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Comparison of Inducible Nitric Oxide Synthase Expression in the Brains of *Listeria monocytogenes*-Infected Cattle, Sheep, and Goats and in Macrophages Stimulated In Vitro

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The expression of inducible nitric oxide synthase (iNOS) was studied in the brains of cattle, sheep, and goat that succumbed to a natural infection with *Listeria monocytogenes*. The lesions in infected brains are characterized by microabscesses, perivascular cuffs, gliosis, glial nodules, and large areas of malacia. Using immunocytochemistry, we detected bacteria in microabscesses, particularly in sheep and goats, and in areas without signs of inflammation, but not in perivascular infiltrates. iNOS was expressed by macrophage (Mϕ)-type cells of microabscesses and glial nodules but rarely by Mϕ in areas of malacia, as determined by immunohistochemistry with iNOS-specific antibodies. iNOS was not detected in perivascular cuffs. Major histocompatibility complex class II molecules (MHC-II), another marker of cell activation, showed a different pattern of distribution. Perivascular cuffs contained high numbers of MHC-II-positive cells, including some with Mϕ characteristics. Microabscesses in sheep and goats showed low expression of MHC-II, particularly in iNOS-expressing cells. In cattle, the expression of markers for activated or recruited phagocytes, the calcium-binding proteins S100A8 and S100A9 (formerly called MRP-8 and MRP-14, respectively), was largely restricted to cells showing weak or undetectable iNOS expression; iNOS-positive Mϕ showed a low expression of S100A8 and S100A9. Thus, iNOS is expressed by a restricted subset of Mϕ in listeric encephalitis. In cultured sheep and goat Mϕ, a low proportion of cells expressed iNOS upon activation by *L. monocytogenes* and gamma interferon, resulting in nitrite generation at least 1 order of magnitude lower than that in similarly treated cattle Mϕ. Since these species differences were much less obvious in vivo, it appears that the well-known species variation in iNOS expression by Mϕ could reflect an in vitro phenomenon.

The term activated macrophages (Mϕ), as originally defined, describes a state in which Mϕ express enhanced microbicidal and antitumoral activity (26). A key effector pathway of activated Mϕ is the induction of a high-output inducible nitric oxide synthase (iNOS) catalyzing the conversion of arginine to citrulline and nitric oxide (NO) (18). NO, a highly reactive nonpolar gas, is a versatile biological mediator with a variety of target molecules (32, 40). Higher vertebrates possess at least three NOS isoenzymes, which catalyze the conversion of arginine to citrulline and NO in a complex reaction involving several cofactors (33). Two NOS isoenzymes show a tissue expression restricted to certain nerve cells and endothelial cells; upon their activation, NO is generated instantly, thereby serving as a neurotransmitter or as a regulator of blood pressure (36, 40). A third isoenzyme, iNOS, is not constitutively expressed but can be induced in a broad variety of tissues, including Mϕ (33). Upon activation by bacterial constituents and/or cytokines, iNOS is induced in murine Mϕ in enzymatically active form, which leads to a high output of NO over extended periods of time. This NO interacts with key enzymes involved in cell division and energy flow, thereby imparting antimicrobial and antitumoral activity (17, 32). In vitro studies with rats and mice showed for a large number of intracellular bacteria and protozoans as well as viral and fungal pathogens (28) that Mϕ-derived NO is an essential mediator of pathogen killing. Evidence for an effect of NO comes from studies with metabolic inhibitors of NOS and on studies with mice genetically deficient for iNOS (27, 28, 48).

The foregoing concept was questioned when it was realized that human Mϕ fail to generate detectable amounts of NO in vitro under conventional activation conditions, despite expression of strong antimicrobial and tumoricidal activity (41). Some studies reported expression of iNOS and/or production of NO by stimulated human Mϕ (see references in references 1 and 28), but the activation requirements reported either could not be confirmed by others or were much more restricted than those reported for rodent Mϕ (5, 24, 41). We and others showed recently that bovine Mϕ derived from bone marrow cell cultures, from monocyte cultures, or from alveolar lavages produce NO when activated by bacterial constituents (1, 2, 23, 29, 51). In contrast, the closely related goat Mϕ resemble human Mϕ in their inability to generate detectable amounts of NO upon activation (1). This finding raises the question of whether a similar species difference is seen in vivo, in foci of microbial infections. In this study, we assessed iNOS expression in brains of cattle, sheep, and goats that succumbed to a natural infection with *Listeria monocytogenes*. The expression of iNOS in the brains of listeric ruminants was compared with that of Mϕ of the same species stimulated in vitro.

In the brain, all major NOS isotypes are either constitutively expressed or induced by appropriate stimulation. They not only fulfill their expected physiological roles but contribute to pathology in a wide variety of diseases, e.g., ischemia, autoimmunity, and microbial infections (8–10, 35, 52). NO is likely to be involved in disorders of consciousness and brain blood flow and in demyelination and cerebral tissue damage (9, 14, 35). A major role has been attributed to NOS activation and NO

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generation in cerebral complications of AIDS (10), in septic encephalitis (8), and in Alzheimer's disease, parkinsonism, and multiple sclerosis (20, 44, 47, 49). The above-mentioned species variation, which extends also to brain cells (19, 37), casts doubts on the relevance of such rodent models for human diseases, and in vitro systems only partially reflect in vivo conditions. The described encephalitis of ruminants due to infection with a gram-positive organism provides a complementary model to learn more about generation, regulation, and effects of NO in the brain of nonrodent species.

Both the ex vivo and in vitro studies reported here suggest that in ruminants, iNOS is expressed by a subset of activated Mϕ. This is one of the first studies looking at iNOS expression in vivo in several nonrodent species and point to both differences and similarities between iNOS induction in Mϕ in vitro and in vivo.

