The anti-leishmanial effect of *Kalanchoe* is mediated by nitric oxide intermediates

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SUMMARY

We have previously shown that oral treatment with the leaf extract of the plant *Kalanchoe pinnata* (Kp) significantly decreases the lesion size and the parasite load in BALB/c mice infected with *Leishmania amazonensis*. Here we report on the mode of action of Kp, particularly on the induction of nitric oxide (NO) production by macrophages. We observed that Kp has no direct inhibitory activity on extracellular promastigotes, but effectively decreases the intracellular amastigote growth in a dose-related fashion. A 58% reduction in amastigote growth induced by 500 μg/ml Kp was associated with a 6-fold increase in the production of NO by the macrophages. IFN-γ synergistically enhanced the NO-stimulating effect of Kp in culture. Co-treatment with the inducible NO synthase enzyme inhibitor L-N^G-monomethyl-arginine abolished the antileishmanial effect of Kp *in vitro* and in *L. amazonensis*-infected BALB/c mice. These results indicate that the protective effect of Kp in leishmaniasis may not be due to a direct effect on the parasite itself but rather to activation of the reactive nitrogen intermediates pathway of macrophages.

Key words: *Kalanchoe pinnata*, nitric oxide, leishmaniasis.

INTRODUCTION

Cutaneous leishmaniasis is an endemic disease caused by the protozoan parasite *Leishmania* which affects at least 12 million people worldwide (WHO, 1991). The disease is characterized by chronic skin lesions generally at the site of the sandfly bite. Macrophages are not only the host cells of *Leishmania* but also effector cells, capable of eliminating the parasites when appropriately activated. When activated by cytokines such as IFN-γ, the product nitric oxide (NO) is generated upon conversion of L-arginine into L-citrulline, a reaction catalysed by the enzyme-inducible nitric oxide synthase (iNOS) (Green *et al*. 1990; reviewed by James, 1995). Production of NO appears to constitute one of the main microbicidal mechanisms of murine macrophages (Liew *et al*. 1990) and has been implicated in the elimination of viruses, bacteria, fungi and protozoa including *Leishmania* (DeGroote & Fang, 1995; Liew *et al*. 1990; Stenger *et al*. 1994). Treatment of mice with NO inhibitors increases lesion sizes and parasite load (Liew *et al*. 1990; Woods *et al*. 1994) and induces prompt reactivation of leishmaniasis in resistant mice (Stenger *et al*. 1996). The mechanisms of action of NO on *Leishmania* is not well established, but it may act together with reactive oxygen species to damage microbial DNA, proteins, and lipids (revised by Fang, 1997).

Kalanchoe pinnata is a medicinal plant used popularly in Brazil, India, China and Africa in the treatment of cutaneous wounds, arthritis and gastric ulcers (Correia, 1926; Kirtikar & Basu, 1975; Perry & Metzger, 1980). Several biological activities of Kp have been experimentally confirmed, such as the antifungal (Misra & Dixit, 1979), anti-inflammatory (Pal & Chaudhuri, 1990), antihistaminic (Nassis, Haebisch & Giesbrecht, 1992) and immunosuppressive (Moraes *et al*. 1994; Rossi-Bergmann *et al*. 1994; Rossi-Bergmann, Costa & Moraes, 1997) activities. We have also demonstrated the anti-leishmanial activity of Kp (Da-Silva *et al*. 1995). In that work, *in vivo* experiments showed that *Leishmania amazonensis*-infected BALB/c mice were successfully protected against chronically growing lesions by oral administrations of the aqueous extract of Kp. After initiation of treatment, the lesion sizes were brought to normal levels and the parasite load was strongly reduced.

In this paper, we investigate the effect of Kp extract on the parasites and on the activation of leishmanial killing by the macrophages, more specifically on the production of NO, in order to establish whether the protection against the disease is direct on the parasites or involves indirect microbicidal mechanisms.

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MATERIALS AND METHODS

Parasites

Leishmania amazonensis (*La*) LV/79 (designation MPRO/BR/72/M1841) promastigotes were used. Parasites were routinely isolated from mouse lesions and maintained as promastigotes in Dulbecco-modified Minimum Essential Medium (DMEM, Sigma Chemical Co., USA) containing 10% heat-inactivated foetal calf serum (HIFCS, Microbiologica, Rio de Janeiro, Brazil) and antibiotics at 26 °C. Subcultures were made in the late log phase of growth and parasites were used at no later than the fourth passage.

