Differential Regulation of Inducible Nitric Oxide Synthase Production in Bovine and Caprine Macrophages

Heiko Adler,* Barbara Adler,* Paola Peveri,* Ernst R. Werner, Helmut Wachter, Ernst Peterhans, and Thomas W. Jungi

Institute of Veterinary Virology, University of Berne, Berne, Switzerland; Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Innsbruck, Austria.

Inducible nitric oxide synthase (iNOS) regulation in human and murine macrophages in vitro differs considerably. In this study, expression of macrophage iNOS in ruminants was addressed. Nitric oxide (NO) output by cattle and goat macrophages was as different as that by human and mouse macrophages. Bovine macrophages activated by heated *Salmonella dublin* or lipopolysaccharide (LPS) expressed high levels of iNOS mRNA, protein, and enzyme activity. Analogously cultured caprine macrophages did not respond to these and other activators by NO generation and iNOS expression. The lack of response was not due to general unresponsiveness to stimuli. Caprine iNOS mRNA was induced by stimulation of caprine macrophages with LPS, as shown by reverse transcription polymerase chain reaction. The level of mRNA expression in activated goat macrophages was lower than in resting bovine macrophages. A caprine 372-bp iNOS mRNA fragment that was sequenced closely resembled the bovine counterpart. This points to species-specific iNOS gene regulation.

The term "activated macrophages," as originally defined, describes a state in which macrophages express enhanced microbicidal activity toward intracellular pathogens and tumoristatic and tumoricidal activity [1, 2]. This cellular state is characterized by induction and up-regulation of transcription, alterations at the posttranscriptional level, and activation of enzyme cascades. Studies in rodents showed that the key biochemical pathway for macrophage activation is the induction of a high-output inducible nitric oxide synthase (iNOS) that is induced by activation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ), either alone or combined [3]. The enzyme iNOS catalyzes the conversion of arginine to citrulline and nitric oxide (NO) [4]. NO, a highly reactive nonpolar gas, is a versatile biologic mediator [5, 6]. The production of high concentrations of NO by activated macrophages leads to an array of biochemical reactions culminating in the inactivation of key enzymes of target pathogens or tumor cells [3, 7].

The above concept was questioned when it was realized that human macrophages do not generate NO under conventional activation conditions. Although a stimulatory regime leading to enhanced antimicrobial or tumoricidal activity was very similar for murine and human mononuclear phagocytes, activation was associated with NO generation in murine but not human macrophages [8, 9]. Some studies reported iNOS induction, NO induction, or both in human monocytes or macrophages [10–14], but the activation conditions reported could not be duplicated by others or were much more restricted than those reported for rodent macrophages. This marked species difference raised the possibility that in evolution, rodent and primate macrophages acquired distinct biochemical pathways mediating antimicrobial and antitumoral activity. It was of interest, therefore, to study the iNOS pathway in other species. We recently showed that bovine bone marrow–derived macrophages and monocyte-derived macrophages produce NO upon stimulation with bacterial constituents, such as LPS [15]. A study on cytokine control of bovine and murine macrophage iNOS showed that iNOS induction is more restricted in bovine macrophages [16]. In the present study, we compared iNOS expression and NO generation in activated macrophages from 2 closely related ruminant species, cattle and goat.

Materials and Methods

Reagents. Recombinant bovine (rbo) IFN-γ and rbo tumor necrosis factor-α (TNF-α) were provided by Ciba-Geigy (Basel, Switzerland). The bioactivity of these cytokines was ascertained as described [16]. rbo granulocyte-macrophage colony-stimulating factor (GM-CSF) was from American Cyanamid (Princeton, NJ). Fetal calf serum (FCS) of low endotoxin content was from Biological Industries (Kibbutz Beth Haemek, Israel), and goat serum was from Sigma (no. 6898; St. Louis). Cell culture media and additives were purchased from Seromed Biochrom (Munich) or from Life Technologies (Basel). Medium with low endotoxin content (<10

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Reprints or correspondence: Dr. T. W. Jungi, Institute of Veterinary Virology, Laenggass-Strasse 122, CH-3012 Berne, Switzerland.