MATERIALS AND METHODS

Reagents. Polyclonal anti-murine iNOS was obtained from Transduction Laboratories, Lexington, Ky., and from Upstate Biotechnology, Inc. (UBI), Lake Placid, N.Y. (catalog no. 06-295). Monoclonal antimurine iNOS was from Transduction Laboratories. Western blot analyses (1) showed that these antibodies stain a double band of the molecular size of iNOS in lysates of activated mouse, cattle, sheep, and goat Mϕ, and the induction pattern was consistent with iNOS expression. The polyclonal antibody from UBI was preferred for immunohistochemistry since no other bands were specifically stained in activated Mϕ and since it could be used on formalin-fixed tissues. Rabbit antibodies against *L. monocytogenes* (serotype 1/4) were purchased from Difco, Detroit, Mich. A panel of monoclonal antibodies recognizing the proteins S100A8 (formerly MRP-8) and S100A9 (formerly MRP-14) (39) was generously provided by Biomedicals AG, Augst, Switzerland. Antibodies reacting strongly with cattle phagocytes in formalin-fixed tissue included those from clone S13.67 (anti-S100A8) and clone S36.48 (anti-S100A9). Antibodies directed against the heterodimeric S100A8-S100A9 (S100A8/A9) complex did not react. None of these antibodies stained cells in fixed goat or sheep tissue. A monoclonal antibody specific for sheep major histocompatibility complex class II molecules (MHC-II), VPM54, was generously provided by B. Blacklaws, Cambridge University, Cambridge, England. It cross-reacts with MHC-II of goat and cattle and is commercially available through Serotec, Oxford, England. Lipopolysaccharide (LPS; *Escherichia coli* O55:B5) was purchased from Sigma, St. Louis, Mo. (catalog no. 2637). *Salmonella dublin* 24-90 and *L. monocytogenes* NCTC 10527 were provided by J. Nicolet, Institute of Veterinary Bacteriology, Berne, Switzerland, and were washed and heat killed (1 h at 60°C). Recombinant bovine gamma interferon (rboIFN-γ) and recombinant bovine tumor necrosis factor alpha (rboTNF-α) were generously provided by Novartis, Basel, Switzerland. Recombinant bovine interleukin-1β (rboIL-1β) was the generous gift of American Cyanamid, Princeton, N.J. Recombinant ovine IFN-γ (rovIFN-γ) was kindly provided by Paul Wood, CSIRO, Parkville, Australia.

Pathological specimens. Animals referred to the Institute of Animal Neurology were necropsied. The formalin-fixed brains were sliced; coronal sections were embedded in paraffin, cut at 4 μm, and mounted on positively charged slides (SuperfrostPlus; Menzel, Braunschweig, Germany). They were deparaffinized and stained with hematoxylin-eosin for histological diagnosis, or they were used for immunocytochemistry. In ruminant listeriosis, histologic examination is of higher diagnostic accuracy than bacteriology.

Cell isolation and culture. (i) Bovine MNC. Bovine blood was collected aseptically in blood transfusion bags (Baxter AG, Dietlikon, Switzerland) and centrifuged at 1,250 × *g*. The upper portion of the pellet was washed three times. Erythrocytes of the cell pellet were lysed in 155 mM NH_4Cl–10 mM $KHCO_3$–0.1 mM EDTA, followed by three washes with citrate buffer (4.8 mM glucose, 3 mM KCl, 30 mM citric acid, 102 mM NaCl [pH 6.5]) and low-speed (300 × *g*) centrifugations to remove the majority of thrombocytes. Mononuclear cells (MNC) of the centrifuged lysate were separated over Ficoll-Hypaque (density of 1.077). The interphase was washed in phosphate-buffered saline (PBS) and resuspended in culture medium, which consisted of Iscove's modified Dulbecco's minimum essential medium (IDMEM; Seromed, Munich, Germany), HEPES (10 mM), sodium pyruvate (1 mM), glutamine (2 mM), nonessential amino acids (1%, vol/vol; Seromed), minimum essential medium vitamin solution (1%, vol/vol; Seromed), penicillin (100 IU/ml), streptomycin (100 μg/ml), neomycin (10 μg/ml), and 20% heated (30 min, 56°C) fetal calf serum (FCS) low in endotoxin (Life Technologies, Basel, Switzerland). MNC were sealed in custom-made bags made from Teflon foil (type 100 A; Du-Pont de Nemours; purchased through Angst & Pfister, Zurich, Switzerland). Bags were sealed with a sealing device (Polystar, Hamburg, Germany) and placed in a humidified incubator (37°C, 5% CO_2). After 1 week, the majority of lymphocytes had disappeared, and monocytes had differentiated into Mϕ (23). Cells were subcultured at day 7 (± 1 day) in flat-bottom 96-well plates for NO determination and cell enzyme-linked immunosorbent assay (ELISA) and in 24-well plates containing a glass coverslip for immunocytochemistry. The medium for subculture was IDMEM with the above-mentioned additives except neomycin and with 5% FCS only. After 2 h of adherence, nonadherent cells (the majority of the remaining lymphocytes) were removed by washing with IDMEM, and cells were stimulated with LPS (1 μg/ml), heat-killed *S. dublin* (200, 20, or 2 μg/ml), or heat-killed *L. monocytogenes* (200, 20, or 2 μg/ml). These stimuli were given either alone or together with rboIFN-γ (10 U/ml) or rovIFN-γ (100 U/ml).

(ii) Sheep MNC. Sheep cells were isolated and cultured accordingly, except that culture medium contained 20% FCS and 20% homologous serum and no neomycin. For subculture, the same medium and the same stimuli as described for bovine cells were used.

(iii) Goat MNC. Goat cells were isolated as described above and were cultured in Teflon bags in RPMI 1640 with the additives described for cattle cells, but with 10% goat serum and 10% FCS and no neomycin. For subculture, the same medium and the same stimuli as described for cattle Mϕ were used.

Immunocytochemistry of tissue specimens. Deparaffinized rehydrated sections were treated with H_2O_2 (1%) and azide (50 mM) for 30 min in order to inactivate endogenous peroxidase and then were overlaid with human immunoglobulin G (IgG; 10 mg/ml) for blocking Fc receptors. Antibodies were overlaid in the presence of saponin (0.05%). The chosen dilutions were 100-fold (anti-iNOS, anti-S100A8, and anti-S100A9) or 25-fold (anti-MHC-II tissue culture supernatant). Specimens were developed by using the ABC staining system (Immuno-Diffusion, Geneva, Switzerland) in the case of iNOS or anti-mouse IgG-peroxidase (Jackson Immunoresearch Laboratories, West Grove, Pa.) in the case of MHC-II, S100A8, and S100A9. The substrates were diaminobenzidine (DAB; Sigma) for iNOS and Vector-SG (Immunodiffusion) for MHC-II and S100A8/A9. Double staining was performed in order to study colocalization of the indicated markers. Specimens which were stained for *L. monocytogenes* were first processed for iNOS staining, followed by treatment with trypsin (2 mg/ml; Difco) in 0.02% $CaCl_2$–50 mM Tris (pH 7.0) for 15 min under gentle agitation at 37°C. Sections were washed twice and exposed to anti-*L. monocytogenes* (200-fold dilution), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson). Fast red-TR (Chroma Gesellschaft, Koengen, Germany) was used as a substrate, and hematoxylin served as a counterstain. In other double-labeling experiments, cells were first stained for iNOS, followed by peroxidase inactivation (see above) and staining for anti-MHC-II. Accordingly, staining for peroxidase, using DAB as a substrate, was combined with staining for phosphatase, using fast red as a substrate. Two procedures were used to rule out that a stronger color completely covered the lighter color of a doubly labeled cell. (i) Three consecutive serial sections were stained, the middle section being double stained and the first and third ones being single stained. (ii) The substrate color combination was inverted. Double labeling was particularly conspicuous for the combination of DAB and Vector-SG. All reagents except primary antibodies were diluted in 20 mM Tris–0.25 M NaCl–0.1% Tween 20 (pH 7.5).

