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Biodegradable microspheres. VIII. Killing of *Leishmania donovani* in cultured macrophages by microparticle-bound primaquine

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Summary

Primaquine covalently bound to polyacryl starch microparticles has been shown to kill *Leishmania donovani* in cultured mouse peritoneal macrophages. The drug was derivatized with a tetrapeptide spacer and the derivative (Ala-Leu-Ala-Leu-PQ) coupled to the microparticles. This drug–carrier complex did not kill free promastigotes in suspension, but was effective against amastigotes in cultured mouse peritoneal macrophages. These results show that lysosomal processing of the drug–carrier complex is necessary in order to liberate the pharmacologically active drug. Also, the possible role of reactive oxygen intermediates for the anti-leishmanial effect was studied.

Introduction

Parasitic infections in man normally require intensive treatment and in particular those afflicting the reticuloendothelial system (RES), e.g. leishmaniasis, are often serious and even fatal when not effectively treated. Effective therapy against such an infection is difficult to achieve, mainly due to the problems of targeting the drugs to the specific compartment, the lysosomal vacuome, where the parasite is taken up. A rational approach to treat leishmaniasis would therefore be to improve the disposition of the anti-leishmanial drugs, so that they are targeted to the RES and the lysosomes there. This can be achieved by using particulate drug delivery systems, which are taken up by cells of the RES in vivo. Some delivery systems, e.g. liposomes and niosomes, have been shown to be effective in animal models (Alving et al., 1978; New et al., 1978; Black et al., 1977; Baillie et al., 1986). (For a recent review, see Croft, 1986.)

In our laboratory, a microparticulate system based on polyacryl starch is currently being investigated to deliver drugs to the RES. The microparticles with entrapped enzyme have so far been shown to be effective in the treatment of an artificial storage disease in mice (Artursson et al., 1984b).

Low molecular weight drugs cannot be entrapped in polyacryl starch microparticles. However, primaquine and trimethoprim, two drugs

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containing amino groups have been covalently bound to the starch matrix via a peptide spacer arm (Laakso et al., 1987b). The drug particle conjugates were stable in serum, but the drugs were released upon exposure to lysosomal enzymes.

In the present paper, the effect of primaquine, known to be toxic to *Leishmania donovani* (Neal and Croft, 1984), has been studied in free and microparticle-bound form. Two test systems have been used: free promastigotes in suspension and amastigotes in cultured mouse peritoneal macrophages. In the latter system, promastigotes are transformed in the lysosomes into the amastigote form.

Materials and Methods

Microparticles

The polyacryl starch microspheres used throughout this study were prepared by polymerization of acryloylated starch in an emulsion, as previously described (Artursson et al., 1984a). Briefly, a solution of acryloylated starch in 5 ml was homogenized together with 300 ml of toluene–chloroform (4 : 1). Ammonium peroxidisulphate (0.08 M) and tetramethylethylenediamine (TEMED) were used to initiate the polymerization. The microparticle composition is characterized by the D-T-C nomenclature. D represents the concentration of acryloylated starch (g/100 ml) in the monomer solution, T represents the total concentration of acrylic groups (g/100 ml) and C represents the relative amount of crosslinking agent (% w/w). In this study, the D-T-C was 10-1.0-0, and 0.5 ml of TEMED was added. To avoid aggregation of the particles, *N*-acryloylalanine was copolymerized according to Laakso and Sjöholm (1987a) in a concentration of 10 mg/ml in the monomer solution, contributing 0.5 to the T-value.

Primaquine (PQ) was coupled to the microspheres in the form of a tetrapeptide derivative Ala-Leu-Ala-Leu-PQ, as described by Laakso et al., (1987b). The particles had a drug content of 3.30×10^{-4} mmol tetrapeptide-PQ per mg dry weight, corresponding to 0.15 mg of PQ diphosphate per mg. The drug content was determined by the radioactivity of the tetrapeptide-PQ complex which was labelled with [^{14}C]alanine used in the synthesis of the tetrapeptide arm.

Chemicals

The following chemicals were used: primaquine disphosphate (PQ) and the benzimidazole dye, Hoechst 33258 from Janssen Chimica (Beerse, Belgium). Nitro blue tetrazolium (NBT) and NADPH from Merck (Darmstadt, F.R.G.); phenazine methosulfate (PMS), superoxide dismutase, catalase and NADH from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Parasites

Leishmania donovani promastigotes (LV 9) were a gift from Dr. David Evans, London School of Hygiene and Tropical Medicine and Dr. Simon Croft, Wellcome Research Laboratories, Beckenham, U.K. The parasites were kept frozen in liquid nitrogen or cultured at 22 °C in Schneider's Drosophila Medium (Gibco, Paisley, U.K.) containing 20% heat-inactivated fetal calf serum (Gibco), 50 U penicillin/ml and 50 μg streptomycin/ml. Half the medium was renewed every second day at a parasite concentration of 10^7 per ml.

