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Antagonistic effects of IL-4 and interferon-gamma (IFN-γ) on inducible nitric oxide synthase expression in bovine macrophages exposed to Gram-positive bacteria

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SUMMARY

Cytokine-mediated modulation of nitric oxide (NO) production by bacteria-stimulated bovine macrophages was studied. When *Salmonella dublin*, as a prototypic Gram-negative organism, was used, NO generation was barely enhanced by recombinant bovine and ovine IFN-γ, but was suppressed by IL-4. *Salmonella dublin*-induced NO generation was not influenced by a panel of nine other cytokines. The panel included IL-1, tumour necrosis factor (TNF) and IFN-α, which are active in a similar mouse macrophage model. The tested cytokines were either homologous or known to interact with bovine cytokine receptors. Recombinant bovine and ovine IFN-γ were the only cytokines which strongly enhanced NO synthesis by macrophages exposed to the Gram-positive organism, *Listeria monocytogenes*. *Listeria*-induced NO generation was strongly suppressed by recombinant human and bovine IL-4, but not by IL-10 and transforming-growth-factor-beta. Thus, two cytokines characterizing a Th1 and a Th2 response up- and down-regulate, respectively, bacteria-induced NO generation in bovine macrophages, whereas nine other cytokines had little activity in this regard. This modulation was reflected in changes in the steady state levels of mRNA coding for inducible nitric oxide synthase. Combinations of IFN-γ and IL-4 suggested that the relative proportion of these cytokines determined whether bacteria-induced NO generation was up- or down-regulated. At saturating IL-4 concentrations, stimulation of bacteria-induced NO generation in macrophages by T cell supernatants was solely dependent on IFN-γ. This was shown by antibody neutralization experiments and by a close correlation between the capacity of supernatants to stimulate NO generation and the IFN-γ content, as determined by immunoassay.

Keywords macrophages cattle inducible nitric oxide synthase NO IL-4 interferon-gamma

INTRODUCTION

The concept that T helper cells differentiate into cells expressing distinct sets of cytokines, leading to different forms of immune responses, is well established [1]. Whether an antigen induces mainly a Th1 (type 1) or a Th2 (type 2) cytokine response in a given individual is dependent on the antigen and on genetic host factors, and may be influenced by additional factors such as the route of immunization, concentration of the antigen, adjuvants and further environmental factors modulating the immune response of the host. Although the cytokine pattern of a Th1 and a Th2 response is complex and may be subject to species differences, two cytokines are the hallmarks of either a Th1 or Th2 response: IFN-γ and IL-4, respectively. This is valid for the mouse [1], and despite certain species differences also for cattle [2,3]. The above cytokines mediate the most typical features of the respective helper cell subsets, namely promotion of inflammation and macrophage activation by Th1 cells, and promotion of antibody, in particular of IgE production by Th2 cells. In addition, they are of importance for antagonistic regulation of T helper cell differentiation itself: IL-4 enhances a Th2 response and suppresses a Th1 response; conversely, IFN-γ enhances a Th1 response and suppresses a Th2 response. This apparent antagonism is also reflected in the effects these cytokines exert on mononuclear phagocytes. Thus, IFN-γ is the most important, if not a unique, macrophage-activating cytokine [4,5]. IL-4 counteracts macrophage activation in many respects [6–11], yet promotes 'alternative immunological macrophage activation', characterized, for example, by up-regulation of mannose receptors and of the CD13 aminopeptidase [12,13], and down-regulation of inflammatory cytokine production [7,14] and CD14 expression [15,16].

Together with transforming growth factor-beta (TGF-β) and IL-10, IL-4 belongs to the cytokines down-regulating induction of inducible nitric oxide synthase (iNOS) and hence NO generation

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by mouse macrophages [11]. IFN-γ, together with IFN-α/β, IL-1 and tumour necrosis factor (TNF), was reported to induce or up-regulate lipopolysaccharide (LPS)-induced iNOS induction and NO generation in mouse macrophages [17]. We were interested to identify the cytokines exerting a stimulatory or a regulatory effect on iNOS expression and NO synthesis in bovine macrophages. We recently showed that these cells are also capable of expressing iNOS and generating NO, although cytokine regulation appeared to be more restricted than that seen in the mouse [18,19]. In this study, the effect of specific cytokines on bovine macrophage iNOS expression was extended. We found that IFN-γ and IL-4 are unique in exerting powerful antagonistic effects on iNOS expression by bovine macrophages exposed to *Listeria monocytogenes* and other Gram-positive organisms. This phenomenon can be exploited to measure the two cytokines in a bioassay, as is demonstrated here for IFN-γ.

MATERIALS AND METHODS

Reagents

The sources of the cytokines used are given in Table 1. Antibodies against IFN-γ were kindly provided by Novartis (Basel, Switzerland). A kit to measure bovine IFN-γ immunoenzymatically was the generous gift of Dr Paul Wood (CSIRO, Parkville, Australia). This kit is routinely used to diagnose Johne's disease in cattle [20]. It is an antibody capture immunoassay specific for bovine IFN-γ. It also recognizes IFN-γ of sheep, goat and buffalo, but neither IFN-γ from pig, deer or man, nor IFN-α/β of cattle [21]. The sensitivity is 25 pg/ml [21].

