Macrophage Activation by Polymeric Nanoparticles of Polyalkylcyanoacrylates: Activity Against Intracellular *Leishmania donovani* Associated with Hydrogen Peroxide Production

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Nanoparticles of polyalkylcyanoacrylates (PACA) can be useful carrier for the targeting of antileishmanial drugs into macrophages and also possess significant antileishmanial activity by themselves. No significant difference in antileishmanial activity could be detected between nanoparticles of five PACAs with differing alkyl side chains, suggesting that the main degradation products of PACA are not involved in their antileishmanial action. The effect of polyisohexylcyanoacrylate (PIHCA) on the induction of the respiratory burst in a macrophage-like cell line (J774G8) was assessed in noninfected macrophages and in macrophages infected with amastigotes of *Leishmania donovani infantum,* by measuring nitroblue tetrazolium (NBT) reduction and hydrogen peroxide production. Phagocytosis of PIHCA nanoparticles led to a respiratory burst, which was more pronounced in infected than in uninfected macrophages. The production of reactive oxygen intermediates associated with the respiratory burst was inhibited by addition of superoxide dismutase and catalase to the cell suspensions. The addition of catalase to the culture medium together with PIHCA nanoparticles significantly reduced the antileishmanial activity of PIHCA. Moreover PIHCA nanoparticles did not induce interleukin-1 release by macrophages. It is suggested that the antileishmanial action of PIHCA and other PACA nanoparticles results from the activation of respiratory burst in macrophages.

KEY WORDS: nanoparticles; polyalkylcyanoacrylates; macrophage activation; *Leishmania donovani;* reactive oxygen intermediates; nitroblue tetrazolium (NBT) reduction; hydrogen peroxide; interleukin-1.

INTRODUCTION

Visceral leishmaniasis is a widespread parasitic disease prevalent in most tropical and subtropical parts of the world. The disease is caused by the protozoan haemoflagellate *Leishmania donovani,* which invades macrophages and survives in the phagolysosomal compartment of its host cell.

Drug targeting using colloidal systems has led to both a reduction in toxicity and an increased efficacy of antileishmanial drugs in visceral leishmaniasis (1). In the group of polymeric synthetic colloidal systems, nanoparticles consisting of polyalkylcyanoacrylate (PACA)[5] have been well characterized with respect to physicochemical properties (size, molecular weight), biodegradability, toxicity, and biodistribution (2–4). These nanoparticles can easily be prepared at an industrial scale (5), while their phagocytosis by macrophages and their localization in both Küpffer cells and spleen macrophages (6,7) render them good candidates for the targeting of drugs against visceral leishmaniasis.

In a previous report, we demonstrated that polyisohexylcyanoacrylate (PIHCA) nanoparticles can be used for the targeting of an 8-aminoquinoline (primaquine) (8). We also showed that the carrier by itself exhibited a significant antileishmanial activity. Indeed, when macrophages infected with *Leishmania donovani* were incubated with nanoparticles of (PIHCA), a significant reduction in parasite burden was measured (ED_{50} = 6 μg/ml) (9). Moreover, preliminary experiments suggest that nanoparticles of PIHCA administered parenterally exert a marked therapeutic effect against leishmaniasis (10). Unloaded polyisobutylcyanoacrylate nanoparticles are also active against bloodstream trypanosomes (11).

The purpose of the present report is to determine the mode of antileishmanial action associated with this carrier. Two main hypothesis were tested:

(1) antileishmanial action results from the intracellular biodegradation of polymer; and
(2) antileishmanial action results from macrophage activation involving reactive oxygen intermediates, which can be assessed by monitoring nitroblue tetrazolium (NBT) intracellular reduction and hydrogen peroxide production and/or interleukin-1.

MATERIALS AND METHODS

Macrophages

The macrophage-like cell line, J774G8, derived from a BALB/c murine reticulum cell sarcoma, previously used in experiments with *Leishmania* (9,12–14), was used in all *in vitro* experiments. Cells of J774G8 were cultivated in RPMI-1640 (Dutch-modified; GIBCO Ltd. Co., Paisley, Scotland), complemented with 10% heat-inactivated fetal calf serum (HIFCS; GIBCO Ltd. Co., Paisley, Scotland) and supplemented with L-glutamine (Flow Laboratories, UK) prior to use. Cells were incubated in an atmosphere containing 5% CO_2, at 37°C.