Cells

Peritoneal macrophages were harvested from untreated BALB/c mice by irrigating the peritoneal cavity with 3 ml ice-cold PBS, as described previously (Artursson et al., 1987). The suspension was centrifuged at $180 \times g$ for 10 min, and the cells were resuspended in culture medium (RPMI 1640, Gibco, Paisley, U.K.) containing 10% heat-inactivated fetal calf serum (Gibco), 50 U penicillin/ml and 50 μg streptomycin/ml. The number of macrophages was adjusted to 2×10^6/ml and 0.5 ml of the suspension added to 12 mm round glass coverslips placed in tissue culture wells. The macrophages were allowed to adhere to the coverslips for 1 h at 37 °C in an atmosphere of 95% air and 5% CO_2, followed by 4 washes with phosphate-buffered saline (PBS) and replacement of culture medium (0.5 ml).

Infection of macrophages

One day after seeding of the macrophages, *L.*

donovani promastigotes were added to the wells in 100 μl culture medium to give a ratio of 1 : 50 between adherent cells and parasites. After 6 h at 37 °C in 95% air and 5% CO_2, the coverslips were removed from the wells, washed in PBS and put in new wells with 0.5 ml fresh culture medium.

Treatment of infected macrophages

One day after infection, 25 μl of different concentrations of soluble or particle-bound primaquine was added to the wells, giving a concentration of PQ-diphosphate ranging from 0.1 to 10 μg/ml. After 2 h the macrophages were washed with PBS, and fresh culture medium was added. After 4 days and one medium renewal (day 2), the coverslips were removed and fixed in acetic acid : methanol (1 : 3) for 10 min.

NBT reduction assay

The absorbance change caused by the reduction of NBT was measured according to Nishikimi et al. (1972) every 20th second for 5 min at 560 nm with a Shimadzu UV-110-02 spectrophotometer. The reactants were dissolved in 0.2 M phosphate buffer pH 8.3 in concentrations as follows: NBT 50 μM, NADPH/NADH 78 μM, PMS 4.35 μM, PQ 4.35 μM, 100 μM or 1 mM. The total volume was 900 μl.

Promastigote viability assay

Flagellar motility has been shown to be a reliable indicator of promastigote viability (Murray, 1981; Pearsson and Steigbigel, 1980). Promastigotes were suspended in PBS containing PQ-diphosphate (free or microparticle-bound) in a concentration ranging from 0 to 500 μg/ml (0–1.1 mM) or PMS in a concentration ranging from 0–3.1 μg/ml (0–10 μM) in the presence of NADH or 0–30.6 μg/ml (0–100 μM) when studied alone. The concentration of NADH was 7.1 μg/ml (10 μM) and the concentration of NADPH 915 μg/ml (1.1 mM).

After 1 h of incubation at 37 °C, 95% air and 5% CO_2, a drop of the suspension was placed on a glass slide and the motility of the promastigotes was determined using phase-contrast microscopy. One hundred promastigotes were examined in each sample.

Staining and counting of intracellular parasites by fluorescence microscopy

The fixed coverslips were stained with Hoechst 33258, a fluorescent dye which binds specifically to DNA (Chen, 1977). The coverslips were mounted and examined in a Zeiss fluorescence microscope (equipped with a BG 38 red suppression filter, a KP 490 interference filter and a K 460 barrier filter).

One hundred macrophages were examined on each coverslip, and the number of parasites was counted. The percentage of parasites killed was expressed as

$$\frac{a-b}{a} \times 100$$

where a = number of parasites in 100 macrophages in control; and b = number of parasites in 100 macrophages in samples.

