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A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice

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SUMMARY

Mice injected in the footpad with carrageenin developed local inflammation which peaked at 48 hr. This delayed-type footpad swelling was significantly reduced in mice injected intraperitoneally (i.p.) with a specific nitric oxide (NO) synthase inhibitor, L-N^{G}monomethyl-arginine (L-NMMA). The draining lymph node (DLN) cells from mice injected 48 hr previously with carrageenin produced significantly higher levels of proliferation and interleukin-1 (IL-1), IL-2, IL-6 and interferon-γ (IFN-γ), but less IL-10, compared to cells from saline-injected controls, when stimulated with concanavalin A (Con A) *in vitro*. Treatment of the carrageenin-injected mice with L-NMMA had little effect on the proliferative response of the DLN cells, but significantly reduced the production of IL-1, IL-2, IL-6 and IFN-γ, and increased the secretion of IL-10. These data demonstrate that NO plays a significant role in local inflammation and the pattern of cytokines induced in this model.

INTRODUCTION

Nitric oxide (NO) is involved in a variety of biological activities, including endothelium-related vascular relaxation, platelet aggregation and neurotransmission, as well as in the anti-microbial and tumoricidal activity of murine macrophages (reviewed in refs 1–3). NO is generated from the oxidation of the terminal guanidino nitrogen atom of L-arginine by the NADPH-dependent enzyme NO synthase, of which there are at least two types (reviewed in refs 2 and 4). One is constitutive, Ca^{2+}/calmodulin-dependent and is expressed mainly in the brain, platelets and endothelial cells. The other is Ca^{2+}/calmodulin-independent and is induced by cytokines and bacterial products. It is expressed by a variety of cell types, including macrophages, neutrophils and smooth muscle.

It is now apparent that NO generated by the inducible enzyme is cytotoxic or cytostatic for a variety of pathogens (reviewed in refs 1, 3, 5 and 6). The mechanisms of target destruction by NO are non-specific and appear to involve both reactions with Fe–S groups, leading to inactivation of enzymes in the mitochondrial electron transport chain, and, at high concentrations, inhibition of DNA replication[7] and the induction of apoptosis.[8,9] Recently, NO has been implicated in a number of pathological conditions, including streptozocin-induced diabetes,[10,11] graft-versus-host disease[12] and experimentally induced neurological diseases.[13] In most cases, the pathologies were significantly reduced through inhibiting NO synthesis *in vivo* by treating the animals with L-N^{G}monomethyl-arginine (L-NMMA).

A role for NO has also been demonstrated in inflammation. In rats, NO is involved in the acute inflammatory response following footpad injection of carrageenin[14] or the topical application of mustard oil.[15] Adjuvant arthritis, a model of chronic inflammation, was also reduced in rats treated with an NO synthase inhibitor.[16,17] In humans, serum from patients with rheumatoid arthritis or osteoarthritis contains significantly higher levels of nitrite compared to those of matched controls.[18] However, the mechanisms involved and the regulation of NO synthesis in these models are obscure.

We have investigated the role of NO in cellular function and cytokine production in a carrageenin-induced model of acute inflammation in mice. We report here that treatment of carrageenin-injected mice with L-NMMA markedly reduced the production of interleukin-1 (IL-1), IL-2, IL-6 and interferon-γ (IFN-γ) by the draining lymph node (DLN) cells *in vitro*. In contrast, the secretion of IL-10 by these cells was significantly elevated.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 6–8 weeks of age, were obtained from Harlan Olac Ltd (Bicester, U.K.). They were housed in temperature-controlled rooms and received food and water *ad libitum*.

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Materials

Lambda-carrageenin and concanavalin A (Con A) were obtained from Sigma (Poole, U.K.). L-NMMA and its enantiomer D-NMMA were kindly provided by Dr H. Hodson (Department of Medicinal Chemistry, Wellcome Research Laboratories, Beckenham, U.K.). Culture medium was RPMI-1640 (Gibco, Paisley, U.K.) containing 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM) and, for cytokine generation, 25 mM HEPES. Mink lung cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. Paired monoclonal antibodies purchased from PharMingen (A.M.S. Biotechnology Ltd, Witney, U.K.) were used for the detection of IL-2, IL-4, IL-6 and IL-10 in enzyme-linked immunosorbent assays (ELISAs). IFN-γ and tumour necrosis factor-α (TNF-α) were detected using a rat anti-murine IFN-γ (R46AT) or rat anti-murine TNF-α (XT22.11) and polyclonal rabbit antibodies, produced in our laboratory by immunization with recombinant IFN-γ or TNF-α. All murine recombinant cytokine standards were obtained from Genzyme (West Malling, U.K.), except IFN-γ and TNF-α which were the kind gift of Dr G. Adolf (Ernst-Boehringer-Institut fur Arzneimittel-Forschung, Vienna, Austria).