Immunocytochemistry of cell cultures. Cells cultured for 18 h on glass coverslips were freed of medium and fixed in paraformaldehyde (2% [wt/vol] in PBS) for 20 min. Cells were washed twice, followed by Fc receptor blockade with human IgG (10 mg/ml) for 20 min, and then antibodies were overlayered as described above except that anti-iNOS, anti-S100A8, and anti-S100A9 were diluted 500-fold and anti-MHC-II was diluted 50-fold. Some sections were stained for iNOS as described above, followed by staining for MHC-II.

Cell ELISA. Twenty-four and 48 h after stimulation, supernatants of 96-well plates were collected for nitrite and nitrate determination (see below), and monolayer cells were fixed with paraformaldehyde (2% in PBS for 20 min) and blocked with human IgG (10 mg/ml). Monolayers were stained for iNOS, using polyclonal anti-iNOS (UBI). The antibody was diluted 500-fold in PBS containing 0.05% saponin. Then goat anti-rabbit IgG was added (100-fold, 1 h), followed by avidine-biotin-alkaline phosphatase complex (100-fold). Thereafter, the soluble substrate *p*-nitrophenyl phosphate was added, and plates were read at predetermined intervals at 405 nm. Parallel cultures were stained with a solution consisting of crystal violet (0.75%)–NaCl (0.25%)–formaldehyde (1.75%) in ethanol (50%, vol/vol), and optical densities (OD) were normalized by expressing OD for iNOS staining in percentage of the OD of crystal violet.

Determination of nitrite and nitrate. Twenty-four and/or 48 h after stimulation, supernatants were collected from 96-well plates, and nitrite was determined by the Griess reaction (2). Nitrate was determined in parallel by incubating supernatants with *Pseudomonas oleovorans* for 90 min at 37°C (final concentration, 0.25% [vol/vol] of packed bacteria), followed by nitrite determination by the Griess reaction. *P. oleovorans* contains a high content of nitrate reductase but no nitrite reductase activity (16) and converts all nitrate of supernatants into nitrite within the indicated time. This was confirmed in each experiment by including both a nitrate and a nitrite standard curve.

Flow cytometry. Mϕ generated in vitro were subjected to flow cytometry, using a panel of bovine surface marker-specific antibodies and a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) as described previously (2, 38).

Procoagulant activity. Procoagulant activity of cultured Mϕ was determined by a kinetic turbidimetric recalcification time assay as described previously (3). Citrated human platelet-depleted plasma was used for all species. This parameter served as an iNOS-independent marker of Mϕ activation.

Monitoring for pyrogen activity. All reagents to which mononuclear phagocytes were exposed during isolation or in cell culture were tested for the absence

of pyrogen activity, using a sensitive turbidimetric kinetic *Limulus* amoebocyte lysate assay (2).

RESULTS

iNOS expression in the brains of ruminants with listeriosis. Formalin-fixed brain pieces from animals that succumbed to listeriosis were sectioned (4 μm) and processed for immunocytochemistry, using either anti-iNOS or anti-*Listeria* antibodies or both combined (Fig. 1). The examined brains showed the expected histopathological picture except that *L. monocytogenes* was seen much more readily than in a previous sheep study (25). Brain lesions were characterized by (i) microabscesses of various sizes with a high Mϕ content and (ii) a large number of perivascular cuffs. Areas of malacia, characterized by swollen axons and edema formation, glial nodules, and diffuse gliosis were also regularly seen. Staining of sheep and goat brains for *L. monocytogenes* was positive in all animals except for one goat (Table 1). Bacteria were found in large numbers within microabscesses but also in areas where no conspicuous foci of inflammation were seen (Fig. 1). All cattle brains examined except two contained far fewer bacteria, or these were even undetectable. Rarely, single bacteria within neurons were seen in all species, but more often, bacteria adjacent to neurons were observed (Fig. 1a). Foci of malacia contained few bacteria, and perivascular cuffs were free of *L. monocytogenes*, a finding consistent with the assumed neurogenic infection route (34). The number of bacteria was higher in microabscesses in goat and sheep brains than in those of cattle, and no bacteria could be detected in three of eight cattle brains. iNOS expression was observed in all cattle, sheep, and goat brains, with the exception of one goat brain that did not stain for *L. monocytogenes* either and one sheep brain (Table 1). This was observed in the large majority of the microabscesses (Fig. 1) and in some glial nodules. The latter contained densely packed cells, some of which stained for iNOS. Plane sections through microabscesses showed several patterns of staining. In the first, the density of iNOS-expressing cells was highest in the center of microabscesses of round or oval shape, and these lesions were clearly confined (Fig. 1e). In a second pattern, iNOS-positive cells formed a ring around a zone characterized by the presence of densely packed iNOS-negative Mϕ, neutrophils, and bacteria (Fig. 1h). In a third pattern, iNOS-expressing cells were loosely distributed over a larger area, and these microabscesses were large and showed several extensions (Fig. 1g). All of these lesions contained bacteria, although in variable numbers. iNOS was not expressed in cells of perivascular cuffs and within the lumina of blood vessels. Occasionally, single brain Mϕ outside foci of inflammation stained positive. All cells staining for iNOS were large and of Mϕ appearance. Neutrophils were negative for iNOS. In some animals, a low proportion of nerve cells stained strongly for iNOS. No sign of cellular infiltration was seen in the neighborhood of these cells. The subcellular distribution of this staining was less homogeneous than in Mϕ. The proportion of iNOS-expressing Mϕ was lower in lesions of sheep and goats than in those of cattle. The staining intensity was variable but was generally stronger in cattle Mϕ than in Mϕ from small ruminants.

