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Differential regulation of cytokine production by nitric oxide

J. MARCINKIEWICZ & B. M. CHAIN* *Department of Immunology, Copernicus School of Medicine Cracow, Krakow, Poland and *Department of Biology (Medawar), University College London, London, U.K.*

SUMMARY

Nitric oxide (NO) has recently been identified as a potent and pleiotropic intracellular mediator produced by and acting on many cells of the body. Although considerable attention has been devoted to the regulation of NO by inflammatory cytokines, and also to the role of NO as an important effector molecule in immune function, there is very little information on the role of this mediator in modulating T-cell-dependent cytokine production. In this study we show that physiological levels of NO (either produced by activated macrophages or by the addition of exogenous NO donors) can selectively down-regulate interleukin-3 (IL-3) production by spleen cells from contact-sensitized mice, while leaving IL-2 activity unaffected. Thus NO may have an important role as an immunomodulatory as well as effector molecule in the immune system.

INTRODUCTION

Nitric oxide (NO), a simple and unstable free radical, has recently been identified as a potent and pleiotropic intracellular mediator produced by and acting on many cells of the body. It acts as an endothelium-derived relaxing factor, a neuromediator and as a defence molecule of the immune system.[1,2] In the immune system both macrophages and neutrophils are the cells responsible for the NO production.[3] Inducible calcium-independent NO synthase in macrophages is produced when these cells are activated by inflammatory cytokines [interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α)]. Activated macrophages have been demonstrated to metabolize the amino acid L-arginine by the oxidative pathway to produce nitric oxide, NO/NO_2^- and citrulline.[4] A large flux of nitrogen oxides causes inhibition of iron sulphur enzymes in target cells, blocking cell reproduction and in some cases causing cell death. This high level of NO synthesis is important in the defence against tumour cells and intracellular micro-organisms.[5] A low output flux of NO modulates vascular smooth muscle and nerve action via activation of guanylate cyclase.[6]

The pleiotropic nature of NO function and its production by activated macrophages raises the possibility that NO also has a role as an immunoregulator.[7] Prostaglandins, by comparison, which have long been known to be key effector molecules in mediating inflammatory reactions, have more recently been shown to regulate the production of several T-cell-derived cytokines selectively.[8-10] There is some indication that such an immunoregulatory role exists. For example, it has recently been shown that the suppression of allogeneic or mitogenic T-cell proliferation can be mediated by NO.[11] It is tempting to speculate that the suppressor activity of macrophages is mediated, at least partially, by NO,[12] perhaps in co-operation with prostaglandins.[13]

NO could contribute to the regulation of immune reaction by modifying the synthesis/release of cytokines, the main lymphocyte immunoregulatory factors. The converse phenomenon, namely the up- and down-regulation of NO synthesis by cytokines, is well documented. Th1 type cytokines (IFN-γ, IL-2) stimulate macrophages to NO production, whereas Th2 cytokines (IL-4 and IL-10) down-regulate NO synthesis.[14] Other cytokines have also been reported to exert inhibitory effects on NO synthesis by IFN-γ-activated macrophages. Macrophage-deactivating factor and the transforming growth factor β family block the ability of IFN-γ to induce release of reactive nitrogen intermediates from murine macrophages.[14,15]

In the present paper we report on the influence of endogenous NO (from activated macrophages) and exogenous NO (from synthetic NO donors) on cytokine (IL-2 and IL-3) release. For these studies we have used spleen cells from mice immunized by contact sensitization with picryl chloride. We have shown previously that lymph node cells from these mice, restimulated *in vitro* with the trinitrophenyl (TNP) hapten, release high levels of IL-2, IFN-γ and IL-3.[16] Recently we have shown that these cytokines are selectively and differentially regulated by eicosanoids produced by activated macrophages.[8,9] It was therefore of interest to carry out a study of the *in vitro* effects of NO and its metabolic inhibitors on cytokine release by the same population of TNP immune cells.

MATERIALS AND METHODS

Animals

Male adult (20–25 g) CBA mice were obtained from Olac (Bicester, U.K.).

Abbreviations: LPS, lipopolysaccharide; L-NMMA, *N*-monomethyl-L-arginine; NO, nitric oxide; TNP, trinitrophenyl.

