorded when *L. donovani* or *L. m. amazonensis* was used as the infecting parasite and the decreased NBT positive cells were similar in each donor rather than related to the species of infected parasite (results not shown).

Discussion

Infection of human monocytes/macrophages by each of the three *Leishmania* species caused a decrease in the oxidative burst as measured by H_2O_2 production, O_2^- production, or reduction of NBT dye. This was consistently found when infected monocytes and control monocytes were compared following IFN-γ treatment. In earlier studies, an increase in the oxidative burst and killing of *Leishmania* following phagocytosis by murine macrophages was reported (Murray 1981). We have not been able to demonstrate this with human monocytes, even using short incubation periods following phago-

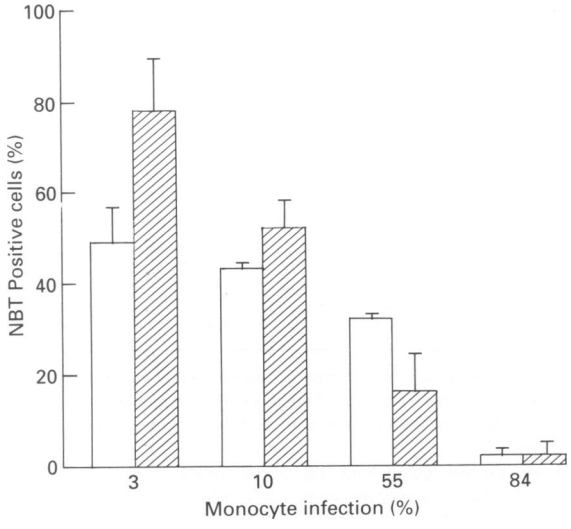

Figure 2. Effect of *L. major* infection on the reduction of NBT by monocyte monolayers. Monocyte monolayers treated with varying amounts of *L. major* parasites were examined after 48 hours in culture for their capacity to reduce NBT dye during a one-hour incubation period. The degree of infection and NBT reduction were quantitated morphologically. Two hundred cells were counted on each cover slip. Results are expressed as the number of NBT positive stained cells. Data presented are from a representative experiment (mean ± s.d., $n = 4$ for each variable). Similar results were obtained in four other experiments. ▨, IFN; □, control.

cytosis of the parasite (Passwell *et al.* 1986a,b). The amount of down regulation of the oxidative burst was clearly dependent on the degree of infection of the monocyte monolayers. The decreased oxidative burst of each infected cell, as observed by the NBT reduction, the direct correlation of increasing level of infection with decreased oxygen metabolites and the necessity for viable promastigotes provide convincing evidence that the intracellular parasite is responsible for the reduced oxidative burst. Findings for monocytes infected with each of the *Leishmania* species were similar, indicating that these *Leishmania* prototypes are capable of decreasing the oxidative burst. A similar downregulation of the oxidative burst in mouse macrophages, treated with lymphokine and infected by *Leishmania enrietti*, has been reported (Buchmuller-Rouillier & Mauel 1987).

The failure of phagocytes from patients with chronic granulomatous disease to kill intracellular amastigotes even after treatment with IFN-γ indicates the important role of the oxidative burst for this function. These results apparently contradict those reported earlier by Murray and Cartelli (1983) who, on the basis of the leishmaniacidal effect following treatment with lymphokine in monocytes derived from patients with chronic granulomatous disease, first postulated that non-oxidative mechanisms were also important in intracellular killing of the parasite. These differences may be explained by the fact that IFN-γ and other molecular species are also effective in inducing non-oxidative pathways. Indeed, although the measured respiratory burst of the phagocyte was reduced in infected monolayers, IFN-γ treatment resulted in a consistent increase in killing of the intracellular parasites (Passwell *et al.* 1986a).

Clearly, the cumulative leishmaniacidal effect of a 72-hour treatment cannot be compared and correlated with the results of the accumulation of oxidative burst products after a one-hour incubation period. Recent attention has been paid to the role of tryptophan degradation and/or an L-arginine dependent mechanism which generates inorganic nitrogen oxide effector molecules. These non-oxidative mechanisms are effective in killing intracellular organisms including *Leishmania* and are induced by IFN-γ independently of the oxidative burst (Green *et al.* 1990; Murray *et al.* 1989). Tumour necrosis factor has been implicated as the major cytokine responsible for induction of this function. Interestingly, IFN-γ induces monocyte/macrophage tumour necrosis factor production, which may be part of the mechanism for its action or these two cytokines may have a synergistic effect (Ding *et al* 1988; Liew *et al.* 1990). Recently a role for membrane bound TNF of antigen specific CD4 lymphocytes, independent of lymphokine secretion, has been invoked by way of direct cell contact in macrophage anti-leishmanial effects (Sypek & Wyler 1991). We were unable to demonstrate an increase in nitric oxide in IFN-γ treated human monocytes (results not shown). Similar findings have now been reported and while this non-oxidative pathway has been shown to be critical for killing of intracellular pathogens by murine macrophages, this was not the case with human phagocytes (Murray & Teitelbaum 1992). Nevertheless, our results suggest that part of the mechanism for the persistence of *Leishmania* within the mononuclear phagocytes and the chronicity of these parasitic diseases is due to the fact that the intracellular parasites scavenge metabolites of the oxidative burst or directly inhibit the normal oxidative burst of these cells (Eilam *et al.* 1985; Meshnick & Eaton 1981; Remaley *et al.* 1985).

