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Phagocytosis and induction of nitric oxide synthase in murine macrophages

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SUMMARY

The murine macrophage cell line, J774, produced little or no detectable levels of nitric oxide (NO) when stimulated with interferon-γ (IFN-γ) alone *in vitro*. However, they expressed high levels of NO synthase and produced large amounts of NO when cultured with IFN-γ in the presence of lipopolysaccharide (LPS). The synergistic action of LPS can be replaced by ingestion by the macrophages of zymosan, *Staphylococcus aureus* or *Leishmania major* in a dose-dependent manner. In contrast, the ingestion of particles such as latex beads or silica in the presence of IFN-γ did not lead to the induction of NO synthase activity. Furthermore, ingestion of ink particles significantly reduced the ability of the macrophages to express NO synthase in response to the optimal stimulation of IFN-γ and LPS. These results therefore demonstrate that phagocytosis *per se* is not sufficient to provide the additional signal for the induction of NO synthase activity in macrophages by IFN-γ, and that the ingestion of certain particles can lead to the paralysis of the expression of this enzyme.

INTRODUCTION

Recent studies have demonstrated a crucial role of nitric oxide (NO) in the anti-microbial and tumouricidal activity of murine macrophages (reviewed in refs 1–5). NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by the NADPH-dependent enzyme NO synthase, of which there are two types (reviewed in ref. 6). One is constitutive, Ca^{2+}/calmodulin dependent and is found mainly in the brain, endothelial cells and platelets. The other is inducible, Ca^{2+} independent and is found in a variety of sites, including smooth muscle and macrophages. The activity of both enzymes can be specifically inhibited by some L-arginine analogues, and the induction of the inducible enzyme can be abrogated by glucocorticoids such as dexamethasone.[6] However, the mechanism of the induction of NO synthase expression is unclear.

Cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor (TNF) are able to induce the expression of NO synthase. However, significant levels of this enzyme are induced by these cytokines only in the presence of lipopolysaccharide (LPS) which presumably provides a co-signal. In addition heat-killed bacillus Calmette–Guérin (BCG),[7] *Listeria*,[8] *Leishmania*[9] or Gram-positive exotoxins[10] can also synergize with these cytokines to induce the expression of NO synthase in murine macrophages. A previous report[11] suggested that phagocytosis, including the ingestion of inert particles such as latex beads, was able to substitute the effect of LPS in the induction of NO synthase activity in murine macrophages. In contast, another study[12] showed that ingestion of zymosan or latex beads was ineffective. As understanding of the signalling pathways is crucial in the regulation of the synthesis of NO, which plays a central role in a variety of biological functions, we investigated the effect of phagocytosis in the induction of NO synthesis in macrophages. Data presented here demonstrate that phagocytosis *per se* is not sufficient as a co-signal for the induction of NO synthase in murine macrophages. Furthermore, ingestion of certain particles can inhibit the induction of this enzyme.

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MATERIALS AND METHODS

Materials

Murine recombinant IFN-γ was kindly provided by Dr G. Adolf (Ernst Boehringer-Institut für Arzneimettel-Forschung, Vienna, Austria). LPS from *Escherichia coli* (026.B6) was obtained from Difco (West Molesey, U.K.). Zymosan (cat. no. Z4250), silica (average diameter 1–5 μm; cat. no. S5631), and polystyrene latex particles (diameter 1.1 μm; cat. no. LB 11) were purchased from Sigma (Poole, U.K.). Zymosan was suspended in water and boiled for 10 min, followed by centrifugation (10 min, 1000 ***g***) and resuspended in medium. In the experiments using opsonized zymosan, it was incubated with fresh normal rabbit serum (10 mg zymosan/ml of plasma) for 30 min at 37°, followed by centrifugation. Black Indian ink was dialysed against phosphate-buffered saline (PBS) overnight before use. The bacteria was grown to stationary phase (about 10^9 bacteria/ml) and killed with formalin. Appropriate dilutions were made on the day of the experiment. All tissue culture reagents were purchased from Gibco (Paisley, U.K.). All other reagents were purchased from Sigma. Culture medium was RPMI-1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomy-