Correspondence: Dr B. M. Chain, Dept. of Biology (Medawar), University College London, Gower St, London WC1 6BT, U.K.

Culture conditions for in vitro *lymphokine production*

Draining lymph node cells or spleen cells collected either from TNP-sensitized animals (topical application of picryl chloride to the skin) or from tolerized mice (intravenous injection of trinitrobenzene sulphonic acid) were cultured at a concentration of 5×10^5/well. These cells were stimulated *in vitro* by co-culture with TNP-coupled spleen cells (10^5/well). After 24 hr 100 μl of supernatant was removed and frozen at -20°. All experimental details for the induction of contact sensitivity (mice sensitization), tolerance and culture conditions have been described previously.[16]

Isolation of macrophages

Peritoneal exudate cells were collected from mice injected intraperitoneally 5 days previously with 2·0 ml of thioglycolate. Macrophages were purified by adherence to plastic for 2 hr (37°, 5% CO_2). After removing non-adherent cells the resultant monolayer was composed of 90–98% macrophages. Adherent cells were removed from plastic by treatment with trypsin and EDTA. Macrophages were cultured in 96-well U-bottom plates in different concentrations of RPMI-1640 containing 5% fetal calf serum (FCS). For activation they were incubated with lipopolysaccharide (LPS) (100 ng/ml) (Sigma, Poole, U.K.) and/or with IFN-γ (100 U/ml) (gift of Dr A. Mudge, University College, London, U.K.). After 20 hr TNP immune cells (from lymph node or spleen) and antigen (TNP-spleen cells) were added to the culture. After a further 24 hr the medium was removed and all supernatants were frozen. In all experiments culture supernatants were collected for measurement of both cytokines and NO.

NO donors

In some cultures immune cells were incubated with NO donors: SIN-1a (a gift from GEA Company, Copenhagen, Denmark) and S-nitroso-glutathione (a gift from Dr M. Radomski, Wellcome Foundation, U.K.) (0·01–1·0 mM). Both these NO donors reacted spontaneously in water to release NO, which was subsequently converted to nitrite (NO_2).[17] The donors were present throughout the period of culture.

Specific inhibition of NO production by macrophages

Endogenous production of NO was inhibited by incubation of stimulated immune cells with the inhibitor of NO synthase—*N*-monomethyl-L-arginine (L-NMMA; 10–100 μM).[18] L-NMMA is an analogue of L-arginine.

Cytokine assay

IL-2 and IL-3 bioactivity assays were carried out using the indicator lines HT-2 and FDCP-2 as described previously.[8,16] Briefly, cytokine levels were assayed by titrating supernatants on the specific indicator cell lines and measuring cell viability with the mitochondrial indicator dye MTT. Mean optical density values, from triplicate cultures, were converted to U/ml by fitting experimental titrations to standard curves obtained using samples of known activity.

Although both cell lines respond to other cytokines, we have shown that the IL-2 activity present in the supernatants of TNP-specific cells is unaffected by antibodies to IL-3 or IL-4 (we do not have a specific blocking antibody for IL-2), and that the IL-3 activity can be specifically blocked by antibodies to IL-3 (ref. 8; J. Marcinkiewicz and B. M. Chain, unpublished results).

Table 1. Production of NO by *in vitro* culture of activated lymphocytes and macrophages

Cell culture*	NO production (μM)†
A—Lymph node cells + antigen	<4
B—Spleen cells + antigen	<4
C—Spleen cells from tolerized mice	<4
D—Thyoglycollate-induced macrophages	9·6 ± 1·3
D + A	15 ± 1·6
D + B	17 ± 1·6
D + C	14 ± 2·7
D + L-NMMA (100 μM)	<4
D + LPS (100 ng/ml)	70 ± 7
D + LPS (100 ng/ml + L-NMMA (100 μM)	6 ± 0·9
D + IFN-γ (100 U/ml)	60 ± 2·3
D + IFN-γ + L-NMMA (100 μM)	7 ± 0·3

* Details of the *in vitro* culture conditions are as follows: (A) 5×10^5 lymph node cells from picryl chloride-sensitized mice (see Materials and Methods) were cultured with 10^5 TNP-coupled spleen cells (as a source of antigen) for 24 hr. (B) As for A except that spleen cells were substituted for lymph node cells. (C) 5×10^5 spleen cells from mice which had been tolerized by intravenous injection of trinitrobenzene sulphonic acid (see Materials and Methods) were cultured with 10^5 TNP-coupled spleen cells for 24 hr. (D) 10^5 peritoneal macrophages from thioglycollate-stimulated mice were cultured for 24 hr in medium only, or with A, B or C above. LPS or IFN-γ was added to some cultures of macrophages prepared as in D above, and the macrophages were cultured for 24 hr prior to collection of supernatants. Where appropriate, the inhibitor of NO synthase, L-NMMA, was present throughout the culture period.