Results

Effect of free and microparticle-bound primaquine on L. donovani promastigotes in suspension

To investigate the susceptibility of extracellular parasites to free and microparticle-bound PQ, the promastigotes were subjected to different con-

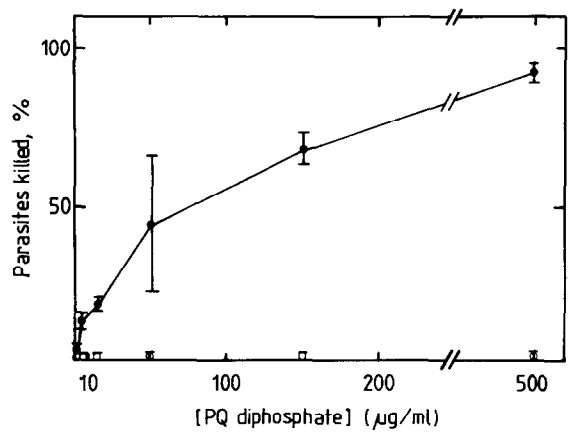

Fig. 1. Effect of free (●) and microparticle-bound (○) PQ on *L. donovani* promastigotes in suspension. The drug content of the microparticles corresponded to 0.15 mg of PQ-disphosphate per mg. Parasite viability was determined as described in Methods.

centrations of drug and drug-carrying microparticles. An increased killing of parasites was seen with increasing concentrations of free drug, the highest concentration giving an almost total killing. The microparticle-bound drug, however, had no effect in this system (Fig. 1).

Effect of free and microparticle-bound PQ on L. donovani in macrophages

The ability of free and microparticle-bound PQ to kill intracellular parasites was studied. Macrophages were infected with promastigotes, cultured for 24 h to allow proper transformation of the parasites to amastigotes, and then exposed to various doses of free and microparticle-bound PQ. After 96 h the number of parasites per 100 macrophages was determined and compared with that of untreated control cultures. This was carried out by fluorescence microscopy after staining the cells with Hoechst 33258. Parasites were easily detected as characteristic patterns of fluorescence surrounding the nuclei (Fig. 2). At the end of the experiment, the control cultures contained 123 (S.D. = 14; $n = 3$) parasites/100 macrophages. The fraction of cells infected was 37% (S.D. = 4.4; $n = 3$). The corresponding figures for the first day after infection (day 0) was 244 (S.D. = 31; $n = 3$) and 67% (S.D. = 1.4; $n = 3$), respectively. The number of adherent cells in control and experimental cultures was the same throughout the experiment.

Both free and microparticle-bound PQ showed a dose-dependent effect on the intracellular parasites (Fig. 3). Interestingly, a more than 100 times lower concentration of PQ in the medium was required to kill the intracellular stages of the parasites as compared with the extracellular ones (Figs. 1 and 3). At the highest PQ concentration