Induction of inflammatory response to carrageenin

Groups of mice were injected subcutaneously in one hind paw with 300 μg carrageenin in a final volume of 50 μl. Control animals were injected with the same volume of saline. Footpad swelling was measured daily using a spring-dial calliper, and expressed as the difference in swelling between the carrageenin-injected paw and the uninjected, contralateral paw. In some experiments, animals were injected intraperitoneally (i.p.) with 5 mg L-NMMA or D-NMMA 2 hr before and 24 and 48 hr after the administration of carrageenin and footpad swelling and monitored as above.

Histopathology

Uninjected and carrageenin-injected paws from both D-NMMA and L-NMMA animals were removed 48 hr after administration. The tissues were fixed in 10% buffered formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

T-cell proliferation assays

DLN were removed 48 hr after administration of carrageenin. Single-cell suspensions were obtained, resuspended at $2{\cdot}5 \times 10^6$ cells/ml, and dispensed at 100 μl/well in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark). Con A (0·5 or 5 μg/ml) was added to each well and the cultures incubated at 37°, in an atmosphere of 5% CO_2, for 48 hr. Cultures, in triplicates, were pulsed with 1 μCi/well [^{3}H]thymidine (Amersham International, Amersham, U.K.) for the final 6 hr of incubation, then harvested and counted in a β-scintillation counter (Pharmacia, Milton Keynes, U.K.).

Generation of cytokines

DLN cells obtained 48 hr after administration of carrageenin were resuspended at 10^6 cells/ml and dispensed at 1 ml/well in 24-well plates. Cultures were stimulated with Con A (5 μg/ml) and cell-free supernatants obtained at 24, 48 and 72 hr post-stimulation. Supernatants were stored at −70°.

Measurement of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α

The levels of these cytokines in culture supernatants were measured by ELISA. Flat-bottomed 96-well microtitre plates were coated with the capture monoclonal antibody (1–4 μg/ml, 50 μl/well). After an overnight incubation at 4°, the plates were blocked with 10% FCS, washed and samples or standards dispensed into each well (100 μl) and incubated at room temperature for 2–3 hr. Bound IL-2, IL-4, IL-6 and IL-10 were detected by the addition of biotinylated anti-cytokine monoclonal antibodies (1–4 μg/ml; 100 μl/well) for 1 hr at 37° and, after washing, by 2 μg/ml extravidin–peroxidase (100 μl/well; Sigma) for 1 hr at 37°. The assay was developed by the addition of 3,3′,5,5′-tetramethyl benzidine (TMB) substrate (100 μl/well; Kirkgaard & Perry Laboratories Inc., Gaithersburg, MD) and read at 630 nm on a dual-wavelength spectrophotometer (Dynatech, Billinghurst, U.K.). Bound IFN-γ and TNF-α were detected by the addition of a polyclonal rabbit anti-murine IFN-γ or TNF-α antibody (5 or 10 μg/ml respectively, 100 μl/well) for 1 hr at room temperature. Finally, sheep anti-rabbit alkaline phosphatase IgG was added at a 1:1000 dilution (100 μl/well; Sigma) for 1 hr at room temperature, the assay developed by the addition of p-nitrophenyl phosphate (1 mg/ml in 1 M Tris, 3 mM $MgCl_2$, pH 9·6, 100 μl/well), and read at 405 nm. The cytokine content of each sample was read off a standard curve established with the appropriate recombinant cytokine.