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[5] Abbreviations used: PACA, polyalkylcyanoacrylates; PMCA, polymethylcyanoacrylate; PECA, polyethylcyanoacrylate; PBCA, polybutylcyanoacrylate; PIBCA, polyisobutylcyanoacrylate; PIHCA, polyisohexylcyanoacrylate; PMA, phorbol myristate acetate; NBT, nitroblue tetrazolium; HRPO, horseradish peroxidase; SOD, superoxide dismutase; HBSS, Hank's balanced salt solution; HIFCS, heat-inactivated fetal calf serum; PBS, phosphate buffered saline; IL-1, interleukin-1; SDM, semidefined medium; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

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For macrophage counting, attached cells were resuspended by multiple suctions in a pipette and counted in a Bürcker cell counter. For infectivity assays cells were cultivated in flat-bottomed 24-well plates, and for respiratory burst assays, in 96-well plates (Flow Laboratories, UK), each well containing 3×10^5 cells/ml.

Parasites

Leishmania donovani infantum [MHOM-67-MA(BE)-ITMAP 263] was isolated by Prof. Le Ray in 1967 from a Moroccan child, temporary in Belgium (Hospital Brugman at Brussels). The strain was stabilized after the second passage in hamster. Amastigotes were transformed *in vitro* to promastigotes and subsequently cultivated in SDM (16) at 28°C. For the counting of promastigotes, a sample was diluted 1/10 with PBS (containing 1% formaldehyde for fixation) and the promastigote number was determined in a Petroff-Hausser cell counter.

Preparation of PACA Nanoparticles

A series of PACA nanoparticles was prepared using a standard polymerization medium (5% glucose, 1% dextran 40, citric acid, pH 3.0) in which different monomers were polymerized. The monomers used were methylcyanoacrylate (MCA), ethylcyanoacrylate (ECA), butylcyanoacrylate (BCA), isobutylcyanoacrylate (IBCA) (all obtained from Loctite Co., Ireland), and isohexylcyanoacrylate (IHCA; Sopar Pharma, Belgium). Polymerization time was 30 min for MCA, 4 hr for ECA, BCA, and IBCA, and 16 hr for IHCA. Nanoparticle suspensions were freeze-dried and stored at −30°C. Nanoparticles were redispersed with sodium bicarbonate (0.02 *M*) prior to use. Particle size and granulometric distribution were measured with a Coulter N4 (2–8).

In Vitro Intracellular Infection and Antileishmanial Activity

J774G8 cells were infected with *Leishmania donovani infantum* by incubating $1.5 \cdot 10^6$ promastigotes/ml and $3 \cdot 10^5$ macrophages/ml, in RPMI-1640 (Dutch-modified) complemented with 10% HIFCS, at 37°C in 5% CO_2 (9). After a 48-hr incubation, which was sufficient to establish an intracellular infection, the culture medium was renewed and antileishmanial agents were added and incubated together with infected cells for another 24 hr (9). Unloaded nanoparticles from different PACA (PMCA, PECA, PBCA, PIBCA, PIHCA) at a final concentration of 12 μg/ml [noncytolytic doses for all PACA (3,15)] were added to assess the influence of the polymer nature. A PIHCA concentration of 48 μg/ml (9) and a catalase concentration of 10 U/100 μl, sufficient to abolish H_2O_2 production completely in short-term incubations, were used to assess the influence of catalase on PIHCA antileishmanial activity. The antileishmanial activity was evaluated on stained microscopic slides. Slides were prepared using 200 μl of resuspended cells per microscopic slide, which were spun (Cytospin, Shandon Ltd.) at 1000 rpm for 3 min, to obtain a single cell layer. The infection was evaluated after May–Grünwald–Giemsa staining by counting the number of amastigotes per macrophage by microscopic examination in oil immersion (magnification, 1000×) (9).

The antileishmanial activity was determined as the percentage reduction in parasite burden (number of amastigotes per infected macrophage) relative to controls and the percentage of infected cells. Results are the mean and standard deviation of five experiments.

Microassay of Nitroblue Tetrazolium (NBT) Reduction

NBT reduction is an irreversible process, yielding formazan after reduction by O_2^-; the test described here measures only intracellular NBT reduction (17).