Mice

BALB/c mice originally purchased from Jackson Laboratory (Bar Harbor, Maine) were bred and maintained at our own facilities. The animals were used at 8–10 weeks old.

Kalanchoe pinnata *extract* (*Kp*)

Fresh leaves of *K. pinnata* were collected from the outskirts of Rio de Janeiro, Brazil, prior to the flowering season. They were crushed in distilled water at 20% (w/v) and heated at 50–60 °C for 30 min. The aqueous extract (Kp) was filtered through filter paper (Whatman No. 1), frozen and lyophilized. The lyophilized powder was dissolved in PBS at 20 mg/ml, filter sterilized using 0·45 μm Millipore membranes, and stored at −20 °C until use.

Anti-promastigote growth

To assess the inhibition of promastigote growth, 10^5 parasites at stationary growth phase were cultured in triplicate in 24-well culture plates (Nunc, Roskilde, Denmark) at 26 °C in DMEM + 10% HIFCS containing the indicated concentrations of Kp. The number of promastigotes were counted daily using a Neubauer chamber.

Anti-amastigote activity

Peritoneal macrophages were obtained from normal BALB/c by lavage with cold DMEM medium. The cells were plated in 8-chamber Lab-Tek slides (Nunc, Naperville, USA) at 2×10^5/well and left to adhere for 1 h at 37 °C/5% CO_2. After removing the non-adherent cells the monolayers were incubated with several concentrations of Kp for 24 h and then infected with promastigotes at 4:1 macrophage for 4 h at 34 °C. Infected macrophages were washed and incubated with Kp for a further 24 h. The monolayers were then stained with Giemsa and at least 100 infected macrophages/sample were counted under optical microscopy.

Alternatively, macrophages infected as above were incubated for 48 h with 500 μg/ml of Kp in the presence or absence of 100 μM of NMMA (L-N^6-monomethyl-arginine, a kind gift of the Wellcome Research Laboratories (Beckenham, UK) after infection. The monolayers were washed, stained and the number of amastigotes counted as described above.

Production of NO

Macrophages were infected as above and incubated 24 h before and after infection with several concentrations of Kp in the presence or absence of 50 U/ml of rIFN-γ in DMEM supplemented with 5% HIFCS. The culture supernatants were collected in the 24 h end and the NO production was measured by assaying for nitrite, using the Griess reaction as described previously (Green *et al*. 1982). The nitrite concentration was calculated from a $NaNO_2$ standard curve. The optical density was measured by spectroscopy at 570 nm wavelength.

To analyse for possible LPS contamination in the Kp preparation, Kp was incubated for 30 min with polymyxin B (Storn, Rosenthal & Swanson, 1997; Lasfargues *et al*. 1989) at 1 μg/ml, a concentration shown to totally inhibit the effect of LPS on NO production, as assessed in preliminary experiments, and then used to treat infected macrophage cultures. The culture conditions and assays were the same as described above.

Infection and treatment of mice

Mice (6/group) were infected in the hind footpad with 4×10^6 La promastigotes. Seven days after infection 2 groups were daily treated with 8 mg Kp in 0·2 ml of distilled water by the oral route (intragastric gavage) for 46 days. At day 15 of infection, one of the groups continued on Kp and started receiving 300 μg NMMA in 10 μl s.c. in the lesion every other day for 38 days. Controls received NMMA only or were left untreated. Lesion sizes were measured with a dial caliper (Mitutoyo, Brazil) every 4–5 days and expressed as the difference between the thickness of the contralateral uninfected footpad.

Statistical analysis

Statistical significance ($P < 0{\cdot}05$) was analysed by Student's *t*-test.