Listeria monocytogenes (strain NCTC 10,527, serotype 4b), *Staphylococcus aureus* (ATCC 29 213), *Bacillus cereus* (ATCC 9634), *B. subtilis* (ATCC 6633) and *Salmonella dublin* (NZL strain 24-90), provided by the Institute of Veterinary Bacteriology, University of Berne, were grown in trypticase soy broth, followed by heat inactivation (2 h at 60°C), repeated washing and resuspension in pyrogen-free saline.

Recombinant bovine IL-4 was produced in *Trypanosoma brucei* (Furger *et al.*, manuscript in preparation). Procyclic forms of *T. brucei* were stably transfected with a construct containing the complete bovine IL-4 coding region [22] or a control construct in which the IL-4 coding region was replaced by that of a trypanosome surface protein [23]. Cells were cultured at 27°C in SDM-79 medium supplemented with 5% heat-inactivated fetal calf serum (FCS) [24]. The supernatant was found to contain high concentrations of IL-4, as evidenced by immunoblotting, and by bioactivity. The latter included the dose-dependent down-regulation of CD14 expression by human monocyte-derived macrophages (A. Furger *et al.*, in preparation). When compared with the bioactivity of rhuIL-4, supernatants were estimated to contain 1·5 mg/*l* bovine IL-4.

Isolation and culture of bovine mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood drawn from healthy female members of a Red-Holstein herd by a modified Ficoll–triosyl procedure. They were resuspended in Iscove's modified Dulbecco's minimum essential medium (IDMEM) containing penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 2·5 μg/ml, glutamine 2 mM, non-essential amino acids for minimum essential medium (MEM) (1% v/v), vitamin solution for MEM (1% v/v), sodium pyruvate 1 mM, HEPES 10 mM, 2-mercaptoethanol (2-ME; 50 μM), G-418 10 μg/ml and FCS (20%) at 4×10^6 PBMC/ml. They were dispensed into custom-made bags of Teflon foil (FEP 100 A; Dupont-de-Nemours, purchased from Angst & Pfister, Zürich, Switzerland), and these were sealed and placed in a humidified atmosphere of 5% CO_2 at 37°C. After 7–10 days, a time after which monocytes had matured to macrophages, the cells were harvested and used for iNOS induction experiments. At this time, 40% of the cells were mature macrophages, as determined by morphology, and viability was > 98%.

Table 1. Overview of cytokines, their abbreviation and origins

Recombinant cytokine	Abbreviation	Source
Bovine IL-1β	rboIL-1	American Cyanamid, Princeton, NJ
Bovine IL-2	rboIL-2	American Cyanamid
Bovine granulocyte macrophage colony-stimulating factor	rboGM-CSF	American Cyanamid
Bovine TNF		Novartis, Basel, Switzerland
Bovine IFN-αI.1	rboIFN-α	Novartis, Basel, Switzerland
Bovine IFN-γ	rboIFN-γ	Novartis, Basel, Switzerland
Ovine IFN-γ	rovIFN-γ	Dr Paul Wood, Parkville, Australia
Human transforming growth factor-beta	rhuTGF-β	Crystal Chem, Chicago, IL
Human IL-1β	rhuIL-1	National Institute of Biological Standards and Control (NIBSC), Potters Bar, UK.
Human IL-2	rhuIL-2	NIBSC and Eurocetus, Amsterdam, The Netherlands
Human IL-4	rhuIL-4	Peprotech, London, UK, and Amersham, Arlington, IL
Human IL-6	rhuIL-6	NIBSC
Human IL-10	rhuIL-10	Peprotech and Genzyme, Cambridge, MA
Human IL-13	rhuIL-13	Peprotech
Human macrophage colony-stimulating factor	rhuM-CSF	Human Genetics Institute, Cambridge, MA
Human tumour necrosis factor	rhuTNF	Chiron (formerly Cetus)
Murine IL-4	rmuIL-4	Peprotech
Murine IFN-γ	rmuIFN-γ	Serotec, Oxford, UK

Freshly isolated PBMC or T cells purified by nylon wool column separation [25] were stimulated with T cell mitogens, and cytokine-enriched supernatants were collected 48 h later. Mitogens included phytohaemagglutinin (PHA; 5 and 25 μg/ml), IL-2 (10 ng/ml), phorbol 12-myristate 13-acetate (PMA; 5 ng/ml), the calcium ionophore A23187 (0·25 μM) either alone or in combinations. Cells were cultured in 25-cm^2 tissue culture flasks at 10^6 PBMC/ml (total volume 5 ml). Supernatants were stored frozen (−20°C) and used later for modulation of NO generation by macrophages, and for determination of IFN-γ by ELISA.