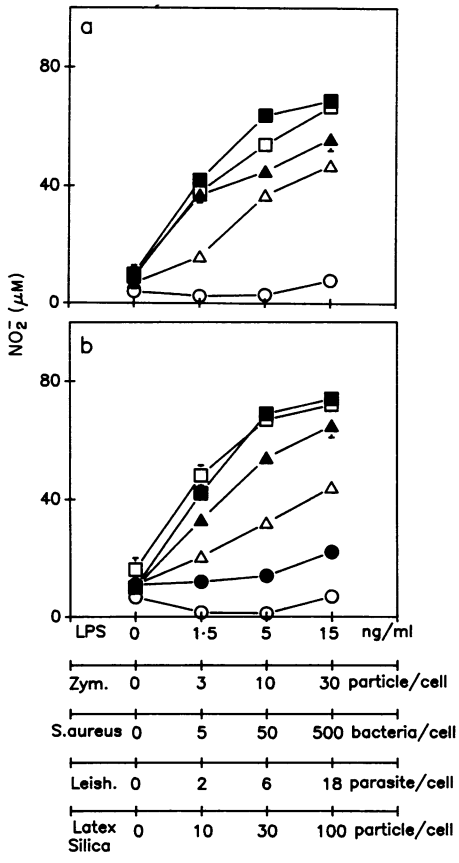

Figure 1. Levels of NO_2^- produced by peritoneal macrophages (a) and J774 cells (b) cultured for 24 hr with IFN-γ (10 U/ml) and silica (○), latex beads (●), *L. major* (△), *S. aureus* (▲), zymosan (□) or LPS (■) in the indicated concentrations. The points 0 on the *x*-axis represent cultures with IFN-γ only. Vertical bars = 1 SEM, $n = 3$. Results are representative of three experiments.

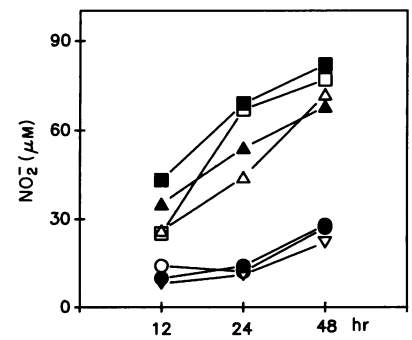

Figure 2. Levels of NO_2^- produced by J774 cells cultured for 12, 24 or 48 hr with IFN-γ [10 U/ml (▽)] alone, silica (30 particles/macrophage) + IFN-γ (○), latex beads (30 particles/macrophage) + IFN-γ (●), *L. major* (18 parasites/macrophage) + IFN-γ (△), *S. aureus* (50 bacteria/macrophage) + IFN-γ (▲), zymosan (10 particles/macrophage) + IFN-γ (□), and LPS (5 ng/ml) + IFN-γ (■). Vertical bars = 1 SEM, $n = 3$. Results are representative of two experiments. Vertical bars are obscured by the symbols.

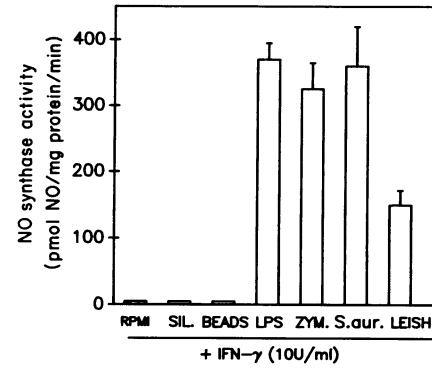

Figure 3. NO synthase activity in J774 cells cultured for 24 hr with medium (RPMI) alone, silica (Sil, 30 particles/ml), latex beads (Beads, 30 particles/macrophage), LPS (5 ng/ml), zymosan (Zym, 10 particles/macrophage), *S. aureus* (S. aur., 50 bacteria/macrophage), and *L. major* (Leish., 18 parasites/macrophages). IFN-γ (10 U/ml) was added into all cultures. Vertical bars = 1 SEM, $n = 3$. Results are representative of two experiments.

cin. The maintenance, cultivation and isolation of the promastigote stage of the parasite *L. major* (LV39) has been described in detail elsewhere.[13] Seven-day stationary phase culture of less than five *in vitro* passages was used. Live parasites were used in all experiments.

Macrophages

The murine macrophage cell line J774 was obtained from the American Tissue Culture Collection [(ATCC) Rockville, MD]. Murine peritoneal macrophages were harvested from CBA mice (Harlan Olac Ltd, Bicester, U.K.) which had been injected i.p. 4 days before with 2 ml of sterile thioglycollate solution (3% w/v in PBS). The cells were maintained in culture medium.