RESULTS

Effect of Kp on intracellular amastigotes

To address the question as to whether the protective effect of Kp extract observed *in vivo* on experimental leishmaniasis (Da-Silva *et al*. 1995) was related to its anti-leishmanial action, macrophages were treated with 0–500 μg/ml of Kp extract before and after

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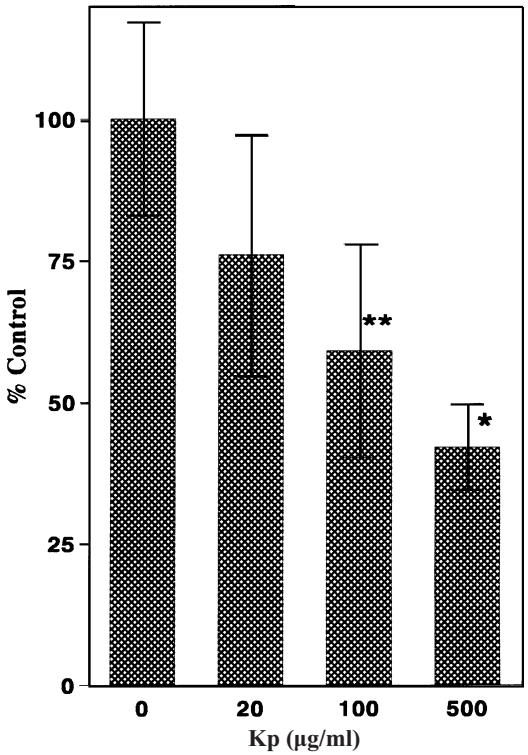

Fig. 1. Effect of Kp on intracellular amastigotes. Peritoneal macrophages of normal mice were incubated with the indicated concentrations of Kp 24 h before and after infection. Monolayers were stained with Giemsa and amastigote numbers were counted under optical microscopy. Controls were infected macrophages incubated with medium alone (5 amastigotes/MO = 100 %). Mean ± S.D. ($n = 8$). Asterisk indicates $P < 0{\cdot}01$ (*) and $P < 0{\cdot}05$ (**) in relation to controls (0 µg/ml).

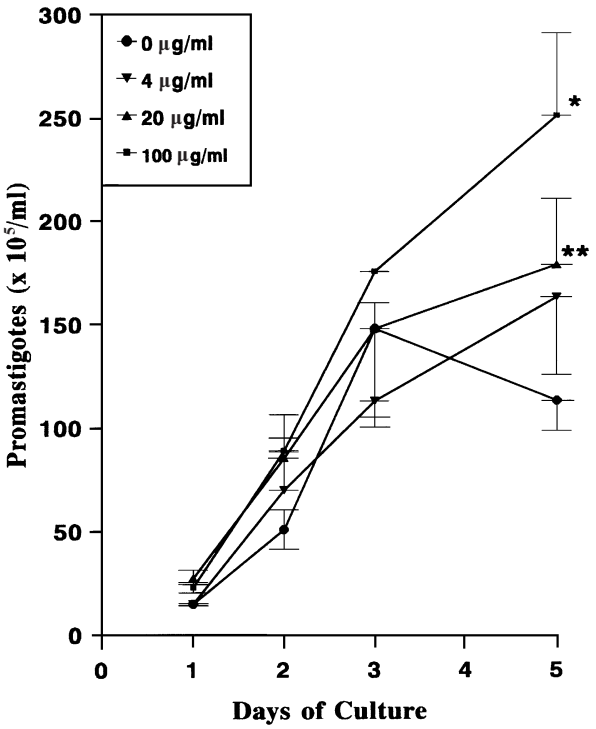

Fig. 2. Effect of Kp on promastigotes. Promastigote forms of La (1×10^5 parasites/ml) were incubated with the indicated concentration of Kp diluted in DMEM plus 10 % HIFCS at 26 °C. The parasite numbers were counted daily in a Neubauer chamber. Mean ± S.D. ($n = 3$). Asterisks indicate $P < 0{\cdot}01$ (*) and $P < 0{\cdot}05$ (**) in relation to controls (0 µg/ml).

leishmanial infection. Fig. 1 shows a dose-related inhibition of intracellular parasite growth, with the number of parasites 42 % of the control at 500 µg/ml Kp. This finding correlates well with the decreased parasite load observed before in Kp-treated mice (Da-Silva *et al*. 1995).

Effect of Kp extract on promastigote forms

To assess if the anti-leishmanial effect of Kp observed in intracellular amastigotes forms was also demonstrated in promastigotes forms, the promastigotes were cultivated in medium with 10 % HIFCS in the presence of several concentrations (0–100 µg/ml) of Kp extract for 5 days at 26 °C. Contrary to what was expected, Kp increased the growth of promastigotes, particularly at the higher concentration of 100 µg/ml, demonstrating a differential effect on the two forms of the parasites (Fig. 2).