Induction and determination of nitrite generation

Monocyte-derived macrophages (MDM) were seeded in 96-well plates ($6–8 \times 10^4$ macrophages/well) and allowed to adhere for 2 h. After removal of non-adherent cells by aspiration, firmly adherent cells were exposed to cytokines or supernatants, followed by the addition of heat-killed bacteria. A concentration of 200 μg wet weight per ml, which was found to be optimal in preliminary experiments, was routinely used. The medium was IDMEM containing 5% pyrogen-free FCS. The total volume varied between 150 and 200 μl. After 24 h of culture, 100 μl supernatant were transferred to another microtitre plate in which the nitrite content of the supernatant was determined by the Griess reaction as described [19]. On each plate, a calibration curve between 300 μM and 1 μM nitrite was used. Data were used only if the nitrite content in an unstimulated culture was < 2 μM.

Northern blotting

Total RNA was isolated with TRIZol (Life Technologies, Basel, Switzerland) directly from MDM subcultured in 25-cm^2 culture flasks. Aliquots (10 μg) were suspended in formamide sample buffer, heated to 65°C and loaded on a 1% agarose-formaldehyde gel. After electrophoresis RNA was blotted onto Tropilon plus positively charged nylon membrane (Tropix, Bedford, MA), UV-crosslinked, prehybridized for 4 h and hybridized with digoxigenin (DIG)-labelled riboprobe specific for bovine iNOS [19] overnight at 68°C. The hybridized blot was washed twice for 5 min in 2 × SSC/0·1% SDS at room temperature, followed by two 15-min washes in 0·1 × SSC/0·1% SDS at 68°C. Staining was done with anti-DIG–alkaline phosphatase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany) according to the instructions of the manufacturer. CDP-Star (Tropix) was used as substrate. A high performance autoradiography film (HyperfilmTM-MP; Amersham, Aylesbury, UK) was exposed for 1 min. Uniformity of RNA loading of the gels was verified by hybridization with a riboprobe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [26].

RESULTS

Effects of cytokines on nitrite generation by bovine MDM

MDM were exposed to *Salm. dublin* or *L. monocytogenes* in the presence of a large panel of cytokines. The tested cytokines were

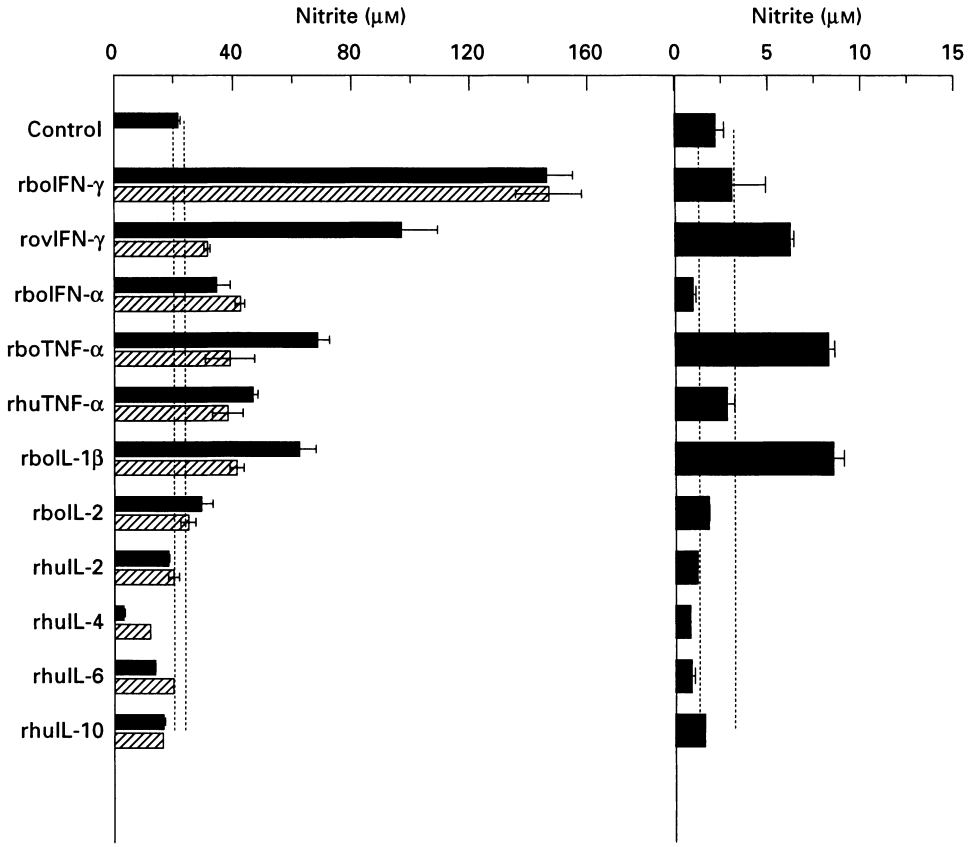

Fig. 1. The effect of cytokines on nitric oxide (NO) synthesis by bovine monocyte-derived macrophages (MDM) (d6) in the presence (left-hand panel) or absence (right-hand panel) of heat-killed *Listeria monocytogenes* (200 μg/ml wet weight). ■, Cytokine concentrations of 100 U/ml (rboIFN-γ, rovIFN-γ), 100 ng/ml (rboIFN-α, rboTNF, rboIL-1, rboIL-2, rhuIL-10), 10 ng/ml (rhuIL-4, rhuIL-6) or 1 μg/ml (rhuIL-2); □, 10-fold lower cytokine concentrations. Nitrite was determined 24 h after stimulation by cytokines and, if applicable, by bacteria. Proportionately higher values were obtained after 48 h (not shown). The figure shows a representative experiment out of three, each performed in triplicate.