Bioassays for IL-1β and transforming growth factor-β (TGF-β)

IL-1 was measured by a bioassay. Briefly, D10-N4 M cells were resuspended at 10^5 cells/ml in RPMI + 10% FCS containing recombinant murine IL-2 (60 U/ml) and Con A (6 μg/ml) and dispensed (100 μl/well) into 96-well flat-bottomed plates containing 100 μl medium plus 50 μl sample. Cultures were pulsed 72 hr later with 1 μCi/well [^{3}H]thymidine for 6 hr, harvested and counted on a β-scintillation counter. The cytokine content of each sample was read off a standard curve established with murine recombinant IL-1β. TGF-β was assayed with a mink lung cell line.[19] Briefly, cells were dispensed at 1×10^5 cells/well and cultured overnight at 37°. Cultures were washed to remove non-adherent cells, samples or standards added in a final volume of 100 μl and incubated overnight at 37°. Cultures were then washed to remove TGF-β, 200 μl complete medium containing 0·5 μCi/well [^{3}H]thymidine added, and incubated for 18 hr before harvesting. The cytokine content of each sample was read off a standard curve established with murine recombinant TGF-β.

Generation and measurement of nitrite from DLN cells, serum and urine

DLN cells were again removed 48 hr after administration of carrageenin, resuspended at 10^6 cells/ml and dispensed at 1 ml/well in 24-well plates. Cultures were stimulated with lipopolysaccharide (LPS; Sigma) (10 μg/ml), LPS (1 μg/ml) plus IFN-γ (200 U/ml) or Con A (5 μg/ml), and cell-free supernatants obtained at 24 and 48 hr post-stimulation. Nitrite levels in the supernatants were measured using the Greiss reaction.[20] Mice were bled before carrageenin administration and at 24 and 48 hr after carrageenin administration and sera were obtained.

Urine was collected at the same time-points. As nitrite was undetectable, nitrate levels were measured by converting nitrate to nitrite using the enzyme nitrate reductase, then measuring nitrite in a chemiluminometer, as previously described.[21]

Statistics

Statistical significance ($P < 0.05$) was analysed by Student's *t*-test. Results are expressed as mean ± SD.

RESULTS

Administration of carrageenin led to a significant increase in footpad size by 24 hr compared to the untreated contralateral paw (Fig. 1a,b). This swelling peaked at 48 hr and was significantly reduced by treatment of animals with L-NMMA (Fig. 1a). Treatment with the inert enantiomer, D-NMMA, had no effect on carrageenin-induced swelling (Fig. 1b). Saline alone did not induce footpad swelling (data not shown).

A small cellular infiltrate was observed in the uninjected contralateral paw of both L- and D-NMMA-treated animals (Fig. 2a,b). Injection of carrageenin resulted in a marked cellular infiltration into the paw. This infiltration was unaffected by prior treatment with L- or D-NMMA (Fig. 2c,d), although oedema, as measured by paw thickness, was greater in the D-NMMA-treated animals (Fig. 1b).

DLN cells were removed 48 hr after injection of carrageenin, at the peak of the inflammatory response, and restimulated *in vitro* with Con A. Cells from animals injected with carrageenin produced a significantly increased proliferative response compared to those from animals given only saline. This response was not significantly altered in animals treated with L-NMMA (Fig. 3). DLN cells removed 48 hr after injection of carrageenin were restimulated *in vitro* with 5 μg/ml Con A, a concentration inducing the optimal proliferative response (Fig. 3). Cells from animals treated with carrageenin produced significantly higher levels of IL-1, IL-2, IL-6 and IFN-γ after 24 hr in culture, compared with animals treated with saline alone (Table 1). Administration of L-NMMA *in vivo* led to a significant decrease in the production of these cytokines by the DLN cells *in vitro* ($P < 0.01$ for IL-2; $P < 0.05$ for IFN-γ; $P < 0.001$ for IL-1 and IL-6; Table 1). Similar results were seen at 48 and 72 hr post-stimulation (data not shown).