Since only a proportion of cells within microabscesses was positive for iNOS, it was of interest to look for expression of other markers typically displayed by Mϕ, e.g., MHC-II. In goats and sheep, MHC-II was strongly expressed by a large proportion of cells within perivascular cuffs, by few cells within blood vessels, and by individual cells scattered over the whole brain. In contrast, microabscesses expressed low numbers of MHC-II-positive cells, and these were more weakly stained than those in perivascular cuffs (Fig. 2). Double staining suggested that in goats and sheep, the cells within microabscesses which stained strongly for MHC-II stained weakly, if at all, for iNOS and vice versa (Fig. 2b and c). In cattle, two types of microabscesses were seen. In one, iNOS-expressing cells were MHC-II negative (not shown). In the other, numerous strongly MHC-II-expressing cells were observed, and some of these also expressed iNOS (Fig. 2a).

That iNOS-positive cells represented a subset of bovine Mϕ within lesions was supported by staining for S100A8 and S100A9, two members of the S100 family of calcium-binding proteins (39). These antigens were reported to be expressed by peripheral blood neutrophils, monocytes, and subsets of tissue Mϕ, particularly those in inflammatory lesions (42), and by activated microglia (4). S100A8 and S100A9 were strongly expressed in the brains of cattle. Numerous Mϕ within microabscesses stained for either S100A8 or S100A9 and occasionally for both (Fig. 2g). Perivascular cuffs also contained cells that expressed either S100A8 or S100A9, and stained activated microglia were scattered over a large area of the brain (Fig. 2i). Double staining suggested that some Mϕ expressed exclusively either S100A8 or S100A9, whereas others expressed both markers simultaneously. When the sections were stained with one color for both S100A8 and S100A9 and with another color for iNOS, it became obvious that Mϕ expressed either high levels of iNOS or high levels of these calcium-binding proteins but rarely both (Fig. 2e and i). Thus, iNOS expression in the brains of ruminants with listeriosis is restricted to a subset of Mϕ within microabscesses.

iNOS expression by activated ruminant Mϕ in vitro. The expression of iNOS in the brains of goats with listeriosis was unexpected, since a previous study showed that goat Mϕ were incapable of producing significant amounts of NO in vitro (1). Monocyte-derived Mϕ from cattle, goat, and sheep were therefore activated by heat-killed *L. monocytogenes*, by IFN-γ, or by both combined or were mock stimulated. After 18 h, they were stained for expression of iNOS, using the polyclonal anti-iNOS antibody (UBI). In a majority of cultures, cattle Mϕ exposed to *L. monocytogenes* and IFN-γ combined were uniformly and strongly stained by anti-iNOS (Fig. 3d). In a minority of cultures, between 70 and 95% of Mϕ were positive (Fig. 4c). Exposure of cattle cells to *L. monocytogenes* alone yielded a subset of strongly stained cells only (between 20 and 50% [Fig. 3c]), and Mϕ exposed to IFN-γ alone or mock stimulated were not stained (Fig. 3b) except in 2 of 18 animals. In contrast, only a minor subset of sheep and goat Mϕ was stained for iNOS, regardless of the combination of stimuli used (Fig. 3e to h and data not shown). The percentage of stained cells varied between 5 and 30. This pattern of reactivity was seen in 16 goats and 5 sheep. Thus, compared with cattle Mϕ, sheep and goat Mϕ encompassed a considerably smaller proportion of cells responding to iNOS induction in vitro.

The restricted expression of iNOS by small ruminant Mϕ, as determined by immunocytochemistry, was confirmed by cell ELISA. Mϕ of all three species expressed measurable iNOS, and the strongest iNOS signal was obtained after stimulation with heat-killed *L. monocytogenes* and IFN-γ combined. However, after correction for the distinct cell densities at the end of the multistep staining procedure, activated Mϕ from small ruminants showed an increase in iNOS staining upon activation that was an order of magnitude smaller than that observed for identically treated bovine Mϕ (data not shown).

To address the heterogeneity of staining, the cell marker-specific antibodies used ex vivo were also tested on Mϕ cultured in vitro. An overwhelming majority of cells expressed