either of homologous origin, or were of human origin and were shown to be active on bovine cells in other systems [27–32]. In confirmation of earlier studies with bone marrow-derived macrophages [19], *Salm. dublin* induced an NO response that was slightly enhanced by coincubation with rboIFN-γ and rovIFN-γ, but not by the other cytokines tested (data not shown). The only cytokine down-regulating the *Salm. dublin*-induced NO response was IL-4 (not shown). Similar experiments were performed with the Gram-positive organism, *L. monocytogenes*. Bovine and ovine IFN-γ strongly enhanced the moderate NO response induced with *L. monocytogenes* alone; IL-1 and TNF had only a weakly enhancing effect (Fig. 1). IL-4, again, was the only cytokine down-regulating *L. monocytogenes*-induced NO generation (Fig. 1). Other cytokines that were tested had little, if any, activity. These included rboIFN-α, rboIL-2, rhuIL-2, rhuIL-6, rhuIL-10, rbo granulocyte-macrophage colony-stimulating factor (rboGM-CSF) and rhu macrophage colony-stimulating factor (rhuM-CSF) (Fig. 1 and data not shown). The strongly up-regulating effect of rboIFN-γ was also observed when macrophages were exposed to other Gram-positive organisms, such as *Staph. aureus*, *B. cereus* or *B. subtilis* (data not shown). To assess further the activity of the cytokines which we found to enhance NO production, and to distinguish their activity from an effect induced by contaminating endotoxin, IFN-γ, IL-1, TNF and LPS were tested for their ability to modulate *L. monocytogenes*-induced NO synthesis before and after heat inactivation. IFN-γ was far superior to IL-1 and TNF in enhancing *L. monocytogenes*-induced NO generation, and heat inactivation led to a reduction of the potency of IFN-γ by two orders of magnitude; in contrast, heat treatment reduced an LPS-induced response only slightly (Fig. 2). rhuIFN-γ and rmuIFN-γ were not active (Fig. 3), confirming the species restriction of this cytokine. The down-modulatory effect observed with human IL-4 was also seen with supernatants from rboIL-4-transfected trypanosomes, which was a potent source of rboIL-4, but not with control supernatants from organisms transfected with the vector alone (Fig. 3). rhuIL-13 had a lower specific activity than rhuIL-4 (Fig. 3). rhuIL-10 and rhuTGF-β had no effect on either *Listeria*-induced (Fig. 3) or *Salmonella*-induced (not shown) NO production.

Antagonistic effects of IL-4 and IFN-γ on induction of NO production by monocyte-derived macrophages

The modulatory activity of IFN-γ and IL-4 was tested at the level of iNOS mRNA expression in macrophages exposed to *L. monocytogenes*. Steady state iNOS mRNA levels induced by bacteria were up-regulated by IFN-γ and down-regulated by IL-4 (Fig. 4). To investigate further the antagonism between IL-4 and IFN-γ on NO synthesis in bacteria-stimulated MDM, concentrations of either IL-4 or IFN-γ were varied in the presence of bacteria and high concentrations of the antagonistic cytokine. Figure 5 shows a representative experiment in which rboIL-4 was used. Regardless

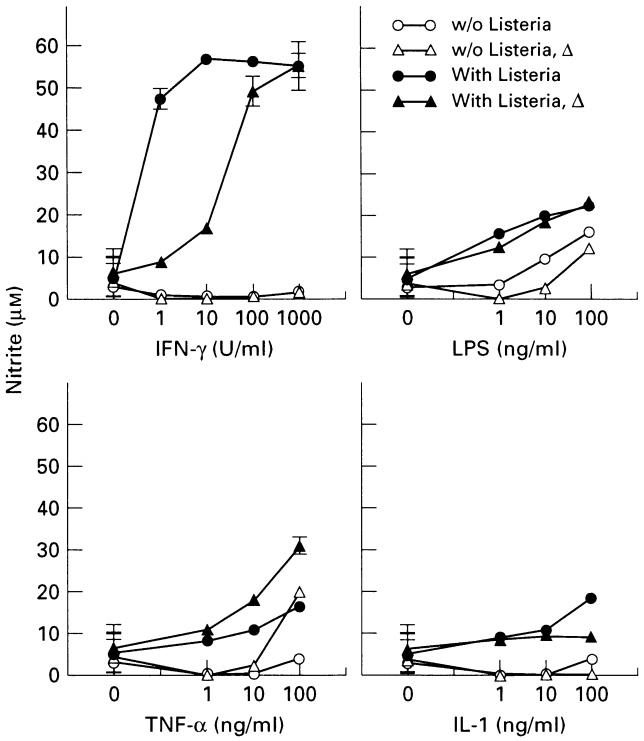

Fig. 2. Dose-dependent induction of nitric oxide (NO) synthesis in bovine macrophages by native (circles) and heat-inactivated (triangles) IFN-γ, IL-1, tumour necrosis factor (TNF) or lipopolysaccharide (LPS) in the presence (closed symbols) or absence (open symbols) of *Listeria monocytogenes* (200 μg/ml). Means ± s.d. of triplicates of a representative experiment are shown.