IL-10 was undetectable in the culture supernatants up to 48 hr post-stimulation (data not shown). At 72 hr, IL-10 was detected at highly significant levels in cultures of cells from control animals injected with saline alone. However, IL-10 remained undetectable in the culture supernatants of cells from mice injected with carrageenin (Table 1). In contrast, DLN cells from mice injected with carrageenin and treated with L-NMMA produced levels of IL-10 comparable with that of cells from the saline-treated controls. IL-4 and TNF-α were not detectable for up to 72 hr in the supernatants of cells cultured with Con A in all the groups. TGF-β was detected in the supernatants of cells from mice injected with carrageenin, but L-NMMA treatment did not significantly alter the levels of TGF-β detected (270 ± 97 pg/ml versus 280 ± 102 pg/ml). DLN cells were unable to produce a significant level of nitrite, regardless of the *in vitro* stimulus used (all groups < 4 μM), and significant levels of nitrate could not be detected in either the serum or urine of any group (data not shown).

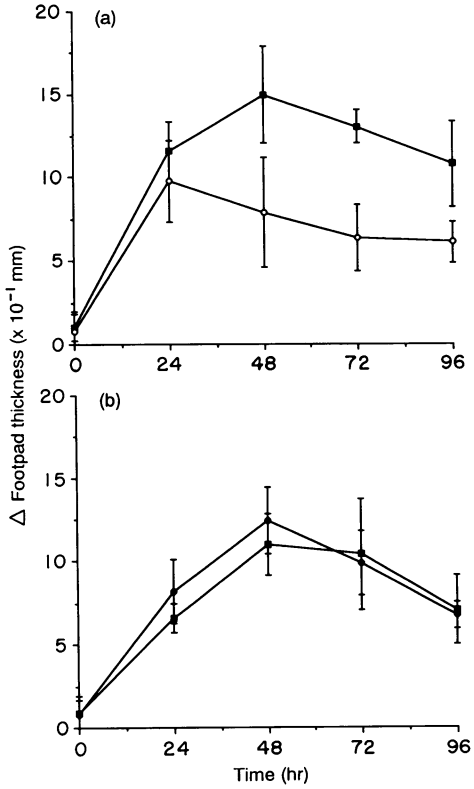

Figure 1. Effect of L- or D-NMMA treatment on the inflammatory response induced by carrageenin. (a) Animals (five per group) were injected with 300 μg carrageenin in the hind paw plus i.p. administration of saline (■) or 5 mg L-NMMA (○) at −2, +24 and +48 hr of carrageenin injection. Paw swelling was measured at 24, 48, 72 and 96 hr following injection of carrageenin. (b) Animals were injected as described, except that some were given 5 mg D-NMMA (●). Results are expressed as the mean ± SD of the difference between the injected paw and the uninjected, contralateral paw, and are representative of three experiments.

DISCUSSION

Data presented here demonstrate that the *in vivo* inhibition of NO production can markedly alter both the inflammatory response and the cytokine secretion pattern of DLN cells in mice undergoing active, local inflammation. Our results implicate NO in the elevation of a number of cytokines involved in local inflammation, including IL-1, IL-2, IL-6 and IFN-γ, and in the inhibition of IL-10. It had, however, little effect on IL-4, TNF-α or TGF-β, or on T-cell proliferation to Con A. These results contrast with findings in chronic inflammation using a model of adjuvant-induced arthritis in rats,[16] where L-NMMA was found to reduce T-cell proliferation, although cytokine responses were not examined. In a murine malaria model, spleen cells from mice infected with *Plasmodium chabaudi chabaudi* produced significant amounts of IL-2 and IFN-γ when stimulated with Con A *in vitro*. The production of these cytokines by the spleen cells was significantly enhanced when the infected mice were treated with L-NMMA *in vivo*.[22] *In vitro*, L-NMMA has also been shown to enhance T-cell proliferation in response to mitogens or alloantigens.[23–25] Thus, the effect of NO on the immune