Effect of Kp extract on nitric oxide (NO) production

To investigate whether the decrease in intracellular amastigote growth was due to the stimulation of antimicrobial activity by macrophages, the nitric oxide production was evaluated, as this is known as their most important leishmanicidal mechanism (Liew *et al*. 1990). Macrophages were incubated with Kp (0–500 µg/ml) with/without 50 U/ml IFN-γ 24 h before and after leishmanial infection. We observed a significant increase in the NO production by infected macrophages cultured in the presence of Kp (Fig. 3 B), an effect which was synergistic with IFN-γ (Fig. 3 A). This result indicates that activation of NO production by macrophages may be responsible for the reduced parasite growth in the presence of Kp.

Control for possible LPS contamination in the Kp

LPS is a potent inducer of NO production by macrophages when associated with a second signal (Cunha *et al*. 1993). To evaluate whether the increased NO production observed in infected macrophages treated with Kp (Fig. 3) was due to LPS contamination, we used Polymyxin B, an antibiotic which neutralizes LPS by binding to its lipid A portion (Storn *et al*. 1977; Lasfargues *et al*. 1989). Then, macrophages were incubated 24 h before and 24 h after leishmanial infection with 500 µg/ml Kp that was treated with 1 µg/ml Polymyxin B for 30 min before use. Pilot experiments demonstrated that this concentration of Polymyxin

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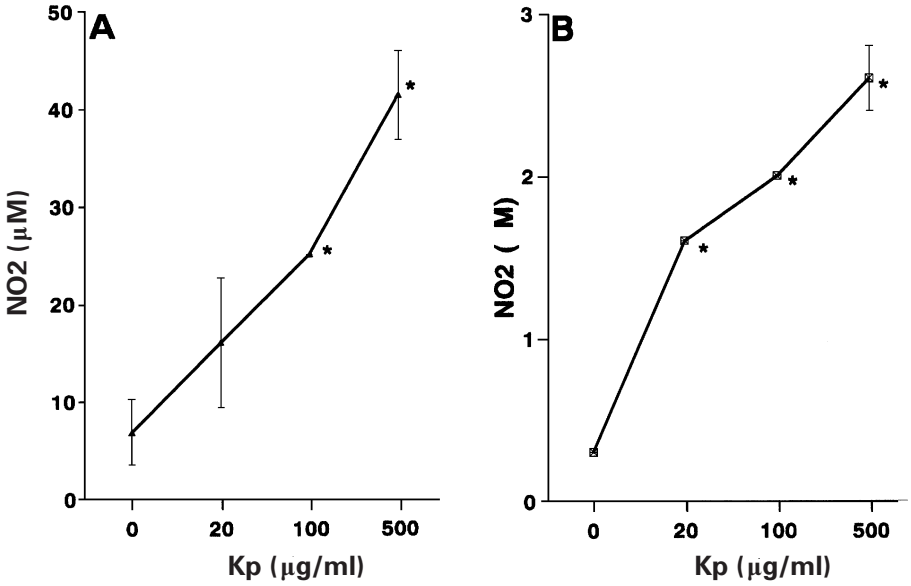

Fig. 3. Effect of Kp on nitric oxide (NO) production. Peritoneal macrophages were incubated with the indicated concentrations of Kp in the presence (A) or absence (B) of 50 U/ml of IFN-γ 24 h before and after La infection. After 24 h, NO production in the supernatants was measured by assaying for nitrite, using the Griess reagent. Nitrite production of infected macrophages incubated with medium only (controls) was $0{\cdot}3 \pm 0{\cdot}03$ μM. Mean $\pm$ S.D. ($n = 3$). Asterisks indicate $P < 0{\cdot}01$ in relation to controls.