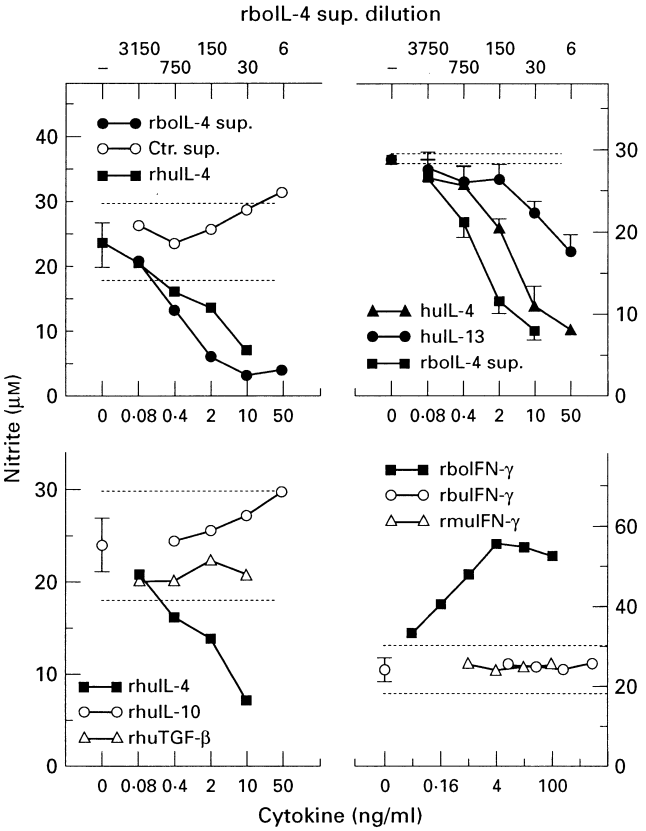

Fig. 3. Cytokine dose-dependent modulation of *Listeria*-induced nitric oxide (NO) production by bovine macrophages. RboIL-4 sup. is the culture supernatant of trypanosomes expressing bovine IL-4, ctr. sup. the culture supernatant from trypanosomes transfected with a control plasmid. The other cytokines were of recombinant human (rhuIL-4, rhuIL-13, rhuIFN-γ), murine (rmuIFN-γ) or bovine (rboIFN-γ) origin, as indicated in Table 1. Dotted lines represent mean nitrite production by macrophages stimulated with *Listeria* alone ± 2 s.d.

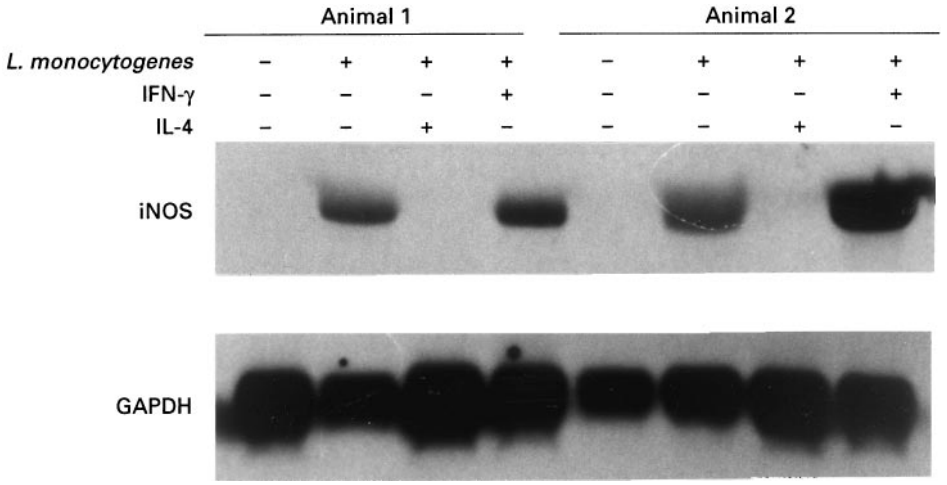

Fig. 4. Inducible nitric oxide synthase **(**iNOS) mRNA expression of bovine monocyte-derived macrophages (MDM) exposed to *Listeria monocytogenes* (200 μg/ml) alone or together with IL-4 (10 ng/ml) or IFN-γ (10 U/ml), as determined by Northern blotting. Hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe served as a loading control. RNA was isolated 20 h after macrophage exposure to bacteria and/or cytokines. The results from two animals are shown.