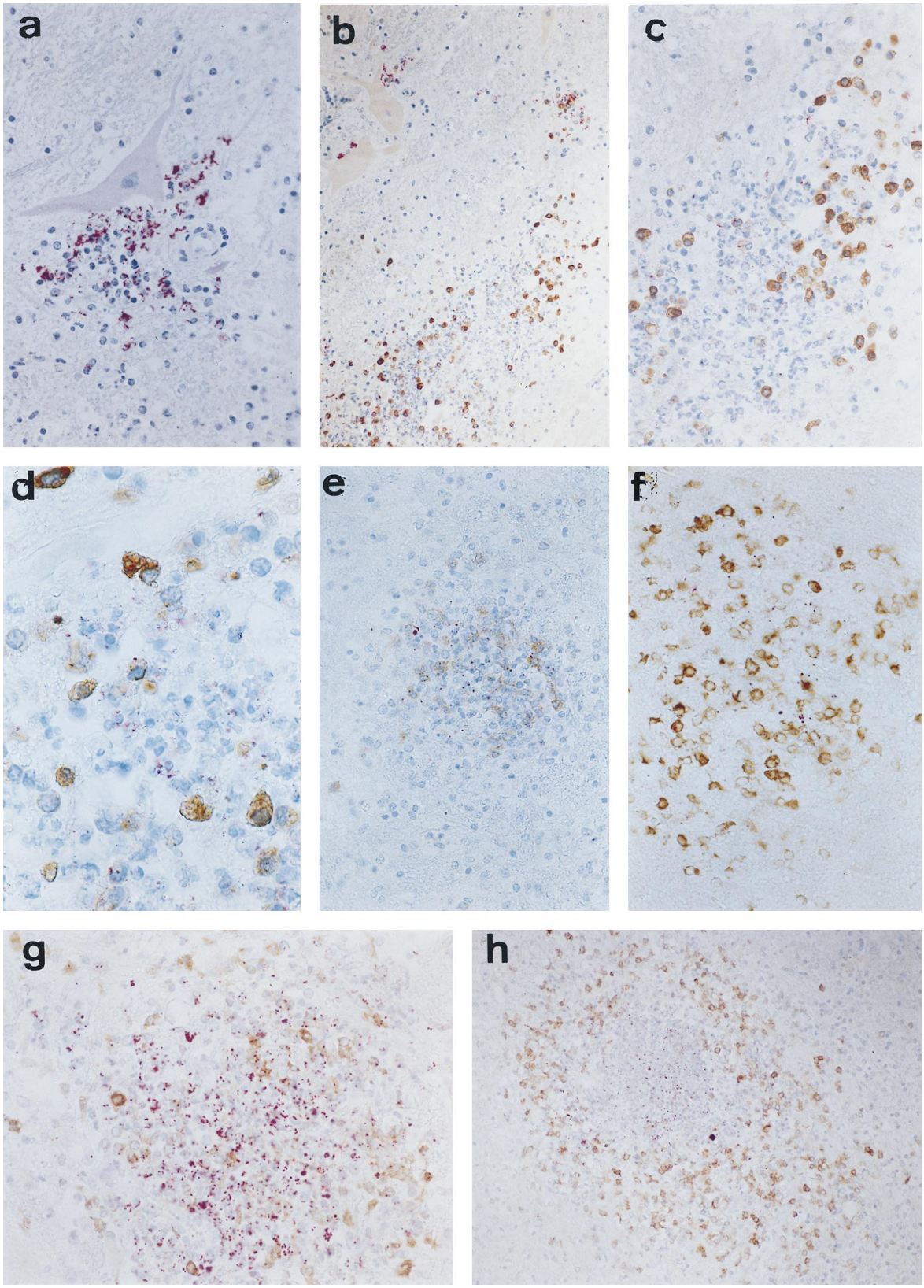

FIG. 1. Detection of *L. monocytogenes* (red) and of iNOS in brains of cattle (a to c), sheep (f), and goat (d, e, g, and h). All specimens were double stained except that in panel a, which was stained for bacteria only. They were lightly counterstained with hematoxylin. Magnifications: ×25 (b), ×40 (a, c, and e to h), and ×100 (d). Expression of iNOS in microabscesses of all three species is shown.

TABLE 1. Expression of iNOS in the brains of ruminants with diagnosis of listeriosis

Species (no. of animals investigated)[a]	No. of animals with:			
	L. monocytogenes in brain	Expression of iNOS in brain	Absence of iNOS despite presence of *L. monocytogenes*	Absence of *L. monocytogenes* despite presence of iNOS
Cattle (8)	5[b]	8	0	3
Goats (10)	9	9	0	0
Sheep (7)	7	6	1	0

[a] For each animal, at least two sldies were stained and inspected.
[b] In three animals, few *L. monocytogenes* organisms were detected.

MHC-II. However, the level of expression varied over a broad range, as determined by immunocytochemistry and by flow cytometry (data not shown). In activated Mϕ from goat and sheep subjected to double staining, strong staining for either iNOS or MHC-II was predominant, suggesting that the subset expressing iNOS at a high level displayed a low density of MHC-II and vice versa (Fig. 4a and b). A similar behavior was seen in the minority of cattle Mϕ populations in which an iNOS-negative subset was preserved, despite maximal activation (Fig. 4c).

The heterogeneity in terms of S100A8 and S100A9 expression in cattle brains with listeric encephalitis prompted us to test for expression of these molecules by Mϕ in vitro. Under the culture conditions described, S100A9 was constitutively expressed by Mϕ, and Mϕ exposure to bacteria, LPS, or rboIFN-γ did not modulate its expression (not shown). In contrast, both nonadherent and adherent cultured Mϕ expressed little S100A8, but when stimulated with bacteria (*L. monocytogenes* or *S. dublin*), LPS, or IFN-γ, a proportion of Mϕ expressed S100A8 (not shown).

NO generation by activated ruminant Mϕ in vitro. Monocyte-derived Mϕ from cattle, goats, and sheep were exposed to heat-killed *L. monocytogenes*, heat-killed *S. dublin*, or LPS in the presence or absence of IFN-γ, and the generation of nitrite and nitrate was determined after 24 and 48 h. Mϕ from cattle showed a strong nitrite response 24 h after stimulation, the strongest stimulus being *L. monocytogenes* and IFN-γ combined (Fig. 5). Optimal cytokine concentrations were 10 U/ml for rboIFN-γ and 100 U/ml for rovIFN-γ; optimal concentrations of bacteria were 200 μg/ml for heat-killed *L. monocytogenes* and 2 μg/ml for *S. dublin*. Nitrite generation was proportional to the cell number per well, at least in the range between 16×10^3 and 80×10^3 Mϕ/well (data not shown). *S. dublin* with or without IFN-γ, LPS with or without IFN-γ, and *L. monocytogenes* without IFN-γ displayed a weaker stimulation, whereas IFN-γ alone did not lead to detectable nitrite accumulation (Fig. 5). Nitrite was the major end product of NO, since the content of nitrite plus nitrate was not significantly larger than that of nitrite alone (Fig. 5). More nitrite accumulated in a 48-h culture than within 24 h, but the patterns of responsiveness were identical at the two time points (data not shown).

Goat Mϕ stimulated in the same manner showed a nitrite or nitrate production within 24 h (Fig. 5) or 48 h (not shown) which was at least 1 order of magnitude lower than that produced by cattle Mϕ. In the majority of animals, it did not reach significant levels (2 μM), thus confirming earlier studies of this laboratory (1). Similarly, cultures of stimulated sheep Mϕ contained little, if any, measurable nitrite or nitrate (Fig. 5). Similar findings were made with other stimuli, e.g., LPS, rboIL-1β, rboTNF-α, or a combination of these agents. However, that Mϕ from these species were also strongly activated was shown by their markedly enhanced procoagulant activity (reference 1 and data not shown).

DISCUSSION

Antimicrobial and antitumoral activity of activated murine Mϕ rests on the expression of iNOS and on the production of NO, as evidenced by experiments with enzyme inhibitors (28) and with genetically iNOS-deficient mice (27, 48). The role of iNOS in these activities is less clear in species in which Mϕ fail to express iNOS by conventional modes of activation, as has been shown for human (41), rabbit (41), pig (45), and goat (1) Mϕ. The latter species is of interest since Mϕ from the closely related cattle display a strong iNOS expression and NO response upon activation (2, 23). The low iNOS expression by activated goat macrophages in vitro (reference 1 and this study) and the lack of evidence for a defect in tetrahydrobiopterin (1) are in favor of species variation at the level of iNOS induction rather than cofactor availability. This species difference prompted us to compare iNOS induction in Mϕ from three ruminant species, goat, sheep, and cattle, in vivo in a bacterial infection and to compare the results with those from Mϕ cell cultures. Listeriosis was an ideal infection to be studied for two reasons. (i) In ruminants, *L. monocytogenes* is a natural infectious agent leading frequently to lethal encephalitis; listeriosis accounted for 20% of the cases referred to the Institute of Neurology, University of Berne, between 1968 and 1978, with about equal representation of the three species. (ii) In vitro, heat-killed *L. monocytogenes* combined with IFN-γ belongs to the strongest inducers of iNOS in bovine Mϕ. The following key findings are noteworthy. (i) In all ruminant species investigated, iNOS is expressed in listeric encephalitis, particularly at sites of bacterial infection. (ii) iNOS expression in vivo is restricted to Mϕ of microabscesses and glial nodules; other foci of inflammation, such as perivascular cuffs and areas of malacia, do not contain iNOS-expressing cells. (iii) iNOS expression is restricted to a Mϕ population low in MHC-II expression (sheep and goat) and low in expression of S100A8 and S100A9 (cattle). (iv) The relatively strong expression of iNOS in vivo, in lesions of goat and sheep, does not reflect the low degree of iNOS induction by relevant stimuli in Mϕ cell cultures from these species. This points to differences in iNOS expression by Mϕ in vitro and in vivo.