Induction of NO synthase activity and NO_2^- synthesis

J774 cells and murine peritoneal macrophages were cultured for 2 hr at 37° in an atmosphere of 5% CO_2 in 12-well Costar plates at 2×10^6 cells in 2 ml of culture medium. Non-adherent cells were then removed by washing and the adherent cells cultured with various stimuli as detailed in the legends to the figures. The activity of NO synthase in macrophage cytosol was determined 12 hr after activation, and NO_2^- concentration in supernatants was determined at 12, 24 and 48 hr. In some experiments IFN-γ (10 U/ml) plus LPS (5 ng/ml) with or without Indian ink (1:2000 dilution) were added to an insert chamber (Costar inserts, cat. no. 3414). This was separated by a semi-permeable membrane from the macrophages placed in the main chamber.

Measurement of NO_2^-

NO_2^- in the culture supernatant was determined by chemiluminescence as described previously.[14]

Measurement of NO synthase activity

Cells were washed with PBS 12 hr after activation, and 250 μl of 0·1 M HEPES buffer (pH 7·4 with 1 mM DTT and 1 mM EDTA) were added to each well. The cells were harvested with a disposable rubber policeman and sonicated. The samples were then centrifuged at 100,000 *g* for 10 min at 4° and the supernatant assayed for NO synthase activity using the oxyhaemoglobin assay as described by Feelisch and Noack.[15] The results were expressed as pmol of NO generated/mg protein/min.

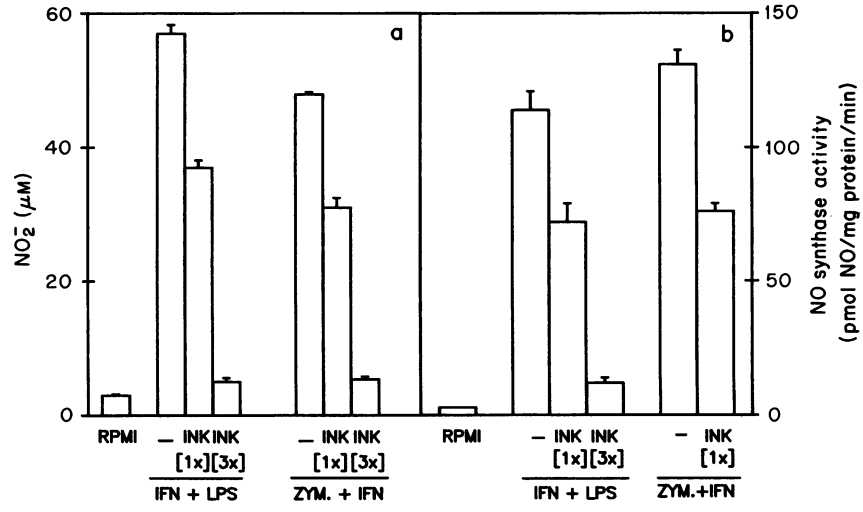

Figure 4. Effect of Indian ink on (a) the synthesis of NO_2^-, and (b) expression of NO synthase by J774 cells stimulated with IFN-γ (10 U/ml) plus LPS (5 ng/ml) or zymosan (10 particles/macrophage). The Indian ink diluted 1:670 (1 ×) or 1:2000 (3 ×) in culture medium, was added at the same time as the stimuli. NO_2^- levels and NO synthase activity were determined 24 hr later. Vertical bars = 1 SEM, $n = 3$. Results are representative of two experiments.

Statistical analysis

Statistical significance ($P < 0.05$) of variance was analysed followed by Bonferroni's *t*-test. Results are expressed as means ± 1 SEM, $n = 3$–6.

RESULTS

LPS, zymosan, killed *S. aureus* or *L. major* were able to synergize with IFN-γ to induce NO synthesis by peritoneal macrophages and J774 cells in a dose-related manner (Fig. 1). In contrast, latex or silica particles induced little or no detectable NO synthesis at up to 100 particles/macrophage in the presence of IFN-γ. None of these stimuli (same concentrations as those in Fig. 1) were able to induce significant NO synthesis in the absence of added IFN-γ (data not shown, $n = 6$). IFN-γ alone at 10 U/ml induced only a low concentration of NO_2^- (Fig. 1). Since similar results were obtained with peritoneal macrophages and J774 cells, J774 cells were used for the rest of the experiments.

For cultures stimulated with IFN-γ plus LPS, zymosan, *S. aureus* or *L. major*, significant levels of NO_2^- were detectable in the supernatants 12 hr after stimulation and increased up to 48 hr (Fig. 2). In contrast, in cultures activated with IFN-γ plus latex or silica particles, there was no significant increase in NO synthesis compared to that induced by IFN-γ alone up to 48 hr after stimulation. Microscopic examination of J774 cells (May–Grunwald stained) revealed that > 80% of the cells had ingested > 3 particles after culturing with 30:1 ratio of silica or latex beads:cell for 2 hr. About 70% of J774 cells contained > 3 zymosan/cell when similarly cultured with 10:1 ratio of zymosan:J774 cells. Thus the inability of latex beads and silica particles to induce NO synthesis is not due to the failure of J774 cells to ingest these particles.