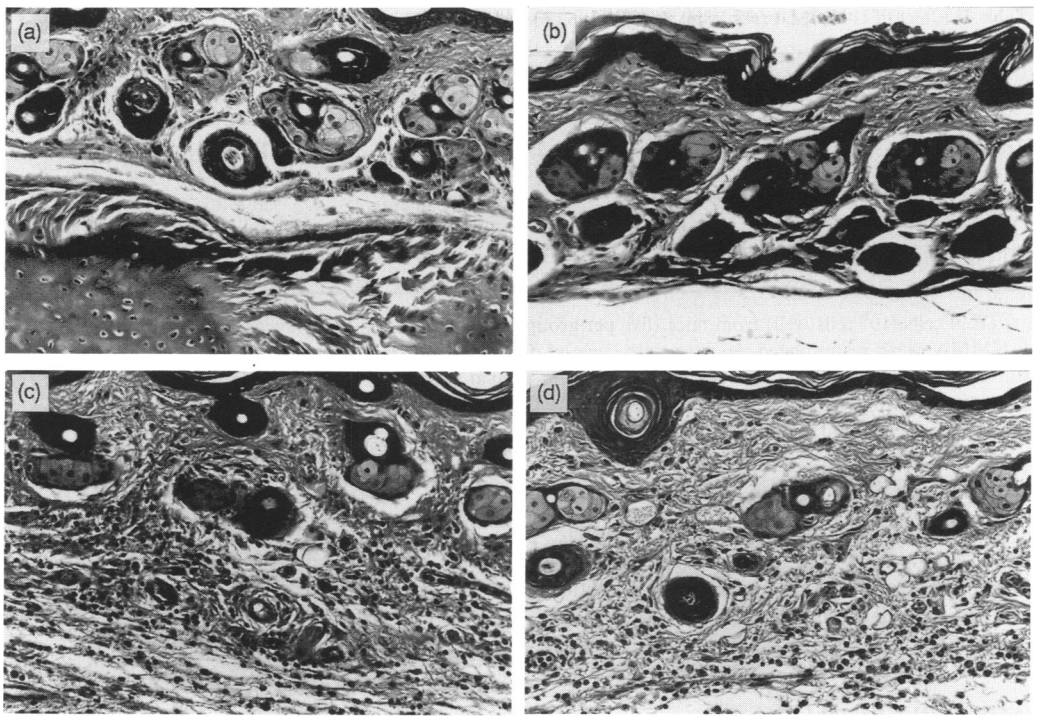

Figure 2. Histology of control or carrageenin-injected footpads following L- or D-NMMA treatment. Footpads were removed 48 hr after injection with 300 μg carrageenin, fixed, sectioned and stained with haematoxylin and eosin. (a) L-NMMA-treated, uninjected paw. (b) D-NMMA-treated, uninjected paw. (c) L-NMMA-treated, carrageenin-injected paw. (d) D-NMMA-treated, carrageenin-injected paw. Original magnification $\times$ 200.

response appears to vary in different systems. A possible explanation for this discrepancy may simply be a dose–response effect of NO in these various models. Indeed, preliminary experiments have shown that T-cell proliferation *in vitro* is enhanced by low doses of NO, but inhibited by high doses of NO (J. Padron and F. Y. Liew, unpublished observations).

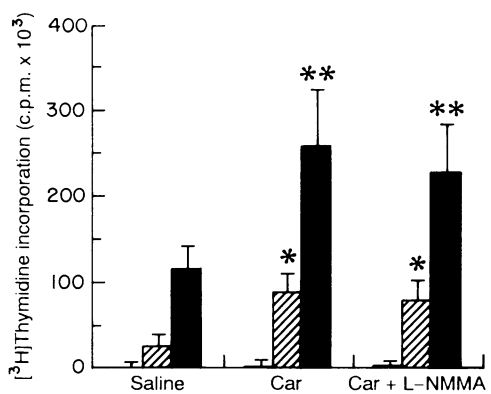

Figure 3. Effect of L-NMMA treatment on proliferation by DLN cells. Animals (five per group) were injected with 300 μg carrageenin in the hind paw plus i.p. administration of saline (Car) or 5 mg L-NMMA (Car + L-NMMA) at -2, $+24$ and $+48$ hr of carrageenin injection. Control animals were injected in the hind paw with saline only (Saline). DLN cells were obtained 48 hr after administration of carrageenin and were either unstimulated (open bars), or stimulated with 0·5 μg/ml (hatched bars) or 5 μg/ml Con A (solid bars). Results are expressed as mean $\pm$ SD, $n = 3$, and are representative of three experiments (*$P < 0{\cdot}001$ versus saline; **$P < 0{\cdot}05$ versus saline). Results are shown for 48 hr cultures, similar results were obtained from cultures incubated for 24 or 72 hr.