* Present affiliations: Dana Farber Cancer Institute, Boston (H.A. and B.A.); Institute of Animal Breeding, University of Berne, Switzerland (P.P.).

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pg/mL LPS bioactivity) was selected. LPS (*Escherichia coli* O55:B5) was obtained from Sigma (no. L2637). *Salmonella dublin* and *Listeria monocytogenes* were provided by the Institute of Veterinary Bacteriology (University of Berne) and were prepared and heat-inactivated (120 min at 60°C) as described [15]. The nitrate reductase (NAD[P]H) from *Aspergillus* species was obtained from Sigma (no. 7265). Monoclonal and polyclonal antibodies specific for mouse macrophage iNOS were from Transduction Laboratories (no. 32020 and 32030; Lexington, KY). In lysates of activated bovine macrophages, monoclonal anti-iNOS stained a band representing iNOS. Polyclonal anti-iNOS strongly stained several other LPS-induced bands but reacted only weakly with bovine iNOS and therefore was not used. D,L-kynurenine and L-tryptophan for calibration of high pressure liquid chromatography (HPLC) were from Serva (Heidelberg, Germany), and acetonitrile and potassium phosphates for preparation of the eluent were from Merck (Darmstadt, Germany).

Culture of bovine and caprine macrophages. Bovine peripheral blood monocyte–derived macrophages (MDMs) were generated by isolation of peripheral blood mononuclear cells by centrifugation over ficoll-hypaque and by selective adherence to tissue-culture flasks. The adherent cells were dislodged after an overnight culture by replacing the medium with PBS and chilling (4°C, 30 min) and vortexing the flasks. Recovered cells were washed once, resuspended in fresh medium, and put into Teflon bags for 6 or 7 days, at which time they had matured to macrophages. The cells were cultured in Iscove's medium without phenol red but containing HEPES (10 m*M*, pH 7.4), L-glutamine (2 m*M*), nonessential amino acids (Life Technologies; 1% vol/vol), MEM vitamin solution (Life Technologies; 1% vol/vol), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (2.5 μg/mL), β-mercaptoethanol (50 μM), and 20% FCS.

Caprine MDMs were prepared and cultured either as described above for bovine MDMs or as follows: Goat mononuclear cells were prepared from buffy coats of citrated blood and separated from neutrophils by a ficoll-hypaque gradient. Contaminating erythrocytes were lysed using a solution of 155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, and 0.1 m*M* EDTA, pH 7.4. The mononuclear cells were washed three times with PBS, and macrophages were derived by culturing the cells for 10–12 days in Teflon bags. The culture medium was RPMI 1640 containing HEPES (10 m*M*, pH 7.4), L-glutamine (2 m*M*), penicillin (100 U/mL), streptomycin (100 μg/mL), β-mercaptoethanol (50 μM), and 10% goat serum. Parallel experiments assured that differences in isolation methods did not influence NO generation by goat or bovine cells.

Bovine and caprine cells that had been cultured in suspension were subcultured in 96- or 24-well culture plates or 25-cm^2 flasks, in which they were exposed to cytokines or bacterial agents (or both). Iscove's medium supplemented as described above and containing 2% FCS was used for subculture.

Nitrite determination. After MDMs (10^5 cells/well in culture medium with 2% FCS) were subcultured for the times indicated, 100 μL of cell-free supernatants or sodium nitrite standard dilutions were mixed with 50 μL of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 1.25% H_3PO_4) and incubated for 10 min at room temperature before NO_2^- concentrations were determined by measuring optical density at 550 nm in an ELISA reader. The detection limit of the test was 2 μM NO_2^-.

Conversion of nitrate into nitrite. Nitrate was converted into nitrite by use of nitrate reductase (Sigma; 0.4 U/well for 18 h in the presence of 500 μM β-NADPH) and then assayed as nitrite. Using these conditions, the detection limit of the test was ~4 μM NO_3^- as determined by the conversion of sodium nitrate standard dilutions, which were included in the test.