Macrophage-like cells (J774G8) were incubated in 96-well microplates (Flow Laboratories, UK). Cell concentration was 3×10^5 per ml, with 100 μl of cell suspension per well. Before assay, the culture medium was removed and replaced by the assay solutions. NBT solution was prepared on the day of assay and the reactants were mixed before addition to the wells. Cells were covered with 100 μl/well of 1 mg NBT/ml solution (Sigma Chemical Co., St. Louis, MO) in phenol-red free HBSS (GIBCO Ltd. Co., Paisley, Scotland) containing the other reactants. One vertical row of eight wells served as a blank, in which cells were preincubated for 10 min at 37°C with iodoacetamide (Sigma Chemical Co.) in HBSS (10 m*M*). Iodoacetamide acts as a sulfhydryl reagent that inhibits the oxidative burst. When the NBT solution was added to the other wells in the plate, the iodoacetamide solution in the reference wells was removed by suction and replaced by a solution containing both 1 mg NBT/ml and 10 m*M* iodoacetamide. Assays were performed using a blank row of wells, a second row with NBT without stimulants (basal level), and a row for each of the stimulants [PIHCA, 48 μg/ml, or phorbol myristate acetate (PMA), 100 ng/ml as a positive control]. For infected macrophages, besides PIHCA, 30 U/well of superoxide dismutase (SOD) was added in a row of wells. Plates were covered with lids and placed at 37°C in a 5% CO_2 humidified incubator for 15, 30, 45, and 60 min. At the desired time, a permanent record of NBT reduction was obtained by removing the NBT solution and fixing the cells in the wells with addition of 100 μl of absolute methanol per well.

The amount of formazan accumulated in cells was quantitated by an enzyme-linked immunosorbent assay (ELISA) reader (Twinreader, Titertek) fitted with a standard 540-nm filter. Results for stimulants were corrected for blanks and are expressed as difference in absorbance units per milligram of cell protein.

Microassay of H_2O_2 Production

The horseradish peroxidase (HRPO)-dependent oxidation of phenol red by H_2O_2, leading to the formation of a compound that, at an alkaline pH, exhibits increased absorbance at 620 nm, was used for assay of H_2O_2 production (17).

After establishing macrophage cultures in microplates, the cells were covered with 100 μl per well of a 0.56 m*M* phenol red–20 U/ml HRPO solution with or without stimulants (PIHCA, 48 μg/ml; PMA, 100 ng/ml, as a positive control). For infected macrophages, a control was set up by adding 10 U/well of catalase to wells containing PIHCA. To one vertical row of wells 110 μl per well of assay solution [made alkaline by adding 1 ml of NaOH (1 *N*) to 10 ml of

phenol red–HRPO solution] was added. This group of wells was used as blanks. Plates were covered with lids and incubated for a time interval of 15, 30, 45, or 60 min at 37°C in an humidified incubator in 5% CO_2. Reaction was interrupted by adding 10 μl of NaOH (1 *N*) per well. After 3 min of equilibration, plates were transferred to the ELISA reader and wells read at 620 nm. Results were expressed as nanomoles of H_2O_2 per milligram of cell protein, using standard curves containing dilutions of H_2O_2 in the assay solution of from 20 to 100 μ*M* (2–10 nmol H_2O_2 per well). The H_2O_2 level was calculated after correction for blank values.

Interleukin-1 Release

Release of interleukin-1 (IL-1) from macrophage cultures incubated with or without PIHCA nanoparticles was evaluated by a modified murine thymocyte assay (18). Thymocytes from BALB/c mice (female, 6 weeks old) were used. Briefly, 1.5×10^6 thymocytes/well were cultured in DMEM supplemented with HEPES–glucose, L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), mercaptoethanol (5×10^5 *M*), HIFCS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (500 μg/ml).

Test samples of macrophage supernatants from cultures of cells infected or not with *Leishmania donovani* and incubated with or without 48 μg/ml PIHCA nanoparticles for 24 hr were incubated at a dilution factor of 4 or 8 in the presence of thymocytes under the conditions described above in the presence of phytohemagglutinin A (1/500 dilution) for maximum response to IL-1 (in a total volume of 200 μl/well). After incubation for 3 days, at 37°C in a 5% CO_2 atmosphere, [^{3}H]thymidine (0.5 μCi/well) was added and the mixture incubated for 6 hr. Then supernatants were removed and cell cultures washed twice with HBSS. Cells were digested by NaOH (1 *N*) overnight (100 μl/well) and the ^{3}H content was determined with a liquid scintillation counter (LKB, Wallac).

The incorporation of [^{3}H]thymidine by thymocytes was correlated with IL-1 content using a calibration in which macrophage supernatants were replaced by IL-1 (Hoffman-Roche, Switzerland) reference dilutions.