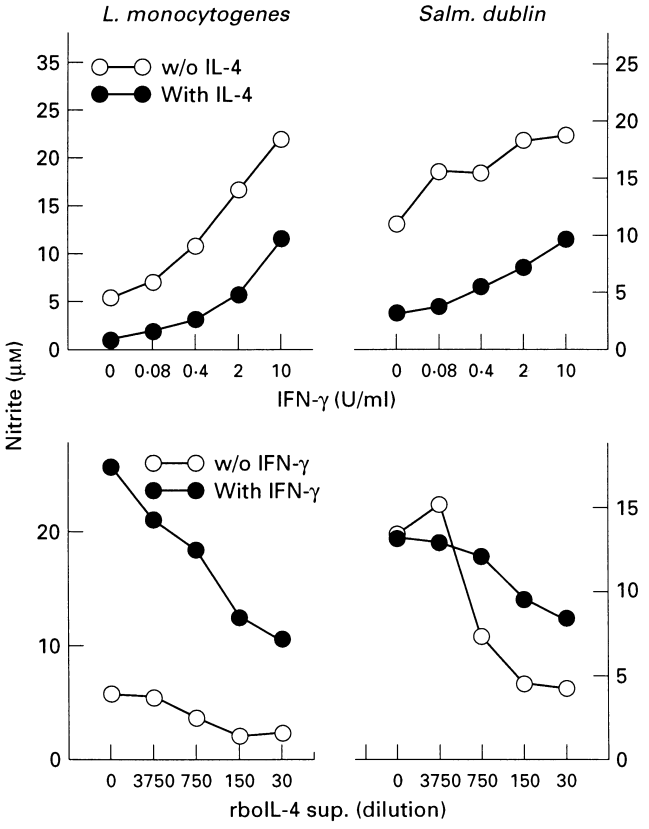

Fig. 5. Dose-dependent modulation of nitric oxide (NO) production by IL-4 or IFN-γ in monocyte-derived macrophages (MDM) exposed to bacteria in the presence or absence of an antagonistic cytokine. Bacteria (*Listeria monocytogenes* or *Salmonella dublin*) were used at 200 μg/ml wet weight. The concentration of rboIL-4 in the rboIFN-γ dose response experiments corresponded to a 50-fold dilution of rboIL-4 containing trypanosome culture supernatant. The concentration of rboIFN-γ in the rboIL-4 dose response experiments was 10 U/ml. No nitrite was generated in the absence of bacteria (not shown).

of whether a high or low IFN-γ concentration was used (resulting in a high or a low NO response in *L. monocytogenes*-stimulated cells), rboIL-4-containing supernatants down-regulated this response in a dose-dependent manner (Fig. 5). Conversely, rboIFN-γ up-regulated NO synthesis in a dose-dependent manner, irrespective of the concentration of IL-4 (Fig. 5).

Evidence that cytokine-induced NO up-regulation is IFN-γ-specific

It was then tested whether the up-regulatory effect of IFN-γ was also observed in supernatants of stimulated T cells, and whether this activity was attributable to IFN-γ alone. In order to separate the up-regulating effect from a potential down-regulation by IL-4, MDM were simultaneously exposed to T cell supernatants and to high concentrations of IL-4, sufficient to saturate IL-4 receptors on MDM. MDM were treated simultaneously with *L. monocytogenes*, IL-4, and with varying concentrations of supernatants from activated PBMC or appropriate control supernatants, and nitrite levels of supernatants collected at 24 h were compared with that of MDM stimulated with IL-4 and bacteria alone. Figure 6a shows that supernatants contained an activity which up-regulated NO production, and the resulting dose–response curves paralleled that achieved with rboIFN-γ. This was consistent with the assumption that these supernatants contained IFN-γ bioactivity. A close correlation was obtained between up-regulation of NO production, and rboIFN-γ content, as determined by an ELISA specific for bovine IFN-γ (Fig. 6b). The assumption that the up-regulating activity was mediated by IFN-γ was borne out in neutralization experiments. Supernatants from mitogen-stimulated T cells, putatively containing natural bovine IFN-γ and many other cytokines at high concentrations, were exposed to polyclonal anti-rboIFN-γ or a preimmune control serum. Table 2 shows that NO-inducing activity was completely neutralized by anti-bovine-IFN-γ, but not by the control serum.

DISCUSSION

Induction of iNOS and NO production in macrophages is subject to an important, but poorly understood species variation [33,34].

Whereas iNOS can be readily induced in murine and bovine macrophages, it is induced in human macrophages under much more restricted activation conditions, and to a low degree only. iNOS induction in bovine and murine macrophages, although of similar magnitude, appears to be differentially regulated. We have reported previously that IFN-γ induces iNOS in the absence of costimulatory factors in murine, but not bovine macrophages, and up-regulates iNOS induced by Gram-negative bacteria or LPS in murine macrophages much more strongly than in bovine counterparts [19]. We now show that iNOS induction by *L. monocytogenes* and other Gram-positive bacteria is strongly enhanced by IFN-γ. This property of IFN-γ appears to be unique, and is not shared by a large test panel of other cytokines. Together with data on other Gram-positive organisms, this qualifies IFN-γ as a unique enhancer of NO synthesis by macrophages exposed to Gram-positive organisms. To our knowledge, similar data on murine macrophages have not been published. Several lines of evidence further support the contention that IFN-γ has a unique role in regulation of NO generation by bovine macrophages. First, the other candidate cytokines most probably contributing to an enhanced NO response, based on murine studies, are IL-1, TNF and IFN-α [17], yet these cytokines had a weak up-regulating effect, at best, in the bovine system. This statement is based on experiments with homologous cytokines shown to be active in other systems [19,35]. Second, the NO-enhancing activity of a crude supernatant from mitogen-activated T cells could be completely neutralized by anti-IFN-γ antibodies. Third, there was a close correlation between NO-enhancing activity of supernatants and their IFN-γ content, as determined by an immunoassay. Thus, although bovine IFN-γ does not induce iNOS by itself, it appears to be the most important cytokine for up-regulating iNOS induced by Gram-positive bacteria (this study), and by zymosan ([36]; T. W. Jungi, unpublished observations). Notably, the combination of the weak iNOS inducer, *Listeria*, and IFN-γ, which is inactive by itself, induces higher levels of NO than LPS or Gram-negative organisms combined with IFN-γ. IFN-γ only slightly up-regulates NO induction by Gram-negative bacteria and high concentrations of LPS, but does enhance NO synthesis induced by low LPS concentration (T. W. Jungi, unpublished).