* NO production was measured by assay of the stable end-product NO_2 as described in Materials and Methods. Results show the average (± SE of the mean) of triplicate measurements.

Nitrite (NO_2) determination

NO is highly unstable in solution and can not therefore be readily assayed. However, NO is converted to the stable nitrite ion in aqueous solution, and NO production is routinely monitored by measuring supernatant nitrite concentrations.[19] Nitrite concentrations were measured using the Griess reagent. Briefly, 100 μl aliquots of culture supernatants were mixed with an equal volume of Griess reagent (1% sulphanilamide/0·1% naphtylethylene diamine dihydrochloride/2·5% H_2PO_3) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated plate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve.

Statistical analysis

All experiments were performed two or three times. In each experiment, all cultures were carried out in triplicate, and statistical significance ($P < 0·05$) between groups within individual experiments was analysed by Student's *t*-test.

RESULTS

NO production by lymphocytes and macrophages

The contact hypersensitivity reaction involves interaction between antigen-specific lymphocytes and activated macrophages. The production of NO (measured as supernatant nitrite) *in vitro* by these two types of cells is shown in Table 1.

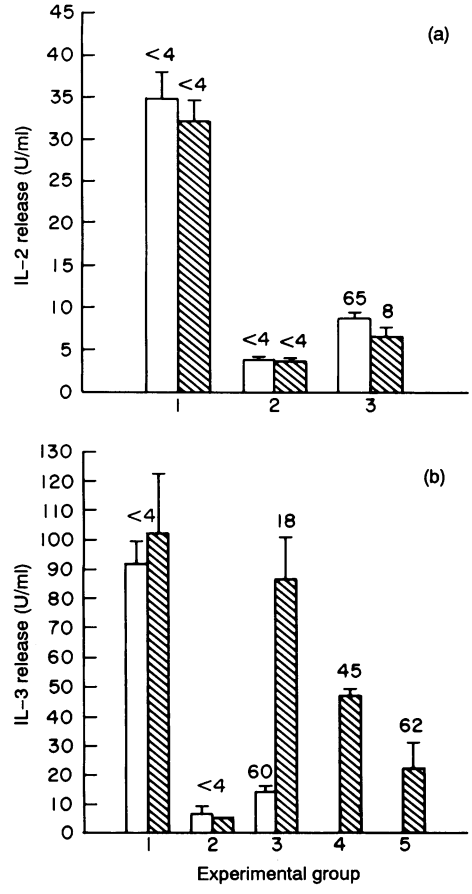

Figure 1. The role of NO in macrophage-dependent suppression of cytokine synthesis. Responder cells from TNP-immunized mice were co-cultured with TNP-spleen cells for 24 hr, before supernatants were collected and analysed for IL-2 (a), IL-3 (b) and NO (as NO_2, shown as nos. above the respective histogram bars). Results represent means + SE of triplicate cultures. Hatched bars represent results from cultures containing the inhibitor of NO synthesis L-NMMA (100 μM). This inhibitor was added at the beginning of the culture and was present throughout the culture period. Experimental groups: (1) 5×10^5 lymph node cells from contact sensitized mice; (2) 5×10^5 spleen cells from TNBS-tolerized mice; (3) 5×10^5 lymph node cells from contact sensitized mice co-cultured with 10^5 LPS-stimulated macrophages; (4) as for (3) but L-NMMA was added at 30 μM; (5) as for (3) but L-NMMA was added at 10 μM.