Detection of iNOS by immunoblotting. Cells were washed in PBS and then disrupted by sonication in a sample buffer containing 6% SDS and 10% mercaptoethanol. The proteins were separated on polyacrylamide gels and electrophoretically transferred to a Hybond C membrane (Amersham, Arlington Heights, IL). The blot was incubated with a monoclonal anti-murine iNOS antibody (Transduction Laboratories) and stained using a peroxidase-conjugated sheep anti-mouse antibody and a chemiluminescence protein detection method (Amersham). Total cellular protein was determined by measuring the optical density at 280 nm.

RNA isolation. For analysis of iNOS mRNA, bovine and caprine MDMs were cultured in 25-cm^2 flasks, and total cellular RNA was isolated from the cell monolayers using a protocol optimized for TRIzol (Life Technologies).

Analysis of mRNA by reverse transcription–polymerase chain reaction (RT-PCR) and Southern blotting. First-strand cDNAs were synthesized by incubating 5 μg of total RNA from bovine or caprine MDMs with 10 n*M* of the appropriate antisense primer in a 50-μL reaction volume containing 50 m*M* TRIS-HCl (pH 8.3), 1 m*M* $MgCl_2$, 75 m*M* KCl, 10 m*M* dithiothreitol, 40 U of RNasin (Promega, Madison, WI), 0.5 m*M* dNTPs, and 8 U of AMV (avian myeloblastosis virus) reverse transcriptase (Promega). The mixtures were incubated for 1.5 h at 42°C. The PCR reaction contained 10 m*M* TRIS-HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% (wt/vol) gelatin, 1% (vol/vol) Triton X-100, 0.25 m*M* dNTPs, 0.2 m*M* sense and antisense primer, 1 U of Taq polymerase (Stehelin, Basel), and 5 mL of RT reaction in a 100-mL volume. A temperature profile of 30 s at 94°C, 1 min at 56°C, and 1 min at 74°C was used for 30 cycles. Bovine iNOS primers (372-bp product) were sense 5′-TAGAGGAACATCTGG-CCAGG-3′ and antisense 5′-TGGCAGGGTCCCCTCTG ATG-3′-[16]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (356-bp product) were sense 5′-GAGATG-ATGACCCTT TTGGC-3′ and antisense 5′-GTGAAGGTCGGA-GTCAACG-3′ [17].

The PCR products were fractionated on agarose gels and blotted onto a positively charged nylon membrane (Boehringer, Mannheim, Germany), and the blot was processed with a digoxigenin-labeled iNOS-specific RNA probe [16] and a digoxigenin-labeled GAPDH-specific RNA probe using the digoxigenin detection kit (Boehringer).

DNA sequencing. The 372-bp iNOS RT-PCR amplification product was cloned into pBluescript SK+ (Stratagene, La Jolla, CA) and was sequenced on both strands by use of the dideoxynucleotide chain termination method with T7 DNA polymerase (Sequenase, United States Biochemicals, Cleveland) and synthetic oligonucleotide primers.

Cytokine assays. Caprine TNF-α activity was determined by a recently described cytotoxicity assay, using porcine PK(15) cells [18]. Cytotoxicity of the PK(15) cells by supernatants of caprine macrophage cultures could be prevented in vitro by a murine monoclonal anti-bovine TNF-α antibody, confirming that this assay is specific for TNF-α [19].

Measurement of procoagulant activity. Expression of procoagulant activity (PCA) by intact macrophages was measured in microtiter plates, using the turbidimetric, kinetic plasma recalcification time assay as described [20].

Detection of tryptophan and kynurenine in cell-free supernatants. The presence of tryptophan and kynurenine was determined by reversed-phase HPLC by use of 1050 HPLC equipment (Hewlett Packard, Vienna) and a 125-mm-long, 4-mm-diameter, 5-mm particle size lichrospher RP-18 column (Merck 50943) equipped with a 4 × 4-mm precolumn of the same material. Cell-free supernatant (50 mL) was injected and eluted isocratically at a flow rate of 0.8 mL/min with 15 m*M* potassium phosphate, pH 6.4, containing 1.8% (vol/vol) acetonitrile. Tryptophan was detected by UV absorption at 280 nm (detection limit, 0.5 m*M*), and kynurenine was detected at 360 nm (detection limit, 0.3 m*M*).

Infection with bovine viral diarrhea virus (BVDV) and staining for viral antigen. BVDV infections of bovine or caprine MDMs and detection of virus-infected cells were performed as previously described [21].