Protein Content

Protein content was determined by the Lowry method using bovine serum albumin (BSA; Sigma Chemical Co.) as protein standard (19).

RESULTS

Influence of Polymer Nature on Antileishmanial Activity

Macrophages infected with *Leishmania donovani infantum* were incubated with 12 μg/ml of the five respective polyalkylcyanoacrylates and their antileishmanial activity was determined (Table I). Results show that there was no significant difference in antileishmanial activity among the five polymers (PMCA, PECA, PBCA, PIBCA, and PIHCA).

NBT Reduction

Reduction of intracellular nitrobluetetrazolium can be used to measure the production of anion superoxide associated with the phagocytosis of the nanoparticles. In noninfected macrophages, the reduction of intracellular NBT induced by PIHCA nanoparticles was not significantly different from that shown by nonstimulated macrophages (basal level) (Fig. 1). In macrophages infected with intracellular amastigotes, the basal level was reduced compared to noninfected macrophages (characteristic feature in *Leishmania* infections), while endocytosis of PIHCA nanoparticles increased the intracellular NBT reduction (Fig. 2). The addition of superoxide dismutase together with the nanoparticles significantly reduced the NBT reduction.

Table I. Particle Size (±SD) and *in Vitro* Antileishmanial Activity of Different Polyalkylcyanoacrylates ($n = 5$)[a]

Addition	Medium size (nm)	Amastigotes/ infected macrophage	% reduction in parasite burden	% of cells infected
None	—	11.2 ± 1.0	—	74.8 ± 4.4
PMCA	388 ± 170	4.7 ± 0.6	58.6 ± 2.3	35.6 ± 5.8
PECA	286 ± 73	4.7 ± 0.6	58.4 ± 3.5	40.0 ± 6.7
PBCA	205 ± 41	4.7 ± 0.5	58.7 ± 1.3	39.2 ± 7.8
PIBCA	196 ± 54	4.9 ± 0.6	57.5 ± 1.7	38.0 ± 4.8
PIHCA	211 ± 38	4.8 ± 0.5	57.7 ± 1.6	34.6 ± 4.0

[a] For details, see Materials and Methods.

Hydrogen Peroxide Production

In both noninfected and infected macrophages, PIHCA nanoparticles caused a significant increase in H_2O_2 production (Figs. 3 and 4). The production of H_2O_2 was higher in infected than noninfected macrophages after 60 min.

The addition of catalase to the incubation medium abolished this response and restored H_2O_2 production back to the basal level (Fig. 4).

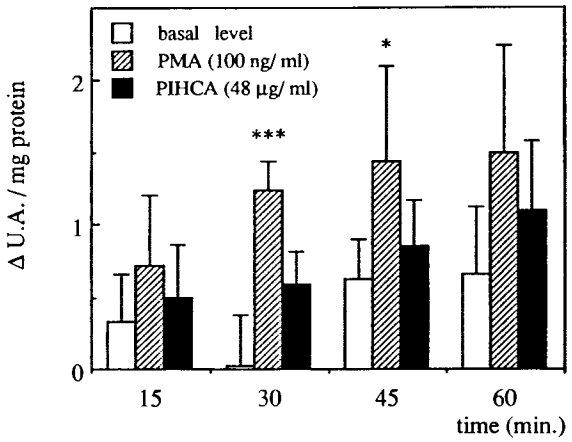

Fig. 1. Intracellular reduction of NBT in noninfected macrophages (J774G8 cells) after incubation with PIHCA (48 μg/ml) or a positive control of PMA (100 ng/ml) (for details, see Materials and Methods). Results are expressed as the mean of five experiments, and Student *t*-test results expressed as differences related to basal level: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ (for details, see Materials and Methods).

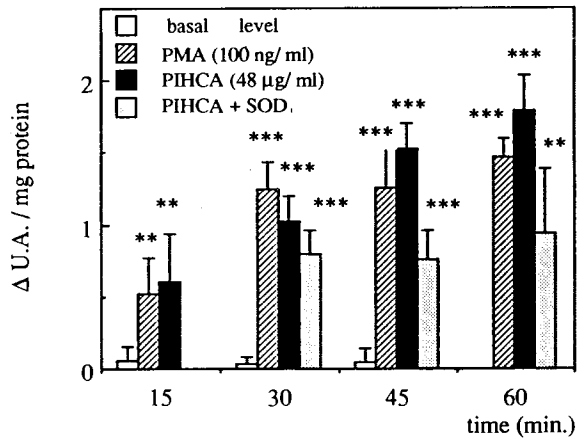

Fig. 2. Intracellular reduction of NBT in macrophages (J774G8 cells) infected with intracellular amastigotes of *Leishmania donovani infantum*, after incubation with PIHCA (48 µg/ml) with or without 300 U/ml of SOD or a positive control of PMA (100 ng/ml) (for details, see Materials and Methods). Results are expressed as the mean of five experiments, and Student *t*-test results expressed as differences related to basal level: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

