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# The Macrophage-Activating Tetrapeptide Tuftsin Induces Nitric Oxide Synthesis and Stimulates Murine Macrophages To Kill *Leishmania* Parasites In Vitro

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**The macrophage-activating tetrapeptide tuftsin was able to activate, in a dose-dependent manner, murine macrophages to express nitric oxide (NO) synthase and to produce NO. Tuftsin required lipopolysaccharides for the optimal induction of NO production and synergized with gamma interferon in the induction of NO synthesis. Tuftsin-dependent NO production was sensitive to inhibition by dexamethasone and the NO synthase specific inhibitor L$^G$*N*-monomethylarginine (L-NMMA). Murine peritoneal macrophages activated by tuftsin were able to kill the amastigotes of the intracellular protozoan parasite *Leishmania major* in vitro.**

Tuftsin is a tetrapeptide (H-Thr-Lys-Pro-Arg-OH), encompassing residues 289 to 292 of the heavy chain of leukokinin (6). It influences a number of macrophage functions including phagocytosis, motility, pinocytosis, and chemotaxis (1, 21). In addition, it can significantly potentiate the bactericidal and tumoricidal activities of macrophages (3, 23). Recent studies have shown that nitric oxide (NO) is a potent antimicrobial agent (15). Nitric oxide is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine (25, 27) by an NADPH-dependent enzyme, NO synthase (NOS). Macrophages stimulated by gamma interferon (IFN-γ) (8), tumor necrosis factor (TNF) (8, 9), and migration inhibition factor (7) express high levels of NOS and produce large amounts of NO. In murine leishmaniasis, NO plays a major effector role in the host resistance to *Leishmania major* in vitro (12, 16) and in vivo (17).

In this study we have investigated the effectiveness of tuftsin on the ability of macrophages to produce NO and to develop leishmanicidal activity.

Female BALB/c mice (10 to 12 weeks old) used in the present study were obtained from inbred colonies maintained at the Institute of General Pathology, University of Palermo, Palermo, Italy. *L. major* MRHO/SU/59/P (LV39) was maintained by continuous passages in BALB/c mice. The maintenance, cultivation, and isolation of the promastigote stage of *L. major* have been described in detail previously (5). Lipopolysaccharides (LPS) were obtained from Sigma (Poole, United Kingdom), recombinant IFN-γ was obtained from Genzyme Corporation (Boston, Mass.), acetate salt tuftsin was obtained from Sigma, and dexamethasone was obtained from Merck Sharp & Dohme (Rahway, N.J.).

Peritoneal exudate cells were collected in complete RPMI 1640 culture medium (Flow Laboratories, Hertfordshire, United Kingdom) from BALB/c mice injected intraperitoneally 3 days previously with 3 ml of a 2% sterilized, hydrolyzed starch solution (BDH Chemicals, Poole, United Kingdom). Adherent cells were cultured with various stimuli, as detailed in the legends to the figures, and at various time intervals culture supernatants were collected for NO and cytokine measurements. After being washed and treated with extraction buffer (250 μl of 0.1 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4] with 1 μM dithiothreitol [Sigma]), the cells were frozen and thawed three times and then harvested with a rubber policeman. They were transferred into Eppendorf tubes and centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was tested for NOS activity.

TNF was determined by enzyme-linked immunosorbent assay commercial kits (Genzyme). $NO_2^-$ in the culture supernatant was determined by the Griess reaction (8). NOS activity was determined by the oxyhemoglobin assay as described by Feelisch and Noack (11), and the results were expressed as picomoles of NOS generated per milligram of protein per minute.

For the leishmanicidal activity assay (4), adherent peritoneal cells plated at $10^5$ cells per 0.1 ml of RPMI in 96-well plates (Nunc, Roskilde, Denmark) were incubated with 100 μl of medium containing various substances as indicated in the legends to the figures. Cultures were incubated for a further 24 h before the addition of $10^5$ *L. major* promastigotes in 100 μl of culture medium to each well. The mixture was cultured for another 72 h and afterwards washed extensively with prewarmed medium. To each well, 100 μl of 0.01% sodium dodecyl sulfate solution was added in serum-free medium at 37°C for 20 to 30 min. Schneider's medium (GIBCO, Ltd, Paisley, Scotland) supplemented with 30% fetal calf serum and L-glutamine was added (100 μl per well), and the cultures were incubated at 28°C for another 72 h. The cultures in three to six replicates were then pulsed with 1 μCi of [$^3$H]thymidine (26 Ci/nmol; Radiochemical Center, Amersham, United Kingdom) per well, and the incorporation of radioactivity by viable parasites after 18 h of further culturing was determined as described above.