Our finding on iNOS expression in ruminant listeriosis is the first demonstration of iNOS expression in bacterial encephalitis in ruminants. From a survey of the emerging literature on expression of iNOS in the brain, it appears that iNOS is more widely expressed in rodents than in ruminants with listeric encephalitis. In the traumatized rat brain, iNOS is expressed mainly by neutrophils and vascular smooth muscle cells (11). Reactive astrocytes express iNOS and presumably produce NO in rats subjected to ischemia (9, 13). The iNOS-positive cells in the brains of listeric animals were clearly Mϕ by morphological criteria. Ramified (resting) microglia, putative precursors of at least some of the Mϕ in the inflamed brain (46), were negative for iNOS, but some of the cells in glial nodules with macrophage appearance were iNOS positive. Neutrophils were negative for iNOS expression. Neutrophils were described to participate in the early elimination of *L. monocytogenes* from the liver in a mouse infection model (12), and rat neutrophils were reported to express NOS in vivo and in vitro (30, 50). Although we cannot exclude the possibility that low iNOS expression went undetected in the paraffin-fixed tissue specimens examined, this would not invalidate our finding that the iNOS expression by Mϕ of microabscesses and glial nodules of all three

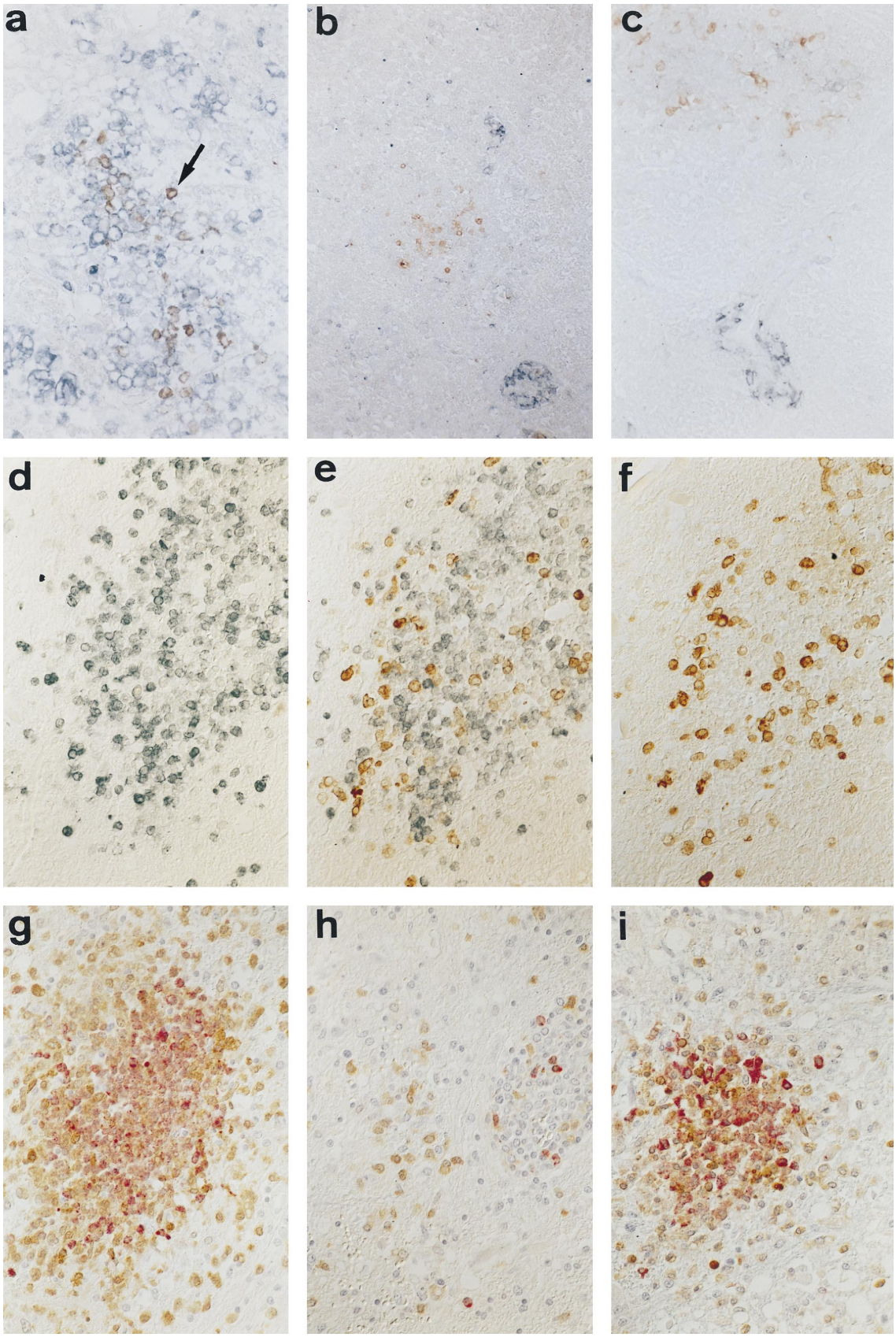

FIG. 2. (a to c) Expression of iNOS (brown) and MHC-II (blue) in brain sections of cattle (a), sheep (b), and goat (c) with listeriosis. (a and b) MHC-II expression restricted to perivascular cuffs and few individual cells and iNOS expression restricted to microabscesses are shown. The arrow points to a double-stained cell to give a color reference. (d to f) Expression of iNOS (brown) and S100A8/A9 in a cerebral microabscess of *Listeria*-infected cattle. Consecutive serial sections are shown, the middle section (e) being double stained. (g and h) Expression of S100A8 (red) and S100A9 (brown) in listeric encephalitis of cattle. A microabscess (g) and a perivascular cuff (h) are shown. (i) Expression of S100A8/A9 (brown) and iNOS (red) by cells of a glial nodule in *L. monocytogenes*-infected cattle.