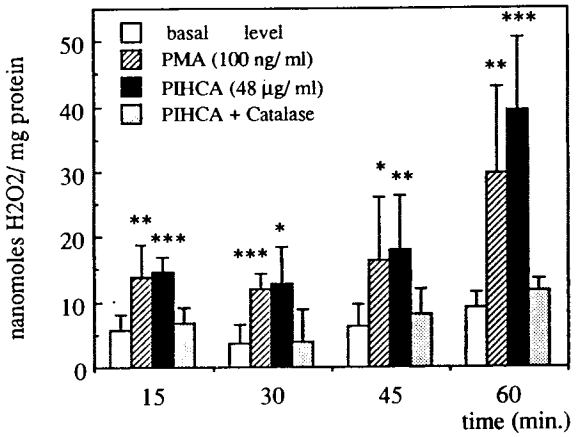

Fig. 4. H_2O_2 production in macrophages (J774G8 cells) infected with intracellular amastigotes of *Leishmania donovani infantum*, after incubation with PIHCA (48 µg/ml) with or without 100 U/ml catalase and a positive control of PMA (100 ng/ml) (for details, see Materials and Methods). Results are expressed as the mean of five experiments, and Student *t*-test results expressed as differences related to basal level: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Effect of Catalase on PIHCA's Antileishmanial Activity

Our short-term incubations (up to 1 hr) suggested that both the production of superoxide radicals and H_2O_2 could account for the antileishmanial action associated with PIHCA nanoparticles. We determined the effect of catalase on the antileishmanial effect of these nanoparticles in long-term experiments (up to 24 hr).

Table II shows that the addition of catalase to the incubation medium significantly reduced the antileishmanial action of PIHCA nanoparticles.

Interleukin-1 Release

Measurement of the release of interleukin-1 showed a significant reduction in IL-1 production by infected macrophages compared with noninfected cells. The presence of PIHCA nanoparticles (48 µg/ml for 24 hr) had no significant effect on IL-1 production in either infected or noninfected cells (Fig. 5).

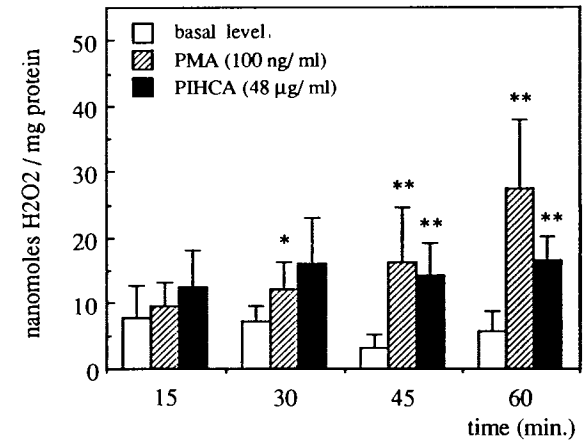

Fig. 3. H_2O_2 production in noninfected macrophages (J774G8 cells) after incubation with PIHCA (48 µg/ml) or a positive control of PMA (100 ng/ml) (for details, see Materials and Methods). Results are expressed as the mean of five experiments, and Student *t*-test results expressed as differences related to basal level: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

DISCUSSION

PIHCA nanoparticles exert antileishmanial activity in both an *in vitro* (9) and an *in vivo* (10) model for intracellular infection of macrophages with *Leishmania donovani infantum*. Two hypotheses were proposed: (i) antileishmanial activity results from the intracellular release of degradation products; and (ii) the antileishmanial activity of macrophages is activated by the polymeric nanoparticles.

Our present experiments argue against the first possibility. Besides a polycyanoacrylic acid chain, the major degradation products of PACA are the alcohols corresponding to the alkyl side chains. Since these alcohols are responsible for the different toxicity of different PACA on various cell lines (3,15), it is unlikely that they induce similar antileishmanial activity. Although an effect of the polyacrylic acid chain cannot be excluded, the composition of these particles probably does not play a significant role in antileishmanial activity.