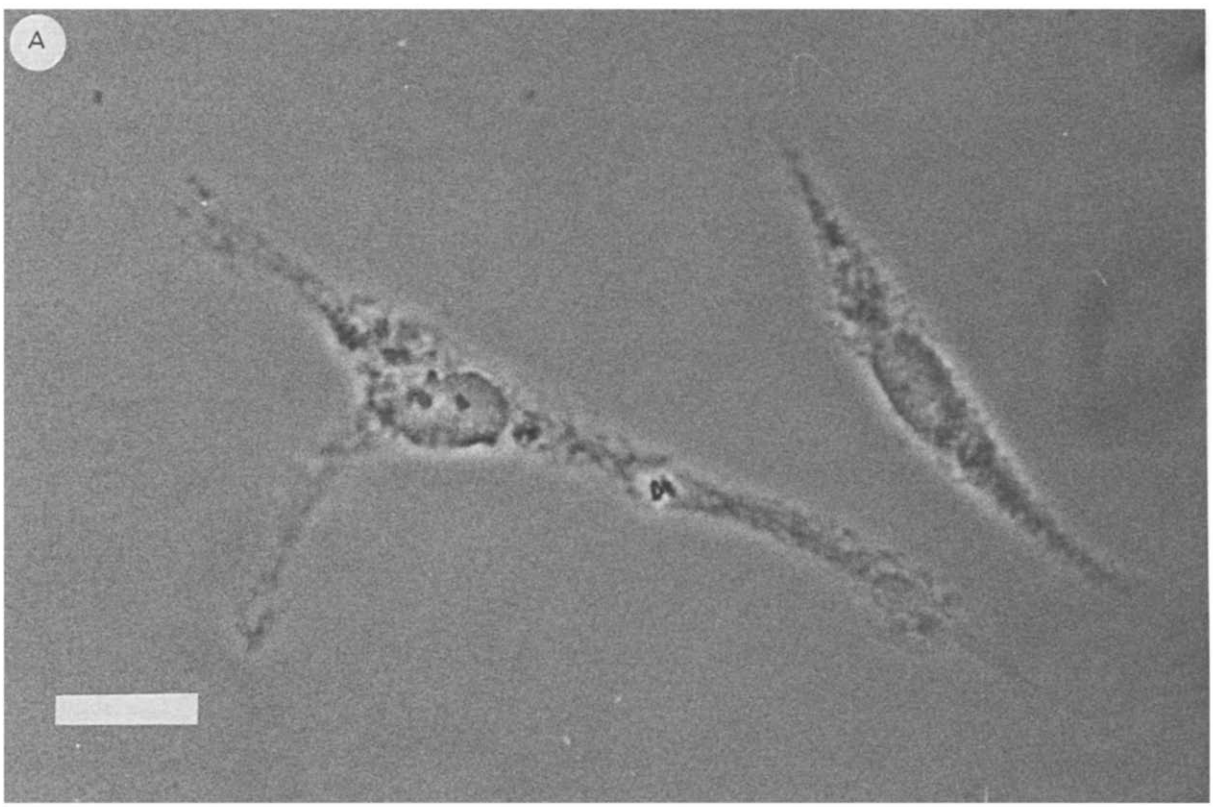

Fig. 2. Micrographs of a *L. donovani*-infected macrophage after staining with Hoechst 33258. Bar = 20 μm. A: phase-contrast microscopy. B: fluorescence microscopy.

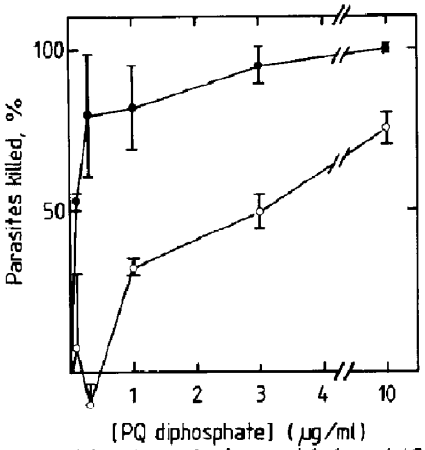

Fig. 3. Effect of free (●) and microparticle-bound (○) PQ on *L. donovani* amastigotes in cultured macrophages. The drug content of the microparticles corresponded to 0.15 mg of PQ-disphosphate per mg. Parasite viability was determined and expressed as described in Methods.

(10 μg/ml) the microparticle associated drug eliminated 70–80% of the parasites from the macrophages, as compared with the 100% elimination obtained with the free drug. These results show that microparticle-bound PQ, although ineffective in the promastigote model, can effectively kill intracellular parasites in the target organelle, i.e. the lysosome.

TABLE 1

Effect of empty microparticles on extracellular and intracellular stages of L. donovani

Drug	Promastigotes killed (%)	Amastigotes killed (%)
none	3 ± 1	0
empty microparticles	6 ± 1	20 ± 5
primaquine microparticles	0	76 ± 5
primaquine	92 ± 3	100

The promastigote and amastigote viability was determined and expressed as described in Methods. The concentration of PQ (free or microparticle-bound) was 500 μg/ml in the promastigote assay and 10 μg/ml in the amastigote assay. The concentration of both types of microparticles was 3.3 mg/ml in the promastigote assay and 0.07 mg/ml in the amastigote assay. The values are the mean ± S.D. ($n = 3$).