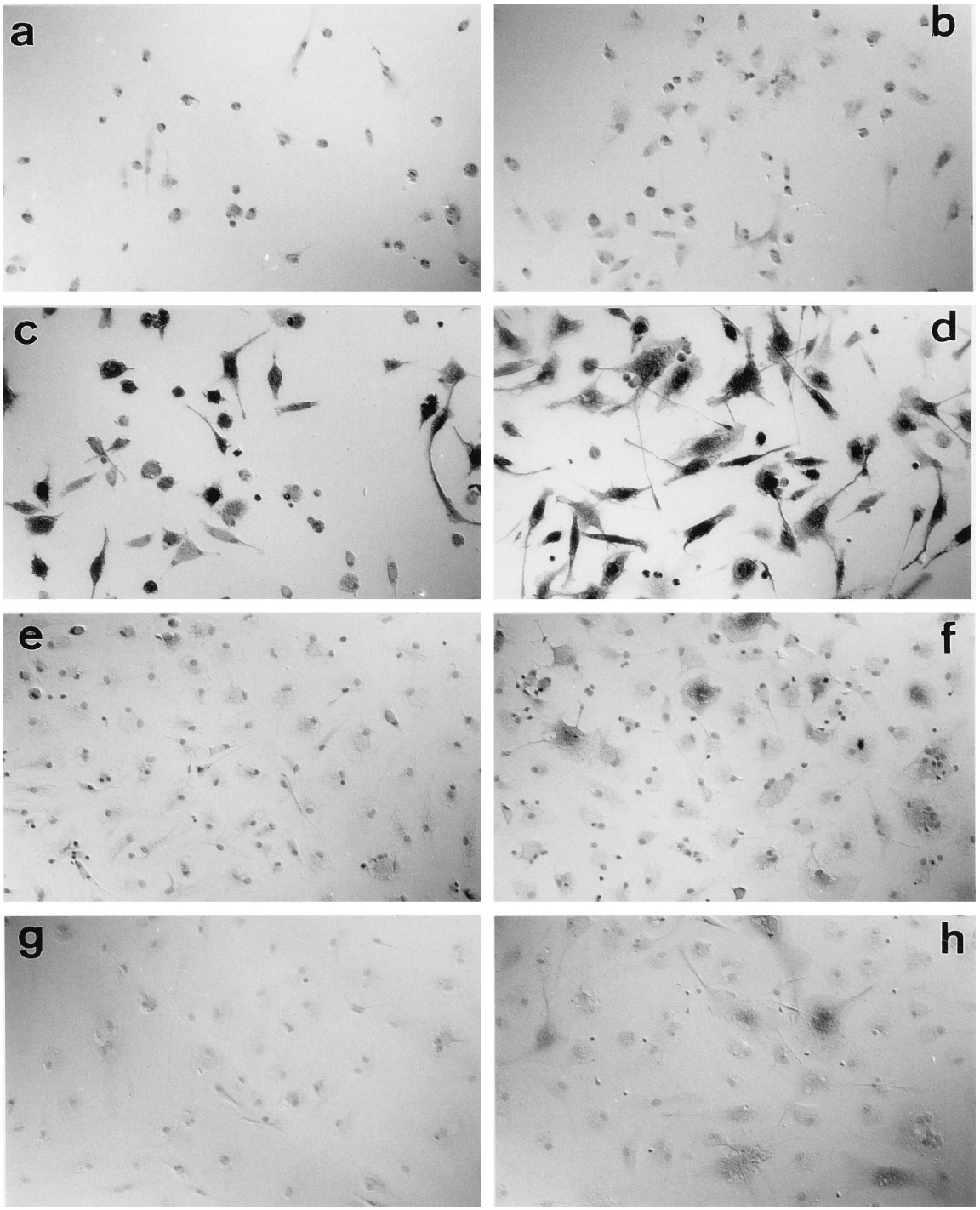

FIG. 3. Expression of iNOS by ruminant Mϕ activated in vitro. Monocyte-derived Mϕ from cattle (a to d), goat (e and f), and sheep (g and h) were either mock stimulated (a, e, and g) or stimulated with IFN-γ (b), with *L. monocytogenes* (c), or with IFN-γ and *L. monocytogenes* combined (d, f, and h). After 18 h, cells were fixed, permeabilized, and stained for iNOS by using the ABC system and DAB as a substrate, followed by counterstaining with hematoxylin.

species is much stronger than that by any other cells of the brains examined. In all species, some neurons also stained with an anti-iNOS serum that does not cross-react with constitutive types of NOS. The significance of this finding which is reminiscent of an in vitro study in the rat (31) remains to be determined.

In listeric encephalitis of ruminants, there was generally a colocalization of the infective agent and iNOS expression. This finding is consistent with our observation that in cattle Mϕ, *L. monocytogenes* and IFN-γ combined provide one of the strongest iNOS-inducing regimens. Notably, both iNOS and bacteria were not detected in perivascular cuffs. This finding is consistent with the notion that a *Listeria* infection of the ruminant brain has a neurogenous origin (34), and it parallels

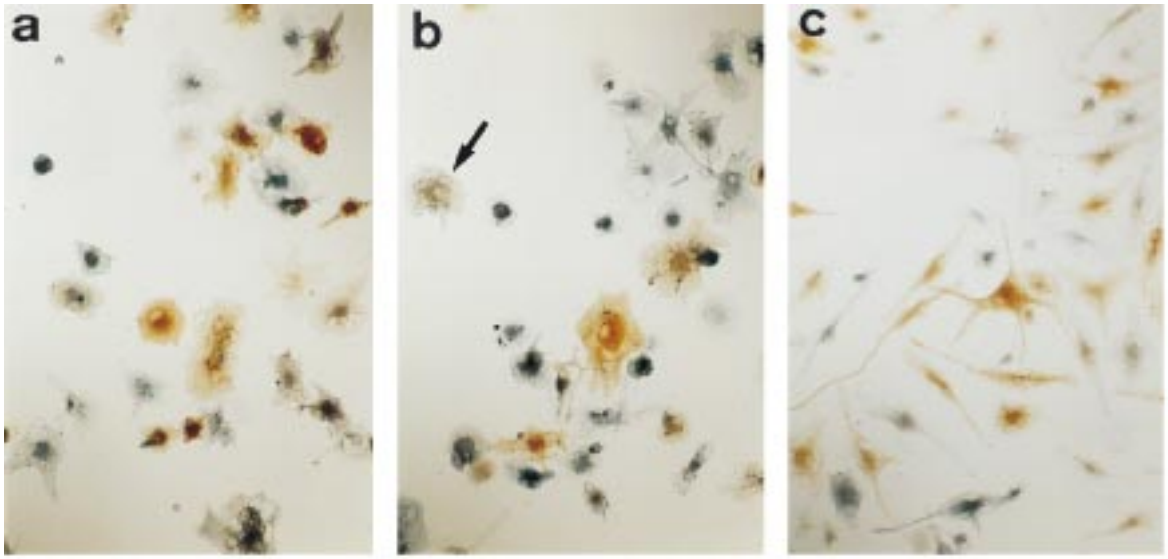

FIG. 4. Expression of iNOS (brown) and MHC-II (blue) by activated Mϕ from sheep (a), goat (b), or cattle (c). Mϕ stimulated with *L. monocytogenes* and IFN-γ for 18 h were stained for iNOS and MHC-II. The arrow indicates a double-labeled cell to give a color reference. (a and b) Specimens showing a relatively low proportion of iNOS-positive cells; (c) a cattle culture showing an unusually low proportion of iNOS-expressing cells.