The generation of toxic oxygen intermediates is the ma-

Table II. Influence of Catalase (100 U/ml) on the Antileishmanial Activity of PIHCA (48 µg/ml) Incubated with J774G8 Cells Infected with *Leishmania donovani infantum* Amastigotes ($n = 5$)[a]

Additions	Amastigotes/ infected macrophage	% reduction in parasite burden	% of infected cells
None	9.1 ± 0.5	—	71.0 ± 10.8
PIHCA	1.8 ± 0.4	79.9 ± 4.1	27.2 ± 5.2
PIHCA + catalase	7.1 ± 0.4	21.9 ± 5.0	58.0 ± 8.2

[a] For details, see Materials and Methods.

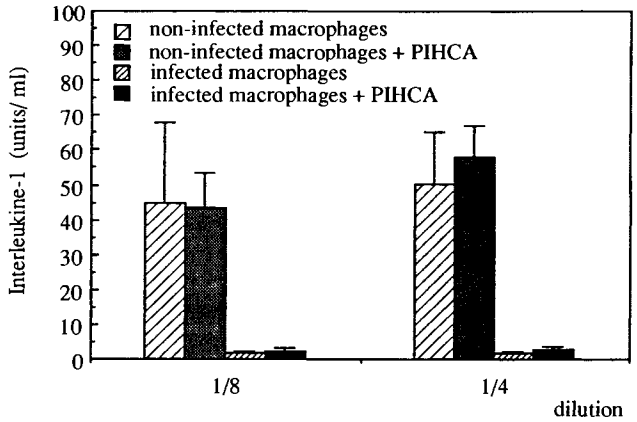

Fig. 5. Interleukin-1 release from infected and noninfected macrophages after incubation with PIHCA (48 μg/ml) for 24 hr. Macrophage supernatants were used at a dilution factor of 8 or 4 in the thymocyte cultures (for details, see Materials and Methods). Results are expressed as the mean of five experiments.

jor mechanism underlying the antiprotozoal activity of mononuclear phagocytes, and hydrogen peroxide alone is sufficient for antileishmanial activity (13,20). Promastigotes show a dose-related susceptibility to reagent and generated hydrogen peroxide. Promastigotes are susceptible to H_2O_2–peroxidase–halide microbicidal mechanisms which are capable of providing host defense against the invading promastigote stage of this pathogen (21,22). This effect is specific for H_2O_2 (23). *Leishmania donovani* amastigotes display the unique ability to evade the triggering of oxidative burst in macrophages primed to generate high levels of microbicidal oxygen intermediates. Not only have the amastigotes developed mechanisms to evade exposure to toxic oxygen intermediates but the host cell gradually loses its capacity to produce these substances after the parasite establishes intracellular residence (24). Amastigote killing depends upon effective generation of hydrogen peroxide (25). However, nonoxidative mechanisms can also play a role in the killing of *Leishmania* (26).

Our experiments indicate that PIHCA nanoparticles induce an activation of the respiratory burst in macrophages, as shown by the increase in the intracellular reduction of NBT and, also, the increased production of hydrogen peroxide.

The enhanced production of reactive oxygen metabolites induced by the respiratory burst associated with phagocytose seems to be the principal cause associated with intracellular antileishmanial activity of PIHCA nanoparticles (Figs. 1–4). Further, this action can be accounted for by hydrogen peroxide production since catalase reduces antileishmanial activity (Table II).

Interleukin-1 is a principal mediator of the host immune response to microbial challenge. Once elaborated, this cytokine initiates a series of inflammatory processes and plays an important role in T-lymphocyte activation. *Leishmania donovani* has the ability both to evade and to suppress the macrophage IL-1 response, and the evasion of signal transduction for IL-1 synthesis may be related to defects in cell-mediated immunity which occur during infections with this organism (27). The results of the present report show that PIHCA nanoparticles do not increase IL-1 release; therefore, involvement of IL-1 in the antileishmanial action of PACA nanoparticles cannot be demonstrated, at least in the *in vitro* model used.

Other macrophage stimulants such as interferon γ were also shown to enhance H_2O_2 release and to induce antileishmanial activity (28). Other drug carriers such as polysaccharides microspheres also induce H_2O_2 and IL-1 release (29,30). The results presented in this report suggest that the antileishmanial activity of PIHCA nanoparticles can be explained by an increase in the respiratory burst in macrophages infected with intracellular amastigotes of *Leishmania donovani infantum*.

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