Limulus amebocyte lysate test. All reagents to which macrophages were exposed were subjected to a kinetic limulus amebocyte lysate assay. When LPS from *E. coli* O55:B5 (Sigma) was used as a standard, the test had a sensitivity of 2 pg/mL. Sensitivity was lower for sera and complex media (threshold of sensitivity, 10–40 pg/mL, depending on the agent). Samples were measured at various concentrations with or without the addition of a 50-pg LPS spike; a spike recovery between 75% and 125% was considered acceptable.

Results

Bovine mononuclear phagocytes produce nitrite upon bacterial stimulation. Bovine and caprine monocytes were allowed to differentiate into macrophages in vitro by use of a nonadherent (Teflon-foil based) culture system. Macrophages were harvested and subcultured under identical conditions in microtiter plates. Bovine and caprine MDMs were either mock-stimulated or stimulated with heat-killed *S. dublin* or purified LPS (*E. coli* O55:B5). Nitrite was determined in 24-h supernatants by use of Griess reagent. Both *S. dublin* and LPS induced high levels of nitrite in supernatants of bovine MDMs, but neither did so in caprine MDMs (table 1, 1st experiment). Nitrite production by bovine MDMs was suppressed by coincubation with N^G-monomethyl-L-arginine (data not shown). Even after prolonged stimulation with *S. dublin,* nitrite was detected in supernatants of bovine but not of caprine MDMs (data not shown).

Unresponsiveness of goat macrophages is restricted to NO generation. The inability of caprine cells to produce nitrite was not due to a general lack of activation by the above-mentioned stimuli, as shown by a variety of functions measured in addition to nitrite generation. Thus, although stimulated caprine macrophages failed to produce NO, they produced copious amounts of TNF-α. On average, supernatants of *S. dublin*–exposed goat cells caused 85% ± 4% cell lysis in a PK(15) cytotoxicity assay (n = 3), whereas supernatants of unstimulated cells failed to lyse these target cells. Both *S. dublin*–stimulated and LPS-stimulated cells displayed enhanced PCA, as determined by a recalcification-time assay (table 1), and similar results were obtained after stimulation with rboIFN-γ, rboTNF-α, *L. monocytogenes* (data not shown), and *L. monocytogenes* and rboIFN-γ combined (table 1). Enhanced PCA is a hallmark of activated macrophages.

Table 1. Generation of NO_2^- by bovine and caprine monocyte-derived macrophages stimulated by *Salmonella dublin* or lipopolysaccharide (LPS).

Stimulant (exposure time)	NO_2^- production within 24 h (μM)*		Recalcification time (s)*	
	Cattle	Goat	Cattle	Goat
Experiment 1				
None	<2	<2	158 ± 12	172 ± 12
S. dublin (24 h)†	10.9 ± 1.7	<2	25 ± 0	25 ± 0
LPS (24 h)‡	4.2 ± 0.5	<2	58 ± 8	65 ± 0
Experiment 2				
None	<2	<2	458 ± 49	405 ± 26
rboIFN-γ (72 h) + *Listeria monocytogenes* (24 h)†	35.7 ± 3.1	<2	132 ± 5.8	62 ± 5.8

NOTE. Bovine or caprine monocyte-derived macrophages were harvested from Teflon bags and subcultured in microtiter plates (10^5 cells/well), in which they were exposed for indicated time to various activating stimuli. Next, nitrite was measured in supernatants, and recalcification time was determined for cell monolayers.

* Means of triplicates ± SD of 2 representative experiments of 19 for goats and >40 for cattle.

† 200 μg/mL.

‡ 1 μg/mL.

We also tested for the presence of an enzyme known to be induced by IFN-γ, indoleamine 2,3-dioxygenase (IDO), which cleaves the pyrrole ring from tryptophan and other indoleamines, thereby generating various tryptophan metabolites of the kynurenine pathway [22]. The IDO pathway appears to be involved in the destruction of some intracellular parasites [23]. IDO activity was evidenced by determining the levels of kynurenine in the supernatants of stimulated and unstimulated macrophages. Decreases in tryptophan and increases in kynurenine levels were noted in supernatants of stimulated but not unstimulated macrophages of either species (table 2). Thus, rboIFN-γ could activate both bovine and caprine macrophages.