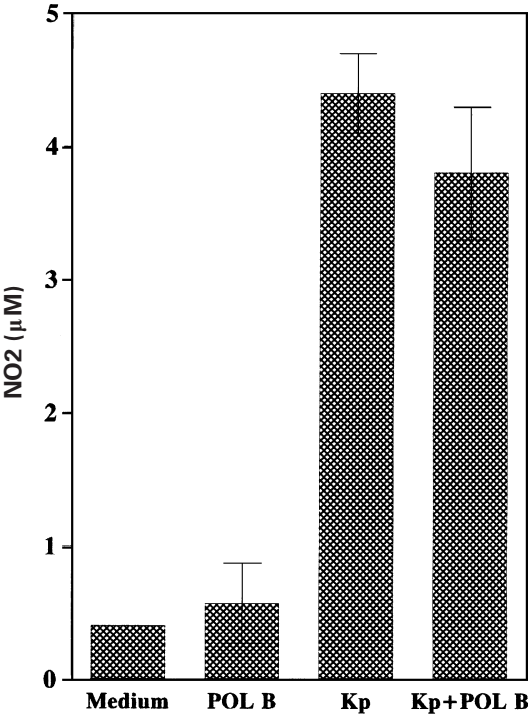

Fig. 4. Assessment of possible LPS contamination of Kp. Normal peritoneal macrophages were incubated 24 h before and after La infection with 500 μg/ml of Kp pre-treated for 30 min with 1 μg/ml Polymyxin B (Pol B). Nitrite in the supernatants was measured by Griess reagent. Controls were infected macrophages incubated with medium or 1 μg/ml of Pol B or 500 μg/ml Kp. Mean $\pm$ S.D. ($n = 3$).

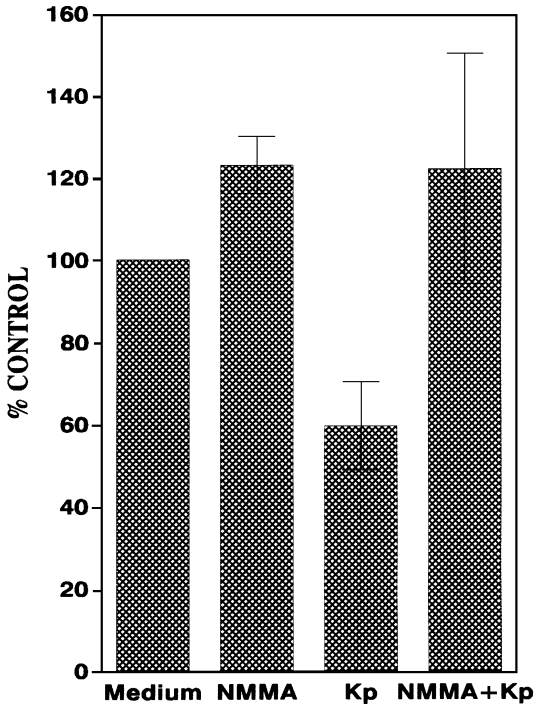

Fig. 5. Effect of the NOS inhibitor NMMA on the anti-leishmanial action of Kp *in vitro*. Macrophages were infected with La and incubated with 500 μg/ml of Kp in the presence or absence of 100 μM NMMA for 48 h. The monolayers were stained with Giemsa and intracellular amastigotes were counted under optical microscopy. Controls were infected macrophages incubated with medium alone (6 amastigotes/macrophage = 100%) or NMMA alone. Mean $\pm$ S.D. ($n = 2$).

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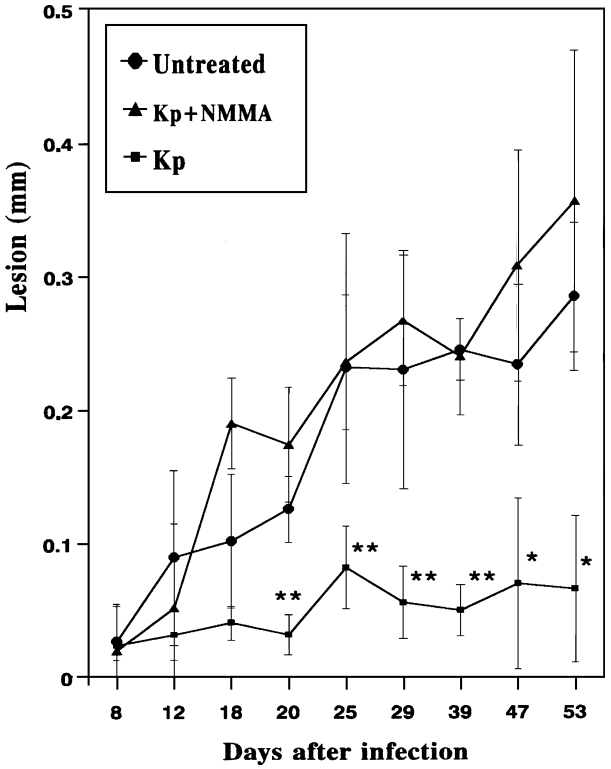