Statistical significance ($P < 0.05$) was analyzed by Student's *t* test. All experiments were carried out three or four times. Data are presented as the means ± 1 standard error of the means of quadruplicate cultures of a single representative experiment.

**Effect of tuftsin on NO synthesis.** Peritoneal macrophages were incubated with different doses of tuftsin (0.1 to 1,000 ng/ml) in the presence of various concentrations of LPS for 72

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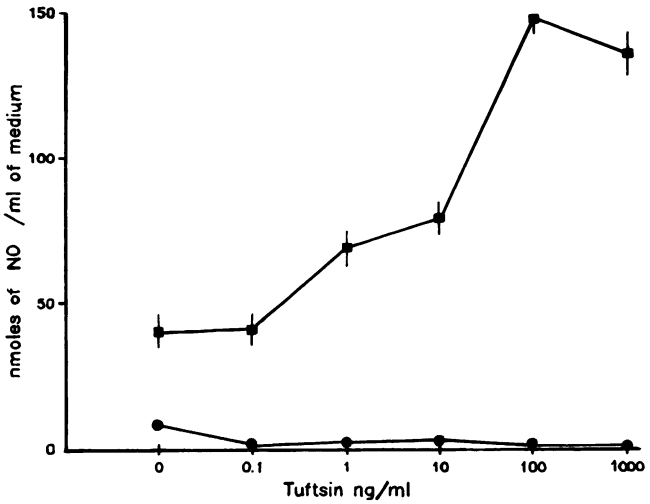


FIG. 1. Levels of $NO_2^-$ produced by peritoneal macrophages after 72 h of culturing with LPS (1 μg/ml [■] or 10 ng/ml [●]) in the presence of different doses of tuftsin.

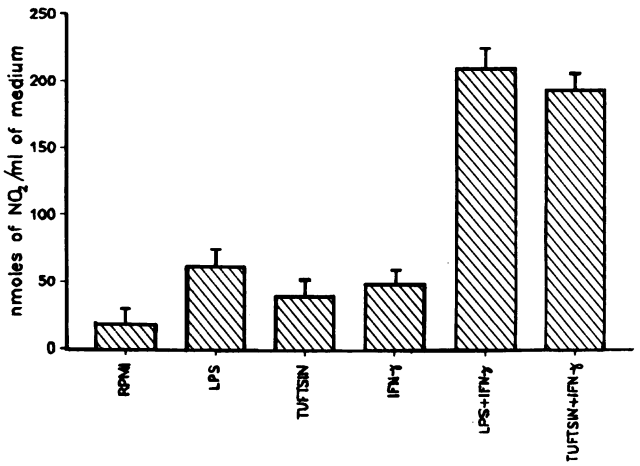


FIG. 2. Tuftsin replaces the effect of LPS in IFN-γ-induced NO synthesis by peritoneal macrophages after 72 h of culturing with IFN-γ (10 U/ml) with or without the addition of either 10 ng of LPS per ml or 100 ng of tuftsin per ml. Results are the means for four experiments. Error bars indicate standard errors of the means.

h, and the supernatants were tested for NO synthesis. Tuftsin enhanced NO production (measured as $NO_2^-$) induced by suboptimal amounts of LPS (1 μg/ml) in a dose-dependent manner. The enhancement levelled off at a tuftsin concentration of >100 ng/ml. However, low concentrations of LPS, or tuftsin alone, were ineffective, indicating dependency on an optimal LPS dose for the induction of NO synthesis by tuftsin (Fig. 1). When peritoneal macrophages were stimulated with optimal amounts of LPS (40 μg/ml), tuftsin (0.1 μg/ml) plus LPS was unable to increase NO production further (means ± standard errors of the means of 150 ± 9 versus 146 ± 6 nmol/ml). The results of accurate titration experiments in vitro, using different doses of tuftsin plus LPS and different incubation times, suggested that tuftsin-enhanced NO production was first detectable 24 h after the addition of tuftsin plus LPS and peaked at 72 h. NO synthesis induced by tuftsin was comparable to that induced by 10 to 20 U of IFN-γ per ml plus 10 ng of LPS per ml (140 ± 8 versus 158 ± 7 nmol/ml).