Conversion of nitrate into nitrite using nitrate reductase. Since NO is an unstable product converted to either nitrite or nitrate, depending on local conditions, the possibility was considered that in cultures of goat macrophages, NO is converted to nitrate rather than nitrite. Supernatants of activated macrophages from both cattle and goats were exposed to nitrate reductase followed by the Griess reaction. In cattle, nitrite was increased 2- to 4-fold by nitrate reductase treatment (data not shown). No increase was obtained with goat supernatants, discounting the possibility that NO was generated but went undetected in simple nitrite determination experiments.

Table 2. Induction of indoleamine 2,3-dioxygenase in cattle and goat macrophages stimulated by recombinant bovine interferon-γ (rboIFN-γ) and *Salmonella dublin*.

	Tryptophan (μM)		Kynurenine (μM)	
Stimulant	Cattle	Goat	Cattle	Goat
None	47.9 ± 1.8*	52.2 ± 10.5	9.3 ± 0.5	0.9 ± 1.7
rboIFN-γ	18.8 ± 15.0	4.4 ± 5.5	22.8 ± 4.1	6.6 ± 1.0
S. dublin	51.8 ± 5.1	48.2 ± 18.9	9.1 ± 1.1	2.0 ± 1.7
rboIFN-γ + *S. dublin*	21.6 ± 10.3	9.1 ± 9.1	21.0 ± 5.4	4.0 ± 2.5

NOTE. Bovine or caprine monocyte-derived macrophages were cultured in Teflon bags (10^6 cells/mL) and stimulated with rboIFN-γ (1000 U/mL for 72 h) and with heat-killed *S. dublin* (200 μg/mL for last 24 h). Tryptophan and kynurenine were determined in cell-free supernatants as described in Materials and Methods.

* Values represent means ± SD (n = 2 for cattle and 4 for goats).

Assessment of various conditions activating goat and cattle mononuclear phagocytes. Since macrophage iNOS induction may not depend solely on LPS but may need additional priming signals, goat macrophages were pretreated with various cytokines (rboIFN-γ, rboTNF-α, and rboGM-CSF) either alone or followed by stimulation with LPS. None of these regimes induced NO generation in goat macrophages (data not shown). Another treatment that induced high levels of NO in bovine macrophages, the combination of rboIFN-γ and *L. monocytogenes*, failed to trigger NO production in goat MDMs (table 1, 2nd experiment).

Infection of bovine macrophages by noncytopathic BVDV primed these cells for enhanced iNOS response upon bacterial stimulation [21]. To test whether a similar priming rendered caprine macrophages responsive to bacterial stimuli, caprine MDMs were infected with noncytopathic BVDV, and virus infection was checked by staining for viral antigen. Although goat cells were readily infected by BVDV, as detected by immunoenzymatic staining of cells for viral antigen, no nitrite was detected in supernatants of virus-infected, *S. dublin*–stimulated goat cells (data not shown).

Because of our earlier observation that the absolute amount of nitrite produced may be dependent on the culture conditions of macrophages and particularly on the serum source used [16], bovine and caprine MDMs were cultured under identical conditions using either FCS or goat serum. Bovine MDMs produced nitrite regardless of the serum source used. In contrast, caprine MDMs did not produce nitrite under either condition (data not shown).

Since iNOS regulation differed in bovine monocytes and macrophages [23a], freshly isolated caprine blood monocytes were also tested for iNOS induction. These cells also failed to produce nitrite in response to rboIFN-γ, rboTNF-α, and rboGM-CSF either alone or followed by stimulation with LPS (data not shown).

Detection of iNOS by immunoblotting. To demonstrate iNOS protein induction, lysates of bovine and caprine MDMs either unstimulated or stimulated with *S. dublin* were immunoblotted by use of a murine iNOS–specific monoclonal antibody (MAb). iNOS protein was detected by the MAb in stimulated bovine but not in unstimulated bovine macrophages or caprine MDMs, regardless of the state of activation (figure 1).