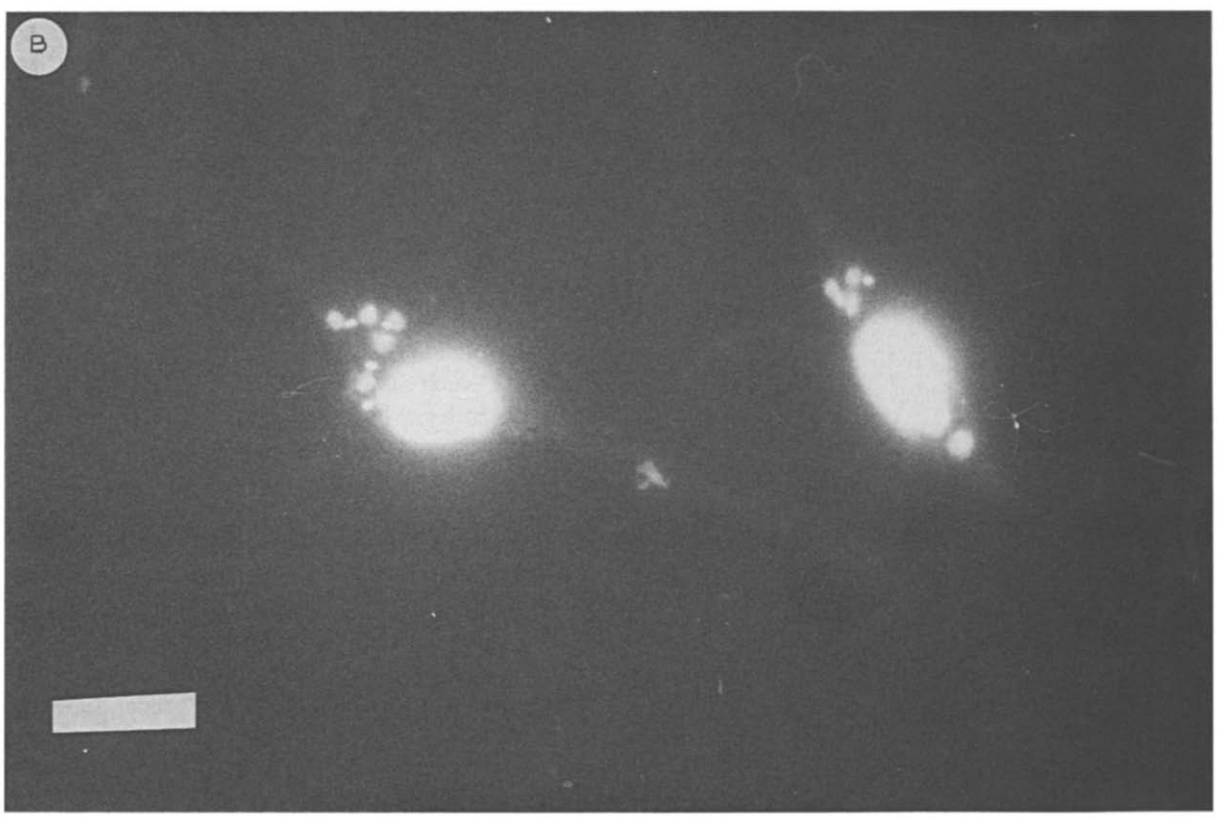

Fig. 2. (contd.).

Effect of "empty" microparticles on promastigote and amastigote survival

Particles without coupled drugs, but otherwise having the same composition as the drug-carrying microparticles, were tested in both the intra- and extracellular parasite systems. The concentration of "empty" microparticles was the same as the highest concentration used of drug-carrying microparticles in each system. No effect on the parasites was seen in the extracellular system with free promastigotes. However, in the macrophages, about 20% of the parasites were killed with "empty" microparticles (Table 1).

The role of oxygen intermediates in the killing of L. donovani

Promastigotes were also incubated with PQ in the presence and absence of NADPH, since the NADPH-mediated reduction of PQ with a subsequent generation of reactive oxygen intermediates (ROI) has been suggested to be a key reaction to explain the toxic effect of the drug on red blood cells (Thornalley et al., 1983).

PMS, a drug which is known to generate ROI in the presence of NADH (Nishikimi et al., 1972) and which has a well documented anti-leishmanial activity (Rabinovitch et al., 1982) was used as a positive control (Table 2). In combination with NADH, the PMS-concentration required to kill 50% of the parasites (LD_{50}) was reduced from 17 μg/ml to 0.1 μg/ml, i.e. 170 times. Parasites incubated with the coenzyme alone showed no decreased viability. The production of ROI by PMS and NADH was demonstrated by incubation of the reactants with Nitro blue tetrazolium (NBT).

NBT has been used to quantify the production of ROI since it forms a blue-coloured reaction product upon exposure to superoxide anion (Nishikimi et al., 1972). As can be seen in Table 2, the mixture of PMS, NADH and NBT gave a reaction product (formazan) which could be followed spectrophotometrically.

The same experiments were repeated with PQ (Table 2). No superoxide anion could be detected in the NBT-reduction assay. More significant, however, is the observation that the addition of electron carriers had no effect on the primaquine dose required to kill 50% of the parasites (Table 2). Moreover, the killing of promastigotes by PQ-NADPH was unaffected (results not shown) by addition of superoxide dismutase (SOD) and catalase (CAT), two enzymes involved in O_2-metabolism, shown to increase (SOD) and decrease (CAT) the PQ-NADPH mediated formation of hydroxyl radicals (Augusto et al., 1986). Thus, the ROI possibly generated by PQ in the presence of NADPH did not increase the efficacy of the drug in the same way as shown for the other electron acceptor, PMS.