J774 cells stimulated with LPS, zymosan or *S. aureus* in combination with IFN-γ expressed high levels of NO synthase 12 hr after stimulation. The level of NO synthase induced by *Leishmania* plus IFN-γ was significantly lower. In contrast, no NO synthase was detectable in J774 cells stimulated with silica or latex beads (up to 30 particles/cell) plus IFN-γ (Fig. 3).

J774 cells expressed high levels of NO synthase and produced large amounts of NO_2^- when activated with IFN-γ (10 U/ml) plus LPS (10 ng/ml). The enzyme activity and NO synthesis was not affected by silica (up to 100 particles/cell) added from the beginning of the culture (data not shown, $n = 3$). In contrast, the addition of Indian ink markedly inhibited the expression of NO synthase in a dose-dependent manner (Fig. 4). This is true whether the cells were activated with IFN-γ plus LPS or zymosan. Macrophages remained viable for the period of the experiment as judged by trypan blue exclusion test (data not shown). However, such inhibition was not evident when the ink was separated from the cells by a semi-permeable membrane. Comparable levels of NO_2^- (17·4 ± 1·8 versus 15·6 ± 1·6 μM $n = 6$) in the culture supernatants were obtained when J774 cells were stimulated with IFN-γ plus LPS with or without the ink placed in an insert chamber separated by a membrane from the cells in the main chamber.

DISCUSSION

In this report we have demonstrated that phagocytosis *per se* does not provide the necessary co-signal for the induction of the expression of NO synthase by IFN-γ. Thus, agents of biological origin, such as LPS, zymosan, *S. aureus* and *L. major* were able to synergize with IFN-γ in the induction of NO synthesis in murine macrophages, whereas ingestion of inert particles such as silica or latex beads were ineffective. This is also the case when these particles were opsonized by pre-incubation with fresh normal rabbit serum (data not shown). These agents were not contaminated with LPS, since polymyxin B did not modify their effect (data not shown). Recently it has been reported[11] that latex beads can synergize with IFN-γ to induce NO synthesis. The reason for the discrepancy between this and our data is not apparent, but may be due to the difference in the numbers of particles added to the cultures. In the previous study[11] approximately 1000 particles/cell was used, which in our case proved to be toxic to the macrophages. Our results using zymosan are also apparently at variance with those of others[12] who reported that

zymosan was not able to provide the co-signal with IFN-γ for the induction of NO synthesis in macrophages. This discrepancy may also be due to the difference in the amount of particles used. We routinely used 3–30 zymosan particles/macrophage compared to approximately 0·2 particles/cell in this previous study[12].

Silica and Indian ink are often used to block macrophage functions *in vitro* and *in vivo*. We therefore tested the ability of these two types of particles to influence the induction of NO synthesis in macrophages. Unlike silica, Indian ink inhibited the expression of NO synthase induced in the macrophages by IFN-γ plus LPS. This inhibitory effect was not due to absorption of IFN-γ or LPS on the ink particles since the same inhibitory effect was seen when the ink was co-cultured with IFN-γ plus zymosan. It is also unlikely that the inhibition was due to the presence of solvent in the ink preparation, because the ink preparation was extensively dialysed before use. Furthermore, the inhibitory effect was not evident when LPS and IFN-γ were added together with the ink inside an insert chamber, separated by a semi-permeable membrane from the macrophages in the main culture well. This membrane was permeable to IFN-γ, LPS and other solutes but not to the ink particles. These results suggest that the inhibitory effect required direct contact between the ink particles and the cells. The results also indicate that the mechanism involved in the blockade of the mononuclear system by both silica and Indian ink is likely to be distinct from the mechanism involved in the inhibition of NO synthase expression, which was only induced by the ink particles.

The nature of the co-stimulatory signal delivered by LPS, zymosan, *S. aureus* and *L. major* is at present not clear. As these are either products of or are themselves pathogenic organisms, it may well be that the mammalian host has evolved an effective defence mechanism, the synthesis of NO, which is only triggered by the presence of immunological stimuli (such as IFN-γ) and the products of pathogens. Such a system would ensure the production of high levels of NO only in the presence of invading pathogens, thus avoiding the potential pathological damage of excessive production of NO in response to the cytokines stimuli alone.

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