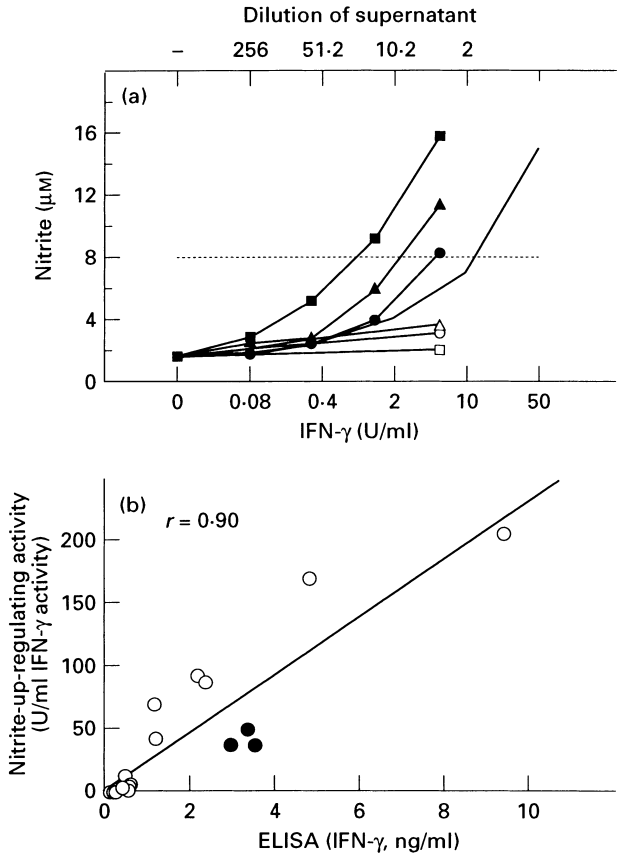

Fig. 6. Correlation between IFN-γ-mediated up-regulation of nitrite generation and IFN-γ immunoreactivity (ELISA). (a) Supernatants from polyclonally stimulated peripheral blood mononuclear cells (PBMC) or purified T cells were tested for up-regulation of nitric oxide (NO) synthesis by monocyte-derived macrophages (MDM) exposed to *Listeria monocytogenes* (200 μg/ml) and IL-4. The latter was a 50-fold dilution of culture supernatant from rboIL-4-secreting trypanosomes. T cells were stimulated for 48 h with phytohaemagglutinin (PHA; ●, 25 μg/ml), PHA and IL-2 (▲; 10 ng/ml), A23187 (0·25 μM) plus phorbol myristate acetate (PMA) (■; 5 ng/ml). Control supernatants were from non-stimulated T cells reconstituted with PHA (○) or PHA plus IL-2 after stimulation (Δ), or from cell-free medium incubated for 48 h with A23187 and PMA (□). Dose–response curves of selected supernatants from stimulated cells and control supernatants are shown. The dotted line reflects half-maximal activation. Nitrite-up-regulatory activity was determined on the basis of the dilution at which half-maximal activity was obtained. - - - -, rboIFN-γ. (b) IFN-γ content, as determined by ELISA (abscissa) was correlated to the respective nitrite up-regulatory activity, as determined by parallel line analysis. The linear regression and the corresponding correlation coefficient are also shown. ○, ●, Supernatants obtained from different donors, and tested in bioassays run at different days.

IL-4 acts as an antagonist to IFN-γ in many systems, and it does so also in the regulation of iNOS expression by bovine macrophages. Thus, IL-4 (and to a lesser extent its sister cytokine, IL-13) down-regulated NO production induced by Gram-negative or Gram-positive bacteria. Both human and bovine recombinant IL-4 were active in a dose-dependent manner. In contrast, two other cytokines reported to have similar activity on murine macrophages were inactive. TGF-β was found to have an effect on bovine macrophages in other regards, namely in down-regulation of procoagulant activity [19]. Human IL-10 from the same source as used here was also shown in another study to be active in a bovine system [31]. Thus, the cytokines other than IL-4/IL-13 which down-regulate NO generation by mouse macrophages fail to do so in our bovine macrophage system.