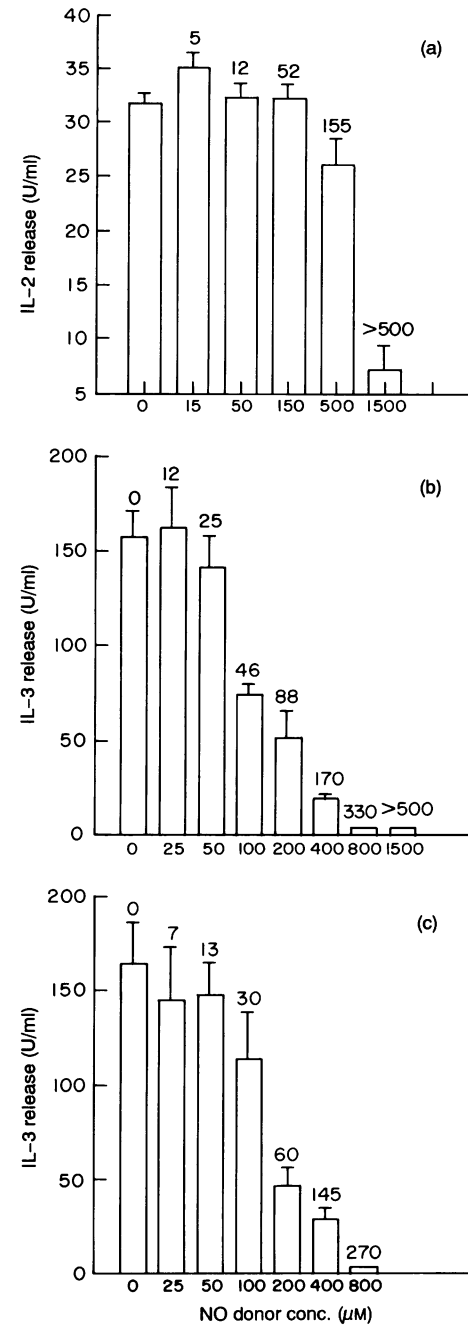

Figure 2. The effect of exogenous NO on cytokine synthesis. Approximately 5×10^5 lymph node cells from contact sensitized mice were incubated with 10^5 TNP-spleen cells in the presence of varying concentrations of NO donors as shown. Culture supernatants were collected after 24 hr and assayed for IL-2 (a) and IL-3 (b, c). The actual levels of NO (measured as NO_2) are shown as nos. above the histogram bars. Results represent mean + SE of triplicate cultures. The NO donor used in (a) and (b) was Sin-1, while in (c) it was *S*-nitroso-glutathione.

Lymphocytes from contact sensitized mice restimulated *in vitro* with antigen or lipopolysaccharide (LPS) failed to generate measurable levels of NO. Similarly, spleen cells from mice which had been rendered tolerant by intravenous antigen,[16] and which contain populations of cells with suppressive activity also failed to generate appreciable levels of NO. In contrast, thyoglycollate-induced macrophages produced low levels of NO (Table 1), and this could be up-regulated by co-culture with either activated or tolerant lymphocytes. Finally, macrophages stimulated with LPS or high concentrations of IFN-γ produced high levels of NO (50–120 μM). In all cases, NO production could be inhibited by 80–90% by adding the L-arginine analogue L-NMMA to the medium. The data presented in Table 1 thus demonstrate that it is possible to manipulate the cellular production of NO in this *in vitro* culture in a number of ways, and thus investigate the effect of NO production on immune function.

Influence of NO synthesis inhibitor on cytokine production

The effect of the NO synthesis inhibitor L-NMMA on IL-2 and IL-3 production is shown in Fig. 1. In view of the very low

spontaneous production of NO by lymph node cells, it was not surprising that the inhibitor had no significant effect on either IL-2 or IL-3 production. Co-culture of lymph node cells with LPS-stimulated macrophages caused profound inhibition of both IL-2 and IL-3 production. However, addition of NMMA demonstrated a striking difference in the underlying mechanisms of this inhibition. L-NMMA had no effect on the macrophage-induced suppression of IL-2 production, although substantially inhibiting NO production. This result implies that factors other than NO are acting to down-regulate IL-2 production in these cultures, although it does not rule out a possible additional effect of NO. In contrast, (Fig. 1b) inhibition of IL-3 production is largely reversed by L-NMMA in a dose-dependent manner. Thus production of NO appears to be a major mediator in the suppression of IL-3 production by activated macrophages.