Detection of iNOS mRNA. Using iNOS-specific primers, a PCR signal was detected in unstimulated bovine but not caprine macrophages. After induction by *S. dublin*, a clear increase in the iNOS mRNA was detected by RT-PCR in bovine macrophages, and a faint signal was visible also in *S. dublin*–stimulated caprine MDMs (figure 2A). To verify the RT-PCR products, the bands were stained with a specific bovine iNOS probe [16] in a Southern blot (figure 2B). This confirmed that the iNOS-specific signal in activated caprine macrophages was stronger than in resting counterparts but weaker than in resting bovine MDMs. Of note, in the latter, NO production could not be detected by the Griess assay.

Cloning and sequencing of a caprine iNOS fragment. The previous experiment suggested that an iNOS transcript was

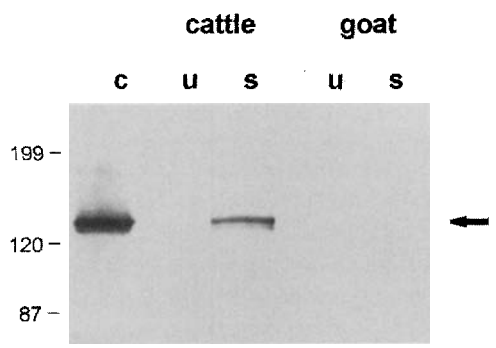

Figure 1. Detection of iNOS by immunoblotting. Monoclonal antibody to murine macrophage iNOS was used for detection in cattle lysates and goat monocyte–derived macrophages. Cells were unstimulated (u) or stimulated (s) with heat-inactivated *Salmonella dublin* (200 μg/mL for 48 h). Cell lysates (10 μg/lane) were fractionated by PAGE, transferred to nitrocellulose, and stained. Antigen was detected by enhanced chemiluminescence. Arrow, position of iNOS (~130 kDa). Protein molecular mass markers are indicated in kDa. Positive control for iNOS (lysate of stimulated mouse macrophages) was loaded in lane c.

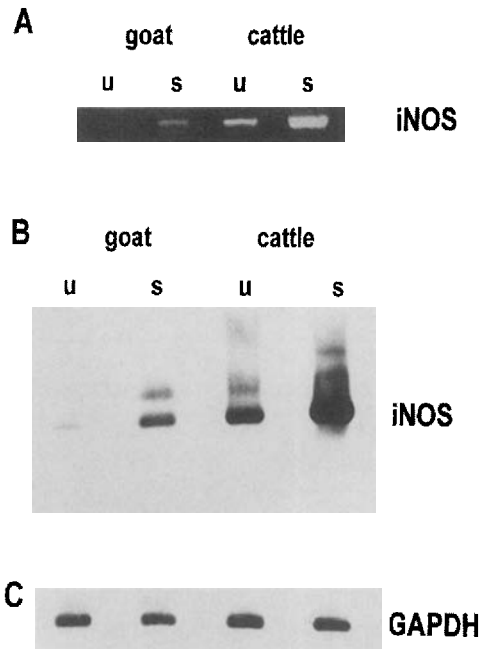

Figure 2. Detection of iNOS mRNA. Total RNA was isolated from unstimulated (u) and stimulated (s) (*Salmonella dublin;* 200 μg/mL) goat and cattle macrophages 6 h after stimulation, reverse transcribed, amplified by polymerase chain reaction (PCR) using iNOS-specific primer pair, and evaluated by PAGE followed by ethidium bromide staining (**A**). iNOS PCR product is 372-bp long. PCR products were blotted onto positively charged nylon membrane, and blot was processed as described in Materials and Methods (**B**). 10 μL of iNOS reverse transcription reaction was used to amplify housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using human GAPDH primers (**C**).

expressed in caprine macrophages, although at a low level only. The 372-bp RT-PCR fragment of the caprine macrophage iNOS was cloned into pBluescript SK+ (Stratagene) and sequenced as described in Materials and Methods to determine the degree of similarity to iNOS of other species. This sequence has been deposited in GenBank (accession no. U29085). The sequence has a 97% similarity with the nucleotide sequence of bovine iNOS [16]. At the amino acid level, only one mismatch was found. An alignment of the bovine iNOS with the cloned and sequenced caprine iNOS fragment is shown in figure 3.