TABLE 2

Requirement of electron carriers for the drug-mediated promastigote toxicity

Drug	Electron carrier	NBT-reduction (Δabs./min)	LD_{50} (μg/ml)
PMS	none	0	17
	NADH	0.02	0.1
	NADPH	0.05	n.d.
Primaquine	none	0	80
	NADH	0	n.d.
	NADPH	0	80

The reduction of NBT was measured as stated in Methods. The effect of the drugs on promastigotes in suspension is expressed as the LD_{50}-value.

Discussion

Several emulsion-based drug delivery systems (DDS), such as liposomes and, more recently, niosomes have been shown to be effective in the treatment of experimental (Black et al., 1977; New et al., 1978; Alving et al., 1978; Lopez-Berestein et al., 1984; Baillie et al., 1986) as well as human (Lopez-Berestein et al., 1985) infectious diseases localized to the macrophages of the reticuloendothelial system. However, the emulsion-based DDS have certain pharmaceutical drawbacks such as low stability and, consequently, various alternative microparticulate DDS have been developed (Tomlinson, 1983). So far, none of the microparticulate DDS has been tried in the treatment of experimental RES-localized infectious diseases. One of these drug carriers, the polyacryl starch

microparticles, has been shown to be effective in the treatment of another category of RES-localized diseases, namely the lysosomal storage diseases (Artursson et al., 1984b). It could therefore be expected that the polyacryl starch microparticles should be suitable for the delivery of antiparasitic drugs to the macrophages of the RES as well.

The primary aim of this work was to study the effect of microparticle-bound PQ on *L. donovani*. For this purpose, a macrophage infection model was developed. Some methodological problems had to be solved before reliable results could be obtained. In accordance with others (Nolan and Herman, 1985), we found that the freshly isolated promastigotes rapidly lost their virulence upon in vitro cultivation. This problem was solved by freezing the newly isolated promastigote cultures in portions. Moreover, a new, simple DNA-staining method was introduced with the fluorescent dye Hoechst 33258 which made it easy to detect the parasites in the macrophages.

Microparticle-bound PQ has no effect on free promastigotes, an observation which can be explained by the fact that the drug remains associated to the particle matrix in aqueous environment. The drug–particle conjugate is equally stable in the presence of serum (Laakso et al., 1987b). Whether this reduced toxicity of the drug–carrier conjugate has implications for the primaquine toxicity to mammalian cells, i.e. erythrocytes (Beutler, 1969) remains to be seen.

The PQ is associated to the carrier through a tetrapeptide spacer arm. Although stable in serum, the tetrapeptide is susceptible to isolated lysosomal enzymes (Laakso et al., 1987b). Therefore, once introduced into the lysosomal compartment PQ should be released in a pharmacologically active form from the microparticle matrix. The finding that the microparticle-associated primaquine was active against intralysosomal parasites supports this statement.

The microparticle drug conjugate was relatively less effective against the intracellular parasites as compared with the free drug. However, the primary aim of this study was not to compare the efficiency of free and particle-associated drug but to investigate if PQ containing microparticles could function as a pharmacologically active drug delivery system. The efficiency of the drug–carrier conjugate relative to that of the free drug can only be investigated in vivo, where the result of the improved targeting of the microparticle-associated drug to the RES can be quantitatively studied. Indeed, preliminary results from a study with another microparticle-associated anti-leishmanial drug (sodium stibogluconate) in an animal model indicates that the particle-associated drug is much more effective than the free drug (Baillie et al., 1987).

Many parasites, including *L. donovani* are susceptible to ROI (Klebanoff, 1975; Murray, 1981). It was recently shown that empty polyacryl starch microparticles could induce the release of hydrogen peroxide from macrophages in vitro (Artursson et al., 1987). Thus, the effect of empty particles on intracellular parasites may be explained by the production of ROI in the macrophages as a response to the interaction with the particle matrix.

This study gave no evidence for the involvement of ROI in the PQ-mediated killing of promastigotes in suspension. However, this does not exclude the possibility of ROI-participation in the *intracellular* antiparasitic effect of PQ, as suggested by Augusto et al. (1986).

In summary, the results presented in this paper show: (1) that microparticle-associated primaquine can effectively kill intracellular but not extracellular *L. donovani*; and (2) that the microparticle matrix itself has some effect on intracellular but not free *L. donovani*.

The microparticles may thus potentiate the antiparasitic effect of the associated drug in two ways; by targeting to the lysosomes and by the inherent anti-leishmanial effect of the microparticle matrix.

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