Influence of exogenous NO on cytokine production

In an alternative approach, NO levels in the culture medium can be manipulated directly by the addition of the two NO donors Sin-1a and *S*-nitroso-glutathione. These two reagents react spontaneously in water to give NO, which is then converted to nitrite.[17] NO concentrations (and hence nitrite) is directly proportional to donor concentration.

The effects of adding exogenous NO (in the form of NO donors) is shown in Fig. 2. IL-2 production is unaffected except at extremely high concentrations of NO (>500 μM). Such concentrations of NO were never achieved in the *in vitro* cultures used in this study, and are unlikely to be significant in immunoregulation. In contrast, IL-3 production is inhibited in a dose-dependent manner by concentrations of NO above 40 μM. In order to confirm that this result was indeed due to NO production, and not, for example, due to superoxide production which is also produced by Sin-1, the effects of the second NO donor, *S*-nitro-glutathione, were tested (Fig. 2c), with similar results. In neither case did the addition of the NO donor lead to significant cell death in the cultures. Addition of sodium nitrite in concentrations up to 500 μM had no effect on either IL-2 or IL-3 production (not shown), confirming that the effects seen were indeed due to NO production, and not to the effect of the NO_2 produced as the end-product of the reaction.

DISCUSSION

The main observation arising from these studies is a differential effect of NO on IL-2 and IL-3 production by TNP-specific spleen cells *in vitro*. Our study does not identify the precise cell type which is the target of NO, although previous studies have indicated that more than 90% of both IL-2 and IL-3 production in these cultures can be attributed to T cells.[16] L-NMMA, an inhibitor of NO production had no effect on cytokine production by TNP-specific lymph node cells but, as we were unable to measure nitrite levels below 4 μM, we can not rule out the possible effects of very low concentrations of this mediator on immune function. However, higher amounts of NO ($NO_2^- > 50$ μM) could be obtained either by adding macrophages stimulated with LPS (endogenous NO) or by using the synthetic NO donors Sin-1a and *S*-nitroso-glutathione. Both compounds spontaneously release NO in an aqueous solution. We could also manipulate the level of endogenous NO by using L-NMMA, an analogue of L-arginine. This analogue is effective as an inhibitor of NO synthesis catalysed by inducible NO synthase in macrophages.

It is well known that activated macrophages (sometimes described as 'suppressor macrophages') inhibit several functions of lymphocytes (e.g. proliferation of mitogen or allo-stimulated T cells). Several mediators which are produced by activated macrophages are responsible for these effects, including eicosanoids and NO.[11,13] We have similarly observed that LPS-activated macrophages significantly inhibit both IL-2 and IL-3 production by TNP-specific T cells from contact sensitized mice. However, there was no correlation between IL-2 synthesis and NO levels in culture medium. In contrast the suppression of IL-3 by macrophages was related to NO (NO dependent) as an inhibition of NO production by L-NMMA could overcome this effect. We have confirmed these observations by incubation of immune cells *in vitro* with NO donors. IL-2 was inhibited only by very high levels of exogenous NO (in concentrations higher than 200 μM). Although it is impossible to measure the levels of NO which may be achieved *in vivo* at the site of inflammation, these levels were higher than those generated by activated macrophages in culture. In contrast 40–50 μM NO inhibited IL-3 production significantly. Comparable levels of endogenous NO were obtained *in vitro* by stimulation of macrophages either with LPS or IFN-γ. These data suggest different mechanisms of IL-2 and IL-3 regulation. Previously we have shown that prostaglandin E_2 (PGE_2) and forskoline suppress IL-2 but not IL-3 production by increasing cAMP production.[8,9] The mechanism of IL-3 suppression may be related to the ability of NO to up-regulate cGMP. As we have found an opposing effect of cAMP and cGMP on IL-2 and IL-3 production, the balance between these two nucleotides may be involved in a fine tuning of cytokine release.

The biological significance of IL-3 inhibition by NO is not clear. However, the balance between IL-3 and IFN-γ is important for macrophage cytotoxicity against intracellular parasites.[20] Over-production of IL-3 by T-helper cells was correlated with an increased susceptibility to *Leishmania* infection. On the other hand IFN-γ is important in activating intracellular killing of this parasite. IFN-γ, a Th1 cytokine, stimulates NO which in turn inhibits IL-3 production. Thus high levels of IL-3 may indicate an ineffective production of NO and contribute to the relationship between IL-3 and increased susceptibility to intracellular parasite infections.