Discussion

The arginine-dependent NO pathway is considered to be an essential part of the antimicrobial and antitumoral arsenal of activated macrophages. This assumption rests on the following lines of evidence generated in rodents: The activation conditions of mouse macrophages leading to antimicrobial and antitumoral activity closely resemble those leading to the generation of NO [24, 25]; arginine analogs serving as metabolic inhibitors of iNOS not only prevent the formation of NO but also antimicrobial and antitumoral effector functions [24–26]; chemical NO generators have potent antimicrobial and antitumoral activity [27, 28]; and iNOS-deficient mice show inefficient antimicrobial resistance [29, 30].

This solid body of evidence contrasts with the controversy as to the role of the iNOS-NO pathway in effector functions of human macrophages. The investigation of macrophage iNOS in other species is therefore of high interest. We previously demonstrated that bovine macrophages can be activated to generate iNOS mRNA and to produce arginine-derived nitrite [15, 16]. The current study shows that there are significant differences in the high-output iNOS pathway between bovine and caprine macrophages. Thus, whereas bovine macrophages could be triggered to express iNOS mRNA and activity by *S. dublin,* LPS, or bacterial constituents combined with IFN-γ, goat macrophages did not generate detectable levels of NO after activation by a broad array of activating agents, including those providing a positive response in bovine counterpart cells.

An analysis of mRNA expression by RT-PCR and Southern blotting showed that levels of mRNA of activated goat macrophages were lower than those of resting bovine macrophages but higher than those of resting goat macrophages. Thus, although the level of mRNA expression increased following activation, the amount of transcript generated was insufficient to maintain generation of enzyme in amounts sufficient to allow detection of nitrite by the Griess assay. This notion was supported by the failure to detect iNOS in activated goat cells by Western blotting. An alternative interpretation is that the MAb, which was raised against murine iNOS, recognizes cattle iNOS but not goat iNOS. We think this unlikely, since the same antibody recognizes mouse, cattle, and human iNOS, and there is a high degree of similarity between species throughout the iNOS genome. Moreover, the absent Western blot signal in goats paralleled the lack of generated nitrite and nitrate, as determined by the Griess assay.

Our observations raise the possibility that macrophages from closely related species differ in iNOS activity when conventionally activated in vitro. Among the species reported, rat [24], mouse [31, 32], cattle [15], and chicken [33] are high responders as regards macrophage iNOS, whereas goat (this study), rabbit [8], pig [34], dog (unpublished data), and human [8] are low responders. This suggests that differences in macrophage iNOS regulation, as manifested in vitro, may have developed more than once, including at times of speciation within ruminants. The mechanism(s) causing these differences is unknown. One possibility is that iNOS is differently regulated at the transcription level. In keeping with this hypothesis, the promoter of murine (macrophage) iNOS and human (hepatocyte) iNOS is clearly distinct. Reporter constructs using elements of the human iNOS promoter appear not to be readily inducible. Similar constructs containing elements of murine iNOS promoters are readily induced by IFN-γ and other agents (Billiar TR, personal communication). The promoters of ruminant iNOS are not known; however, it is unlikely that promoters

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bovine  iNOS  (  1)  GTGGAAGCAGTAACAAAGGAGATAGAAACAACAGGAACCTACCAGCTGAC
caprine iNOS  (  1)  --------G-----------------------------------------

bovine  iNOS  ( 51)  GGGAGATGAGCTCATCTTCGCCACCAAGCAGGCCTGGCGCAACGCCCCCC
caprine iNOS  ( 51)  ------------T-----T-------------------------------

bovine  iNOS  (101)  GCTGCATCGGAAGGATCCAGTGGTCGAACCTGCAGGTCTTTGACGCCCGG
caprine iNOS  (101)  ----------G-----------------------------------------

bovine  iNOS  (151)  AGCTGTTCCACGGCCCAGGAAATGTTCGAACACATCTGCAGACACGTGCG
caprine iNOS  (151)  -------------------------T-----------------------

bovine  iNOS  (201)  TTATGCCACCAGCAACGGCAACATCAGGTCGGCCATCACTGTGTTCCCCC
caprine iNOS  (201)  ---C-------A---T----------------------C----------

bovine  iNOS  (251)  AGCGGAGCGATGGGAAGCATGACTTCCGGGTCTGGAACGCCCAGCTCATC
caprine iNOS  (251)  ------------------------------------T------------

bovine  iNOS  (301)  CGCTATGCCGGCTACCAGATGCCAGATGGCAG
caprine iNOS  (301)  --------------------------------
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Figure 3. Alignment of nucleotide sequence of caprine-iNOS fragment with bovine iNOS sequence [16].