Several findings regarding the effect of IFN-γ on the killing of intracellular *Leishmania* should be emphasized. First, as in previous studies with human and animal systems *in vitro*, and with various forms of the organism, some of the phagocytes in culture did not contain amastigotes and appeared resistant to infection.

Massive infection could be achieved only when very large numbers of parasites were used to infect the monocytes. Second, infected monocytes generally contained more than one amastigote. Third, IFN-γ consistently increased the leishmaniacidal effect of monocytes in culture, but usually did not completely eradicate all of the intracellular amastigotes. We and others have shown that mitogen-induced lymphokine depleted of IFN-γ also exhibits leishmaniacidal effects, indicating that molecular species other than IFN-γ also have a leishmaniacidal capacity (Passwell *et al.* 1986b). Indeed, recent studies with other recombinant cytokines of T-lymphocyte origin, particularly GM-CSF, have demonstrated leishmaniacidal effects *in vitro* (Ho *et al.* 1989). Combined treatments with IFN-γ and purified or recombinant interleukins have shown that the resistance of murine macrophages to infection with *L. major* was enhanced by IL-2 and GM-CSF (Belosevic *et al.* 1988). Various combinations of known interleukins and as yet other identified molecular species may show additive effects with IFN-γ, and thus explain the incomplete killing of amastigotes with IFN-γ alone following infection of the phagocyte.

The varied forms of leishmaniasis are clearly dependent on the species causing the infection, but variations in host susceptibility also account for the rarer forms of disseminated skin disease. It is still not clear what percentage of individuals exposed to the parasite establish immunity and do not develop disease. All the peripheral blood monocyte monolayers that we have tested from normal donors showed increased leishmaniacidal activity and oxidative burst following IFN-γ treatment, although minor innate donor differences exist. This suggests that these defence mechanisms are unlikely to be implicated as the host factors which determine whether infecting parasites cause a subclinical infection, a benign skin disorder or a disseminated visceral form of the disease. Other immunological mechanisms, such as the failure of appropriate antigen presentation due to downregulation of Class II products and/or abnormal T-cell effector mechanisms, have been implicated in this regard (Belosevic *et al.* 1988; Heinzel *et al.* 1989; Huszer *et al.* 1987; Modlin *et al.* 1985; Reiner *et al.* 1987; Jaffe *et al.* 1994).

Acknowledgements

This investigation received financial support from the United Nations Developmental Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Disease and John D. and Catherine T. MacArthur Foundation.

References

Belosevic M., Davis L.E., Meltzer M.S. & Nacy C.A. (1988) Regulation of activated macrophage antimicrobial activities. Identification of lymphocytes that cooperate with IFN-gamma for induction of resistance to infection. *J. Immunol.* **141,** 890.

Buchmuller Y. & Mauel J. (1979) Studies on the mechanisms of macrophage activation. II Parasite destruction in macrophages activated by supernatants from Concanavalin A-stimulated lymphocytes. *J. Exp. Med.* **150,** 359.

Buchmuller-Rouillier Y. & Mauel J. (1987) Leishmania Spp impair macrophage respiratory burst. *Infect. Immun.* **55,** 587.

Ding A.H., Nathan C.F. & Stuehr D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immun.* **141,** 2407.

Eilam Y., El-On J. & Spira D.T. (1985) Leishmania major: excreted factor, calcium ions and the survival of amastigotes. *Exp. Parasitol.* **59,** 161.

Green S.J., Meltzer M.S., Hibbs Jr. J.B. & Nacy C.A. (1990) Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-Arginine-dependent killing mechanism. *J. Immunol.* **144,** 278.

Haidaris C.G. & Bonaventure P.E. (1982) A role for oxygen-dependent mechanisms in killing of *Leishmania donovani* tissue forms by activated macrophages. *J. Immunol.* **129,** 850.

Heinzel F.R., Sadick M.D., Holiday B.J., Koffman R.L. & Locksley R.M. (1989) Reciprocal expression of Interferon-γ or Interleukin-4 during the resolution or progression of murine leishmaniasis. Evidence of expansion of distinct helper T cell subsets. *J. Exp. Med.* **169,** 59.

Ho J.L., Reid S. & Wick E.W. (1989) Effect of macrophage colony stimulating factor on intra macrophage survival of *Leishmania mexicana amazonensis. Clin. Res.* **37,** 431A.

Huszer M., Shor R., Trau H., Gazit E. & Passwell J.H. (1987) The T cell phenotypes in the lesion of patients with cutaneous leishmaniasis. *Clin. Exp. Dermatol.* **12,** 103.