Some of the cytokines tested here, e.g. IFN-γ, IL-1 and TNF, were previously tested on NO synthesis by bovine alveolar macrophages [37]. In that study, an additive effect of the cytokines was observed after 72 h of culture; the nitrite levels at 24 h after stimulation were in the same range as those reported here, i.e. an order of magnitude lower than those obtained by combining *Listeria* with IFN-γ, and high concentrations of IL-1 and TNF (100 ng/ml) were required to demonstrate an effect [37]. In confirmation of this and our earlier study [19], IL-4, but not TGF-β, was reported to down-regulate LPS-induced NO synthesis.

The cytokine-mediated regulation of iNOS expression in bovine macrophages suggests that Th1 cells up-regulate iNOS via IFN-γ, whereas Th2 cells down-regulate iNOS via IL-4. This antagonism was shown to operate at the level of iNOS mRNA expression (Fig. 4), and it was also operative when the two cytokines were applied in combination (Fig. 5). Thus, a high iNOS response under the influence of IFN-γ could be decreased

Table 2. The capacity of T cell supernatants to up-regulate nitric oxide (NO) production in bovine macrophages is neutralized by IFN-γ-specific antibodies

Co-stimulus	Dilution/conc. (U/ml)	Antiserum*	Dilution	Nitrite (μM)†
None		None		28·1 + 1·4
rboIFN-γ	25	None		67·1 + 2·4
	5	None		59·7 + 1·8
	1	None		47·0 + 1·8
	0·2	None		34·0 + 0·5
	0·04	None		30·6 + 2·0
Act. T cell sup.‡	1:20	None		61·3 + 3·3
	1:80	None		56·1 + 1·9
	1:320	None		47·3 + 1·4
	1:1280	None		38·8 + 1·0
Act. T cell sup.	1:20	Anti-boIFN-γ	1:40	18·0 + 0·3
	1:20	Anti-boIFN-γ	1:160	21·9 + 1·8
	1:20	Anti-boIFN-γ	1:640	26·1 + 1·4
	1:20	Anti-boIFN-γ	1:2560	35·0 + 1·5
	1:20	Rabbit serum	1:40	59·5 + 4·5
	1:20	Rabbit serum	1:80	60·3 + 0·8

*Antibodies were incubated with supernatants for 15 min at 37°C before addition to macrophages.
†Means of triplicates (a representative experiment out of three is shown).
‡Supernatants from peripheral blood mononuclear cells (PBMC) stimulated for 48 h with A23187 (0·25 μM) and phorbol myristate acetate (PMA) (10 nM).

by IL-4 in a dose-dependent manner, and a low iNOS response under the influence of IL-4 could be enhanced by IFN-γ in a dose-dependent manner. This suggests that the level of iNOS expression ultimately depends on a IFN-γ/IL-4 balance. Our finding has implications for measurement of IFN-γ and IL-4 by bioassay. Correlative data and antibody neutralization experiments clearly showed that NO generation by bovine macrophages is an appropriate bioassay system to measure bovine IFN-γ. Samples suspected to contain variable levels of IL-4 can still be measured if they are assayed in the presence of saturating concentrations of exogenous IL-4. Although NO production by monocytoid cell lines has been proposed as an IFN-γ assay in the mouse [38], a simultaneous involvement of other modulating cytokines is much more difficult to achieve in the mouse, since at least four down-regulatory cytokines (IL-4, IL-13, IL-10 and TGF-β) and three additional stimulatory cytokines (IL-1, TNF, IFN-α/β) are known. In cattle, however, macrophage modulation of bacterially induced NO generation by T cell supernatants represents a measure of IFN-γ bioactivity. In a similar vein, NO synthesis by macrophages might also be considered as an indicator for bovine (or human) IL-4, under appropriate test conditions. The final proof could not be provided, however, due to lack of neutralizing antibodies and an immunoassay for bovine IL-4. It is anticipated that with the help of the present macrophage test system, rboIL-4 purification and antibody production will be facilitated, thereby promoting the development of ruminant IL-4 immunoassays.

Understanding the principle of iNOS regulation in macrophages in response to bacterial infection and its biological significance is hampered by the species variation. According to this study, cytokines have a limited, yet clearly defined role in regulating iNOS expression triggered by bacterial components. With the exception of LPS, the exact bacterial constituents inducing iNOS in macrophages have not been identified. Knowledge of the exact nature of the surface receptors linking bacterial recognition to iNOS induction is also limited, but it is to be expected that pattern recognition receptors are involved [39]. Fine tuning of this crude recognition system can occur by the opposing activities of IFN-γ and IL-4, which, in general, are produced by restricted sets of T cells, having perceived an antigenic stimulus in a unique manner. This raises the question as to the conditions under which T cells are instructed to dampen or enhance NO generation by macrophages. It is reasonable to assume that intracellular organisms induce conditions favouring enhancement. This will probably be mediated by IL-12 [40]. Why iNOS expression needs to be restricted in other host defence situations, e.g. when Th2 cells are induced, is not clear. It may be due to the detrimental effects NO can exert, such as immunosuppression, mutagenesis, superoxide-dependent peroxynitrite formation, blood pressure reduction and neuronal disturbance. Cattle may provide a model in which both aspects of this double-edged sword, long-lasting induction as well as cytokine-mediated restriction in expression, can be studied in more detail.

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