In conclusion, our study demonstrates for the first time that NO can act to regulate cytokine production by antigen-specific spleen cells. Further studies are in progress to define both the molecular mechanisms responsible for these effects, and the role of NO in immunoregulation.

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REFERENCES

1. MONCADA S., PALMER R.M.J. & HIGGS E.A. (1989) Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. *Biochem. Pharmacol.* **138,** 1709.
2. COLLIER J. & VALLANCE P. (1989) Second messenger role for NO widens to nervous and immune system. *TIPS*, **123,** 427.

3. Hibbs J.B., Taintr R.R., Vavrin Z. & Rachlin E.M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. biophys. Res. Comm.* **157,** 87.
4. Marletta M.A., Yonn P.S., Lyengar R., Leaf C.D. & Wishnok J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27,** 8706.
5. Liew F.Y. & Cox F.E.G. (1991) Non-specific defence mechanisms—the role of nitric oxide. *Immunoparasitol. Today*, **2,** A17.
6. Palmer R.M.J., Ferridge A.G. & Moncada S. (1987) Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. *Nature*, **257,** 524.
7. Kolb H. (1992) A pathogenic factor in autoimmunity: nitric oxide. *Immunol. Today*, **13,** 157.
8. Marcinkiewicz J., Radziszewski W. & Chain B.M. (1992) Prostaglandin E_2 differentially regulates the production of IL-2 and IL-3 by murine immune T cells. *Folia Histochem. Cytobiol.* **30,** 1.
9. Marcinkiewicz J. & Chain B.M. (1993) Differential cytokine regulation by eicosanoids in the contact hypersensitivity reaction to TNP. *Cell Immunol.* (in press).
10. Betz M. & Fox B.S. (1991) Prostaglandin E_2 inhibits production of Th1 lymphokines but not Th2 lymphokines. *J. Immunol.* **146,** 108.
11. Hoffmann R.A., Langrehr J.M., Billiar T.R., Curran R.D. & Simmons R.L. (1990) Allo-antigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J. Immunol.* **145,** 2220.
12. Mills C.D. (1991) Molecular basis of 'suppressor' macrophages. Arginine metabolism via nitric oxide synthetase pathway. *J. Immunol.* **146,** 2719.
13. Marcinkiewicz J. (1991) *In vitro* cytokine release by activated murine peritoneal macrophages: role of prostaglandins in the differential regulation of TNF-α, IL-1 and IL-6. *Cytokine*, **3,** 327.
14. Liew F.Y., Lee Y., Severn A., Millot S., Schmidt J., Salter M. & Moncada S. (1991) A possible novel pathway of regulation by murine T helper type-2 (TH2) of a TH1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.* **21,** 2489.
15. Nacy C.A., Nelson B.J., Meltzer M.S. & Green S.J. (1992) Cytokines that regulate macrophage production of nitrogen oxides and expression of anti-leishmanial activities. *39th Forum in Immunol.* 573.
16. Marcinkiewicz J. & Chain B.M. (1989) Antigen specific inhibition of IL-2 and IL-3 production in contact sensitivity to TNP. *Immunology*, **68,** 185.
17. Radomski M., Rees D.D., Dutra A. & Moncada S. (1992) S-nitroglutathione inhibits platelet activation *in vitro* and *in vivo*. *Br. J. Pharmacol.* **107,** 745.
18. Rees D.D., Palmer R.M.J., Schulz R., Hodson H.F. & Moncada S. (1990) Characterisation of three inhibitors of endothelial NO synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.* **101,** 746.
19. Nicholas D.J.D. & Nason A. (1957) Determination of nitrates and nitrites. In: *Methods in Enzymology* (eds S. F. Colowick and N. O. Kaplan), Vol. 3, p. 981. Academic Press, New York.
20. Liew F.Y., Millot S., Li Y., Lelchuk R., Chan W.L. & Ziltener H. (1989) Macrophage activation by interferon gamma from host protective T cells is inhibited by IL-3 and IL-4 produced by disease promoting T cells in leishmaniasis. *Eur. J. Immunol.* **19,** 1227.