**Tuftsin replaces the effect of LPS in IFN-γ-induced NO synthesis.** Inasmuch as IFN-γ-induced NO synthesis is well defined in the murine macrophage system and IFN-γ requires LPS for the optimal induction of NO production, we investigated whether tuftsin could replace the effect of LPS in IFN-γ-induced NO synthesis. Peritoneal cells stimulated with IFN-γ (10 U/ml) plus tuftsin (100 ng/ml) produced levels of NO comparable to the levels produced by cells stimulated with IFN-γ (10 U/ml) plus LPS (10 ng/ml) (Fig. 2). Therefore, taken together, the above results suggest that tuftsin can synergize with IFN-γ and replace the effect of LPS. To determine whether the synergistic effect of tuftsin with IFN-γ and LPS was consistent with the effect on other cytokines, peritoneal cells were treated with tuftsin (100 ng/ml) plus LPS (1 μg/ml), and culture supernatants were collected for TNF measurement. The data indicated that tuftsin significantly increased TNF secretion with respect to data for untreated controls (1,100 ± 100 versus 760 ± 60 pg/ml).

**Sensitivity of tuftsin-induced NO synthesis to dexamethasone and L-NMMA treatment.** To investigate whether the tuftsin effect on NO synthesis was mediated by the activation of NOS, peritoneal exudate cells were incubated with LPS in the absence or presence of 100 ng of tuftsin per ml in the presence of 20 μM dexamethasone or 500 μM L-$N^G$-monomethylarginine (L-NMMA). Dexamethasone treatment significantly inhibited the induction of NOS and NO production by tuftsin plus LPS (4 ± 0.5 versus 120 ± 6 pmol/mg of protein per min and 5 ± 2 versus 70 ± 3 nmol of $NO_2^-$ per ml, respectively). Furthermore, while L-NMMA, the substrate inhibitor of NOS, abrogated NO secretion induced by tuftsin (0.5 ± 0.5 versus 72 ± 6 nmol/ml of $NO_2^-$ per ml), L-arginine treatment reversed the inhibitory effect of L-NMMA (55 ± 10 versus 72 ± 6 nmol of $NO_2^-$ per ml).

**Effect of tuftsin on the leishmanicidal activity.** As the macrophage killing of the intracellular *L. major* is mediated by NO, we next examined the effect of tuftsin on the viability of amastigotes within infected macrophages. Peritoneal exudate cells activated with LPS (1 μg/ml) or IFN-γ (10 U/ml) were incubated with increased doses of tuftsin and infected with *L. major* promastigotes 24 h later. Parasite survival was estimated by [$^3$H]thymidine incorporation by residual parasites. Macrophages treated with 10 or 100 ng of tuftsin per ml acquired enhanced ability to kill intracellular parasites in the presence of both LPS and IFN-γ, although the effect was more intense with the latter (Fig. 3). The indirect effect of tuftsin was confirmed by the observation that, at the concentration used, the compound did not have any direct effect on the viability of the parasite in vitro. Uninfected macrophages cultured with the various concentrations of the compounds used for the same period remained viable (>99%) as examined by a dye exclusion assay.

The results presented in this paper confirm that tuftsin exerts antimicrobial effects in vitro through the stimulation of phagocytic cells, particularly the macrophages (1, 3, 6, 21, 23, 26). The microbicidal effects of this compound has been explained so far on the basis of its capacity to augment considerably the production of TNF (32) and three highly reactive oxygen compounds, superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^\cdot$) (13, 14, 30). We now report for the first time that tuftsin in the presence of low concentrations of LPS can activate macrophages to express high levels of NOS and synthesize large amounts of NO in vitro, and these cells thus become effective in killing intracellular pathogens such as *L. major*. The relative dependence on