Fig. 6. Effect of NO inhibitor NMMA on the protective effect of Kp *in vivo*. BALB/c mice were infected in the footpad with 4×10^6 La promastigotes. After 7 days 2 groups were daily treated with 8 mg Kp by the oral route. After 15 days, one of the groups continued on Kp only while the other started receiving also 300 μg NMMA s.c. on alternate days, as indicated. Controls were left untreated. Lesion size was measured with a dial calliper. Mean $\pm$ S.E. ($n = 6$). Asterisks indicate $P < 0{\cdot}01$ (*) and $P < 0{\cdot}001$ (**) in relation to controls.

B was enough to block the NO-inducing effect of up to 10 ng/ml LPS. Higher antibiotic concentrations showed toxicity for the macrophages, as observed by cell rounding (data not shown). The supernatants of cultures were then assessed for nitrite concentration. The results in Fig. 4 show that optimal concentration of Polymyxin B did not affect NO production induced by Kp, demonstrating that this effect was not due to LPS contamination.

Inhibition of NO reverts the anti-leishmanial effect of Kp in vitro

To investigate whether NO induction was important in the anti-leishmanial effect of Kp, infected macrophages were incubated with Kp in the presence or absence of the NO inhibitor NMMA for 48 h. Controls were infected macrophages incubated with medium or NMMA only. Fig. 5 shows that the decrease of amastigote growth with Kp was reverted with NMMA. This result indicates that induction of NO production by macrophages is an important mode of anti-leishmanial action of Kp.

Importance of NO in the protective effect of Kp in vivo

To analyse the importance of NO in the protective effect of Kp previously demonstrated in experimental leishmaniasis (Da-Silva *et al*. 1995), BALB/c mice were infected with La and at day 7 of infection were orally treated by daily doses of 8 mg Kp. One week after initiation of treatment, 1 of the groups received intralesional injections of 300 μg NMMA (an inhibitor of NO production) every other day. We confirmed that lesion growth in the group treated with Kp was significantly ($P < 0{\cdot}01$) suppressed in relation to untreated controls. However, when simultaneously treated with NMMA, Kp was ineffective in controlling lesion growth (Fig. 6). This result indicates the importance of NO induction in the mode of action of Kp in experimental leishmaniasis. Another control group treated with intralesional NMMA alone had lesion sizes similar to the untreated group (data not shown for clarity), indicating that NMMA itself did not alter the normal course of infection.

DISCUSSION

We had previously demonstrated that the aqueous extract of Kp given by the oral route suppressed cellular immune responses and protected mice infected with La, reducing the lesion size and the number of parasites for at least 30 days after treatment withdrawal (Da-Silva *et al*. 1995). In the present work, we attempted to delineate the mode of action of Kp against the parasites, with emphasis on the production of NO.

The reduction of intracellular infection by Kp was accompanied by an increased production of NO, suggesting activation of microbicidal mechanisms of macrophages. Despite being active on its own, Kp could also further enhance the NO-inducing activity of IFN-γ in a synergistic manner. This finding is quite relevant. An important mechanism evolved by the parasites to evade the host immune defences is to lower the capacity of macrophages to mount an effective NO production and thus prevent their destruction. The assumption that the inhibition of parasite growth by Kp occurs through activation of macrophages is supported by the observation that Kp does not inhibit axenically growing promastigotes. On the contrary, it even enhances their growth in a dose-related way. The growth stimulation may be related to the high levels of glucose in the Kp extract (Lucas & Machado, 1946), which is the major energy source for promastigotes (Tielens & Van Hellemond, 1998).