of caprine and bovine iNOS are as different as those of murine and human iNOS. This does not exclude that subtle differences in the promoter region are responsible for the low levels of iNOS transcript found in caprine cells. In humans, even subtle allelic differences in the TNF-α promoter code for differences in TNF-α expression upon infection with *Plasmodium falciparum,* the high responders being at risk for contracting cerebral malaria more readily than the low responders [35].

A primary concern regarding the different iNOS activity in caprine macrophages was the ability of stimuli to actually activate caprine MDMs (e.g., by induction of other functions). Three functions characteristic for activated macrophages were expressed by goat macrophages in the absence of iNOS expression: up-regulation of PCA, expression of TNF-α, and up-regulation of IDO. The latter two have been implicated in protection against a number of microorganisms [36, 37] and tumors [38]. Of interest, although species variation has been shown for expression of IDO [38–41], goat and cattle do not appear to differ in this respect. Thus, the inability of caprine macrophages to respond to activating stimuli, such as bacterial constituents, cytokines, or a combination of the two, is specifically restricted to iNOS expression, as also reported for human macrophages.

Another possible explanation for different iNOS activity in bovine and caprine macrophages is that cofactors (e.g., tetrahydrobiopterin levels) differ in the two respective cell types. We regard this to be unlikely for two reasons: The difference in iNOS activity is manifest also at the level of mRNA and protein expression, as determined by immunoblotting, and preliminary experiments aimed at determining the levels of various biopterin metabolites did not reveal conspicuous differences between goat and cattle macrophages.

Yet another interpretation of the reported results is that the differences observed in vitro differ from macrophage effector function in vivo. This would imply that under in vitro conditions, important aspects of macrophage functions cannot be reproduced in cells of some species but easily can be reproduced in cells of closely related species, even when adhering to identical in vitro conditions. In a similar vein, iNOS expression is observed in bovine bone marrow–derived macrophages under more restricted activation conditions than in murine counterparts [16, 42], and mononuclear phagocytes of distinct differentiation stages show differences in iNOS regulation [23a]. The reasons for species- and differentiation stage–specific iNOS induction in macrophages are not known. It will be of interest to compare macrophage iNOS expression or activity in vivo in tissues harboring intracellular pathogens.

Regardless of whether nonresponder macrophages can or cannot be induced to express iNOS and produce NO under the "right" conditions, an intriguing question is how activated macrophages of nonresponder species promote antimicrobial and antitumoral activity under conventional activation conditions in vitro (i.e., in the absence of iNOS expression). Antimicrobial activity of macrophages may rest on more than one pathway, depending on the microorganism, the type of macrophage being infected, and other conditions. In several models of infection by intracellular pathogens, arginine- or NO-dependent pathways are essential or not essential, depending on the circumstances. These models include pathogens such as *L. monocytogenes* [43–46], *Francisella tularensis* [47, 48], and *Toxoplasma gondii* [36, 49, 50]. For example, human glioblastoma cells infected by *T. gondii* control this parasite in an IFN-γ– and tryptophan-dependent but arginine-independent fashion [23].

Much of the evidence for an essential role of iNOS rests on studies with metabolic inhibitors (arginine analogues). Inhibitors of any kind are prone to have side effects, and since arginine is an essential amino acid, arginine analogues might compete with arginine in vital cellular processes unrelated to macrophage effector function as such. Having at our disposition

macrophages from 2 closely related species, which express distinct levels of iNOS when cultivated under identical culture conditions, it will now be interesting to compare antimicrobial effector functions in an inhibitor-free setting in these two in vitro models.

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