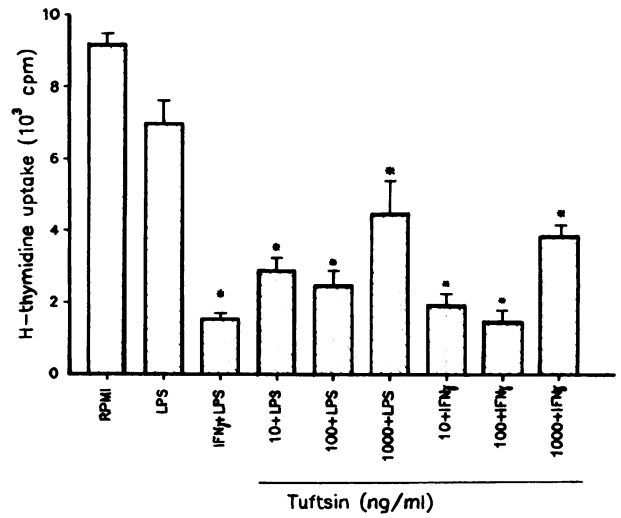


FIG. 3. Effect of tuftsin on leishmanicidal activity in vitro. Peritoneal cells were treated with LPS alone (1 μg/ml), IFN-γ (10 U/ml) plus LPS (10 ng/ml), and various doses of tuftsin in the presence of 1 μg of LPS per ml or 10 U of IFN-γ per ml for 24 h and then infected with *L. major* promastigotes. Results are the means from three experiments. Error bars indicate the standard errors of the means. *, $P < 0.05$.

LPS for the induction of NO synthesis by tuftsin is similar to that by IFN-γ (7), with which the tetrapeptide can synergize. Altogether, the results indicate that tuftsin acts as a costimulator. On the other hand, this potential effect has already been demonstrated in studies on in vitro myelopoiesis, in which tuftsin exercised a costimulatory activity with colony-stimulating factor 1 (20). However, since the compound can replace the effect of LPS in IFN-γ-induced NOS expression and NO synthesis, it may be possible that tuftsin-activated macrophages secrete other substances that act as cofactors with tuftsin in the induction of NO synthesis. It is also possible that the effect of tuftsin is to increase TNF secretion which, in turn, synergizes with LPS and IFN-γ to induce NOS. We have demonstrated here that tuftsin-mediated NO synthesis is inhibited by the addition of dexamethasone, in a manner similar to that observed for migration inhibition factor and IFN-γ-induced NO synthesis (7). Since dexamethasone affects transcriptional, translational, and posttranslational processes involved with protein synthesis, processing, and secretion (2, 10), the macrophage activation for NO synthesis by tuftsin, IFN-γ, and MIF may share common steps. The induction of NO synthesis by tuftsin is sensitive to the action of the substrate analog inhibitor of NO synthase, L-NMMA. The biological significance of the effect of tuftsin on NO synthesis was shown by our leishmanicidal activity experiments. The compound was able to potentiate the ability of macrophages from the highly susceptible BALB/c mice to kill *L. major* amastigotes. The susceptibility to *L. major* in a murine model has been shown to be related to the relative inability of the host macrophages to synthesize an adequate amount of NO (16). The results which we obtained indicate that tuftsin stimulates the microbicidal activity of macrophages, increasing the threshold level of NO necessary for the effective elimination of intracellular parasites (16). Tuftsin activity has also been confirmed in vivo (18, 24, 28). It has been demonstrated that macrophages from mice treated with tuftsin and infected with *Lysteria monocytogenes* had 10-times-higher killing ability than did untreated controls (18). Similar results have also been obtained with *Candida albicans* infection (24). Furthermore, tuftsin was used in the treatment of rabbit microbial keratitis, and it was demonstrated to be valuable as an adjunct to antibiotic therapy (28). Thus, tuftsin appears to have a potential as a drug for the treatment of diseases caused by intracellular pathogens, and its use should be further explored, particularly by testing stabilized tuftsin (31). In fact, enzymatic degradation of tuftsin strongly reduces its stimulating effect on immunocompetent cells (29, 31) and produces biologically active tripeptides (H-Thr-Lys-Pro-OH and H-Lys-Pro-Arg-OH) that can inhibit tuftsin activity (29, 31).

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