The mechanism by which NO can affect T-cell activation and cytokine production is at present unknown. However, it is clear that its effects may vary, depending on the experimental model. Although L-NMMA can reduce regional and microvascular blood flow and vascular permeability,[26–28] there was no reduction in the number of cells entering the site of inflammation from the bloodstream. However, L-NMMA treatment did reduce oedema formation, as measured by a marked reduction in footpad swelling. Therefore, the generalized reduction in the level of cytokines produced locally cannot be explained by a reduction in cellular infiltration into the paw. In addition, a differential production of IFN-γ and IL-10 was also detected, with IFN-γ levels high in those groups in which IL-10 was undetectable and vice versa, as could be expected from the differential regulation of these cytokines.[29,30]

In the present system, nitrite was not detected in the culture supernatant of the draining lymph node cells, nor was nitrite or nitrate detected in the serum and urine of the carrageenin-injected mice. This may reflect the insensitivity of the assay used, which has a lower limit of 4 μM. However, it is likely that local production of NO within the paw is sufficient to modulate the inflammatory response. In an arthritic model, nitrite was detected in the synovial joint during the early, acute phase of inflammation.[17] Some cytokines, such as IFN-γ, IL-1, TNF-α and IL-6, are involved in the induction of NO synthesis, while others, e.g. IL-10, IL-4 and TGF-β, can inhibit NO production (reviewed in 3). A recent report has suggested that endogenous NO production can down-regulate the levels of IL-6 secreted by rat Kuppfer cells following activation with IFN-γ and LPS.[31]

Table 1. Effect of L-NMMA treatment on cytokine production by DLN cells restimulated *in vitro* with Con A

Treatment	Cytokines (pg/ml)				
	IL-1	IL-2	IL-6	IFN-γ	IL-10
Saline	16 ± 0·5	1090 ± 35	0 ± 0	50 ± 17	2000 ± 622
Carrageenin	111 ± 33	21,500 ± 1212	800 ± 121	13,400 ± 1386	0 ± 0
Carrageenin + L-NMMA	20 ± 7	15,000 ± 1559	0 ± 0	7200 ± 866	2960 ± 866

DLN cells (10^6 cells/well) from mice (five per group) treated with saline, carrageenin or carrageenin plus L-NMMA were restimulated *in vitro* with 5 μg/ml Con A. Cytokine levels were measured 24 hr post-stimulation, with the exception of IL-10 which was measured at 72 hr post-stimulation. Results are expressed as the mean ± SD of triplicate cultures and are representative of two experiments. Cytokines were undetectable in the culture supernatants of unstimulated cells.

Therefore, it is possible that NO generated locally during activation can inhibit cytokines produced later in the cascade. Unravelling of this mechanism will shed important light on our understanding of the biology of NO and may have therapeutic implications against some of the most important inflammatory diseases.