Jaffe C.L., Shor R., Trau H., Apte R.N., Gazit E. & Passwell J.H. (1994) Presentation of Leishmania antigens on human monocytes identified with Leishmania specific monoclonal antibodies. (Submitted for publication).

Liew F.Y., Li Y. & Millott S. (1990) Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. *J. Immun.* **145,** 4306.

Meshnick S.R. & Eaton J.W. (1981) Leishmanial superoxide dismutase: a possible target for chemotherapy. *Biochem. Biophys. Res. Commun.* **102,** 970.

Modlin R.L., Tapia F.J., Bloom B.R., Gallinto M.E., Costes M., Rondon A.J., Rea T.H. & Convit J. (1985) In situ characterization of the cellular immune response in American cutaneous leishmaniasis. *Clin. Exp. Immunol.* **60,** 241.

Murray H.W. (1981a) Susceptibility of Leishmania to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* **153,** 1302.

Murray H.W. (1981b) Interaction of Leishmania with a macrophage cell line. Correlation between intracellular killing and generation of oxygen intermediates. *J. Exp. Med.* **153,** 1690.

Murray H.W. & Cartelli D.M. (1983) Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and independent leishmaniacidal activity. *J. Clin. Inv.* **72,** 32.

Murray H.W., Masur W.H. & Keithly J.S. (1982) Cell mediated immune response in experimental visceral leishmaniasis. I Correlation between resistance to *Leishmania donovani* and lymphocyte-generating capacity. *J. Immunol.* **129,** 344.

Murray H.W., Rubin R.Y. & Rothermal C.D. (1983) Killing of intracellular *Leishmania donovani* by lympokine-stimulated human mononuclear phagocytes. Evidence that interferon-γ is the activating lymphokine. *J. Clin. Inv.* **72,** 1506.

Murray H.W., Szuro-Sudol A., Wellner D., Oca M.J., Granger A.M., Libby D.M., Rothermel C.D. & Rubin B.Y. (1989) Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophates. *Infect. Immun.* **57,** 845.

Murray H.W. & Teitelbaum R.F. (1992) L. Arginine dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. *J. Infect. Dis.* **165,** 513–517.

Nacy C.A., Fortier A.H., Meltzer M.S., Buchmeier N.A. & Schreiber R.D. (1985) Macrophage activation to kill *Leishmania major* activation of macrophages for intracellular destruction of amastigotes can be induced by both recombinant interferon-γ and non-interferon lymphokines. *J. Immunol.* **135,** 3505.

Nacy C.A., Meltzer M.S., Leonard E.J. & Wyler D.J. (1981) Intracellular replication and lymphokine-induced destruction of *Leishmania tropica* in C_3H/HeN-mouse macrophages. *J. Immunol.* **127,** 2381.

Nathan C.F., Murray H.W., Wiebe M.E. & Rubin B.Y. (1983) Identification of interferon-γ as the lymphokine which activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med.* **158,** 670.

Passwell J.H., Shor R., Keren G., Messer G. & Elon Y. (1984) Comparison of the effect of various stimuli on the leishmaniacidal capacity of human monocytes *in vitro*. *Clin. Exp. Immunol.* **56,** 553.

Passwell J.H., Shor R. & Shoham J. (1986a) The enhancing effect of interferon-γ and α on the killing of *Leishmania tropica major* in human mononuclear phagocytes *in vitro*. *J. Immunol.* **136,** 3062.

Passwell J.H., Shor R., Gazit E. & Shoham J. (1986b) The effect of Con A induced lymphokines from the T lymphocyte subpopulations on human leishmaniacidal capacity and H_2O_2 production. *Immun.* **59,** 245.

Pearson R.D., Wheeler D.A. Harrison L.H. & Kay H.D. (1983) The immunobiology of leishmaniasis. *Rev. Infect. Dis.* **5,** 907.

Pick E. & Keisari Y. (1980) A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. *J. Immunol. Methods.* **38,** 161.

Pick E. & Mizel D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods.* **46,** 211.

Reiner M.E., Ng W. & McMaster W.R. (1987) Parasite-accessory cell interactions in murine leishmaniasis. II *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *J. Immunol.* **138,** 1926.

Remaley A.T., Das S., Campbell P.I., LaRocca G.M., Pope M.T. & Glew R.H. (1985) Characterization of *Leishmania donovani* acid phosphatase. *J. Biol. Chem.* **260,** 880.

Schnur L.F., Zuckerman A. & Greenblatt C.L. (1972) Leishmanial serotypes as distinguished by the gel diffusion of factors excreted *in vitro* and *in vivo*. *Isr. J. Med. Sci.* **8,** 932.

Sypek J.P. & Wyler D.J. (1991) Antileishmania defense in macrophages triggered by tumor necrosis factor expressed on CD4 + T lymphocyte plasma membrane. *J. Exp. Med.* **174,** 755.