Both amastigotes and particularly the promastigotes are sensitive to the damaging effects

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of NO (Mauel & Ransijn, 1997), which acts probably by triggering iron loss from enzyme(s) with iron-sulphur prosthetic groups, in particular aconitase (Lemesre *et al*. 1997) or by regulating the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity of the parasites (Bourguignon, Alves & GiovanniDeSimone, 1997). NO donors are likely to become an important new class of anti-leishmanial drugs (Zeina, Banfield & Al Assad 1997; Lopez-Jaramillo *et al*. 1998). However, the observation that only intracellular parasites were inhibited by Kp suggests that the extract may not serve as an NO donor. NO donors such as sodium nitroprusside may require an acidic pH. This condition is provided with higher concentrations of Kp (500 μg/ml) when the culture pH is slightly acidic (pH 6), with no decrease in the promastigote growth. Also, the possibility that putative NO donors in Kp interacted with serum proteins in the promastigote cultures to form nitrosyl groups should be considered, but the same serum concentration was applied to macrophage cultures. Rather, Kp may possess macrophage-activating substances unrelated to lipid A, which is the active site of the potent NO-inducer, LPS. This was evidenced by the lack of effect of lipid A-neutralizing Polymyxin B co-cultured with Kp prior to addition to macrophages. The importance of nitrogen oxidative intermediates in the host control of leishmaniasis is supported by the observations that disruption of iNOS gene renders mice highly susceptible to *L. major* infection (Liew, Wei & Proudfoot, 1997), particularly in the first stages of infection (Huang *et al*. 1998). We used an inhibitor of the NOS enzyme, the L-NMMA, to determine the importance of NO in the mode of action of Kp and found that treatment of infected mice with L-NMMA reverted the protective effect of Kp *in vivo*.

Increased production of IL-10 and IL-4 cytokines reflects a bad prognosis in mice infected with *L. major*. This may be due to inhibition of the NO production by macrophages with consequent inhibition of intracellular killing of the parasites (Vouldoukis *et al*. 1997). The possibility that Kp selectively affects IL-4 and IL-10-producing TH2, favouring IFN-γ producing NK and TH1 cells *in vivo* is not yet known, but modulation of TH1 and TH2 cells should be considered, once Kp has been shown to suppress certain T cell and B cell functions, such as unresponsiveness to specific antigen and lower production of specific antibodies in mice immunized with ovoalbumin (Rossi-Bergmann *et al*. 1994). Moreover, increased production of NO has been associated with suppression of immune responses in different systems (Kawabe *et al*. 1992; Fecho *et al*. 1994; Stefani, Muller & Louis, 1994; Abrahamsohn & Coffman, 1995) and may involve apoptotic cell death, as reported for murine trypanosomiasis (Martins *et al*. 1998). On the other hand, in higher concentrations NO may regulate the immune responses mediated by TH1 cells (Taylor-Robinson, 1997) which will in turn produce IFN-γ with feedback increase in the NO production by macrophages (Munder, Eichmann & Modolell, 1998). General immunosuppression is associated with protection in susceptible mice infected with *Leishmania* (Howard, Hale & Liew, 1981; Behforouz & Wenger, 1988; Silva, Bertho & Mendonça, 1994). Therefore, it seems possible that the increased production of NO by Kp could protect mice against progressive leishmaniasis by acting in 2 directions: (i) by inducing direct parasite killing by the macrophages and (ii) by suppressing unwanted immune responses.

Kp treatment was effective even 7 days after initiation of infection, when the lesions were already established. *In vitro* experiments demonstrate that the addition of 500 μg/ml Kp 4 h after infection of macrophages reduced the intracellular parasite load by 42%, a rate slightly lower than observed when the cells were incubated with the same concentration of Kp *prior* to infection to achieve a maximum effect, i.e. 60%. Altogether, these findings indicate that pre-activation of macrophages before infection is not really necessary, but may improve its anti-leishmanial effect. It is possible therefore that its effect *in vivo* is further enhanced if it is prophylactically administered before infection.

We are currently attempting to identify the active substance(s) in Kp which has IFN-γ or LPS-like effects with respect to NO-induction by macrophages and consequent leishmanial killing. Our previous observations that oral doses of Kp higher than employed in the present study do not significantly alter the mortality rate nor the serum levels of enzymes related to kidney and liver toxicity (Dantas *et al*. 1994) suggests that Kp acts in a much safer manner than LPS *in vivo*, and that its potential use as an adjuvant for the treatment of leishmaniasis should be explored, for example as an affordable substitute for IFN-γ in the therapy of the disease (reviewed by Berman, 1997) or the long-sought 'non-toxic LPS'.

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