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REFERENCES

1. Liew F.Y. & Cox F.E.G. (1991) Nonspecific defence mechanism: the role of nitric oxide. In: *Immunoparasitology Today* (eds C. Ash and R.B. Gallagher), p. A17. Elsevier Trends Journals, Cambridge.
2. Moncada S., Palmer R.M.J. & Higgs E.A. (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43,** 109.
3. Green S.J. & Nacy C.A. (1993) Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. *Curr. Opin. Infect. Dis.* **6,** 384.
4. Marletta M.A. (1993) Nitric oxide synthase structure and mechanism. *J. biol. Chem.* **268,** 12231.
5. Hibbs J.B., Taintor R.R., Vavrin Z., Granger D.L., Drapier J.-C., Amber I.J. & Lancaster J.R. (1990) Synthesis of nitric oxide from a terminal guanidino atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In: *Nitric Oxide from L-Arginine: A Bioregulatory System* (eds S. Moncada and E. A. Higgs), p. 189. Excerpta Medica, Elsevier, Amsterdam.
6. Nathan C. (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6,** 3051.
7. Nathan C.F. & Hibbs J.B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* **3,** 65.
8. Sarih M., Souvannavong V. & Adam A. (1993) Nitric oxide synthase induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* **191,** 503.
9. Albina J.E., Cui S., Mateo R.B. & Reichner J.S. (1993) Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* **150,** 5080.
10. Lukic M.L., Stosic-Grujicic S., Ostojic N., Chan W.L. & Liew F.Y. (1991) Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. *Biochem. Biophys. Res. Commun.* **178,** 913.
11. Kolb H., Kiesel U., Kroncke K.-D. & Kolb-Bachofen V. (1991) Suppression of low dose streptozotocin induced diabetes in mice by administration of a nitric oxide synthase inhibitor. *Life Sci.* **49,** 213.
12. Garside P., Hutton A.K., Severn A., Liew F.Y. & Mowat A.McI. (1992) Nitric oxide mediates intestinal pathology in graft-vs-host disease. *Eur. J. Immunol.* **22,** 2141.
13. Koprowski H., Zheng Y.M., Heber-Katz E., Fraser N., Rorke L., Fu Z.F., Hanlon C. & Dietzschold B. (1993) *In vivo* expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. natl. Acad. Sci. U.S.A.* **90,** 3024.
14. Ialenti A., Ianaro A., Moncada S. & Di Rosa M. (1992) Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.* **211,** 177.
15. Lippe I.T., Stabentheiner A. & Holzer P. (1993) Participation of nitric oxide in the mustard oil-induced neurogenic inflammation of the rat paw skin. *Eur. J. Pharmacol.* **232,** 113.
16. Ialenti A., Moncada S. & Di Rosa M. (1993) Modulation of adjuvant arthritis by endogenous nitric oxide. *Br. J. Pharmacol.* **110,** 2.
17. McCartney-Francis M., Allen H.B., Mizel D.E., Albina J.E., Xie Q.-W., Nathan C.F. & Wahl S.M. (1993) Suppression of arthritis by an inhibitor of nitric oxide synthase. *J. exp. Med.* **178,** 749.
18. Farrell A.J., Blake D.R., Palmer R.M.J. & Moncada S. (1992) Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann. Rheum. Dis.* **51,** 1219.
19. Like B. & Massague J. (1986) The antiproliferative effect of type β transforming growth factor occurs at a level distal from receptors for growth-activating factors. *J. biol. Chem.* **261,** 13426.
20. Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S. & Tannenbaum S.R. (1982) Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* **126,** 131.
21. Palmer R.M.J., Ferrige A.G. & Moncada S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* **327,** 524.
22. Taylor-Robinson A.W., Liew F.Y., Severn A., Xu D., McSorley S.J., Garside P., Padron J. & Phillips R.S. (1994) Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cell. *Eur. J. Immunol.* **24,** 980.
23. Hoffman R.A., Langrehr J.M., Billiar T.R., Curran R.D. & Simmons R.L. (1990) Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J. Immunol.* **145,** 2220.
24. Albina J.E., Abate J.A. & Henry W.L. (1991) Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation. Role of IFN-gamma in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* **147,** 144.

25. MILLS C.D. (1991) Molecular basis of 'suppressor' macrophages. Arginine metabolism via the nitric oxide synthetase pathway. *J. Immunol.* **146,** 2719.
26. WHITTLE B.J.R., LOPEZ-BELMONTE J. & REES D.D. (1989) Modulation of vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.* **98,** 646.
27. GARDINER S.M., COMPTON A.M., BENNETT T., PALMER R.M.J. & MONCADA S. (1990) Regional haemodynamic changes during oral ingestion of N^G-nitro-L-arginine methyl ester in conscious Brattleboro rats. *Br. J. Pharmacol.* **101,** 10.
28. HUGHES S.R., WILLIAMS T.J. & BRAIN S.D. (1990) Evidence that endogenous nitric oxide modulates oedema formation induced by substance P. *Eur. J. Pharmacol.* **191,** 481.
29. FIORENTINO D.F., ZLOTNIK A., VIERA P., MOSMANN T.R., HOWARD M., MOORE K.W. & O'GARRA A. (1991) IL-10 acts on the antigen presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* **146,** 3444.
30. MOSMANN T.R.& MOORE K.W. (1991) The role of IL-10 in crossregulation of Th1 and Th2 responses In: *Immunoparasitology Today* (eds C. Ash and R. B. Gallagher), p. A49. Elsevier Trends Journals, Cambridge.
31. STADLER J., HARBRECHT B.G., DI SILVIO M., CURRAN R.D., JORDAN M.L., SIMMONS R.L. & BILLIAR T.R. (1993) Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J. Leukocyte Biol.* **53,** 165.