our in vitro observation that cattle Mϕ show little iNOS expression when stimulated by cytokines alone, whereas a broad panel of gram-positive bacteria alone do induce iNOS (2, 3, 21a). Bacteria observed at sites without accumulation of inflammatory cells might represent an early stage of neurogenous bacterial colonization (34), preceding an inflammatory host response. Inflammatory foci with undetectable bacteria (one goat and three cattle) may represent late stages of host defense in which bacteria were already cleared by Mϕ.

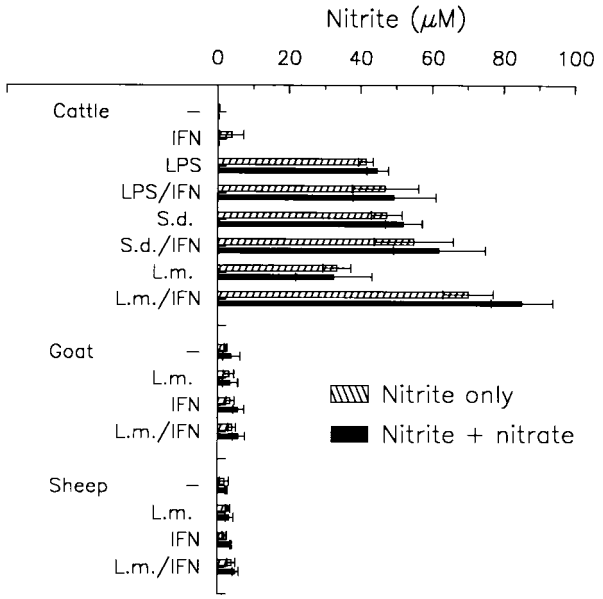

FIG. 5. Generation of nitrite and nitrate by Mϕ of cattle, sheep, and goats. Monocyte-derived Mϕ were subcultured in microtiter plates (60×10^3 to 80×10^3 Mϕ/well) and stimulated as indicated. Nitrite and nitrite plus nitrate combined were determined 24 h after stimulation. Stimuli were heat-killed *L. monocytogenes* (L.m.; 200 μg/ml), heat-killed *S. dublin* (S.d.; 200 μg/ml), LPS (1 μg/ml), and IFN (rboIFN-γ, 10 U/ml). Means ± standard errors of the means of four to eight experiments, each performed in triplicate, are shown.

In contrast to colocalization of iNOS and listerial antigen expression, the expression of iNOS and of MHC-II overlapped to a small extent only. This may be surprising at first glance, since MHC-II is up-regulated by IFN-γ in many species, including ruminants (2, 6, 25a, 43). In vitro studies with bovine Mϕ suggest that IFN-γ is the most important, if not the only, cytokine up-regulating iNOS expression (22). However, in contrast to mouse Mϕ, IFN-γ alone does not induce iNOS in bovine Mϕ in the absence of bacterial antigen, and *Listeria* cells could not be detected in perivascular cuffs. More difficult to explain is the relatively low expression of MHC-II in microabscesses of goats and sheep, in which *L. monocytogenes* is overtly present. Likewise, small ruminant Mϕ exposed in vitro to *L. monocytogenes* and IFN-γ leads to a bimodal expression of either iNOS or MHC-II. In brains of sheep with listeriosis, CD4 cells and CD8 cells, both putative sources of IFN-γ, were detected in perivascular cuffs and in lower number in the periphery of microabscesses (25), but no information on the level of IFN-γ expression at these sites is available. Further in vitro and in vivo work is required to delineate the additional requirements leading to the acquisition of an $iNOS^{high}$ $MHC\text{-}II^{low}$ or an $iNOS^{low}$ $MHC\text{-}II^{high}$ phenotype.

Cattle Mϕ in microabscesses were heterogeneous with regard to iNOS expression and staining for S100A8 and S100A9. These Ca-binding proteins with largely unknown functions (for reviews, see references 39 and 42) are expressed by phagocytes in the periphery and in tissues, particularly by Mϕ within foci of inflammation, and by activated microglia (4). For the purpose of this study, S100A8 and S100A9 characterized two distinct Mϕ subsets which partially overlap. Positive cells for either marker are found in microabscesses (majority of cells stained), in perivascular cuffs, and scattered as single cells over a large area of the brain. Intriguingly, a sizable proportion of cells within microabscesses expressing S100A8 and/or S100A9 do not express iNOS and vice versa, suggesting that these traits are independently regulated. This was confirmed by in vitro experiments showing that S100A9 is constitutively expressed by cultured Mϕ, whereas S100A8 is not expressed but is induced in a portion of the Mϕ by bacteria or IFN-γ. Undoubtedly,

more work is required to understand function and regulation of these calcium-binding proteins in inflammatory cells.

Expressed iNOS is enzymatically active, and in vitro findings indicate a proportionality between iNOS expression by ruminant Mϕ and NO generation. Many reports point to an involvement of NO generated in the central nervous system and pathological sequelae (for reviews, see references 9, 32, and 40). The role of cerebral NO generation in ruminant listeriosis remains to be studied. Another question is whether iNOS-mediated NO generation is essential for the elimination of *L. monocytogenes* in the brain. The killing of *L. monocytogenes* in vitro was shown to depend on NO in some studies and to be relatively NO independent in others (see references in reference 15). In still another report, murine but not human Mϕ killed *Listeria* cells in an arginine-dependent manner (7). A contribution of neutrophils to early elimination of *L. monocytogenes* from the infected mouse liver (12) points to additional mechanisms of bacterial elimination, e.g., by reactive oxygen species (21). In this study, iNOS was more strongly expressed in bovine than in ovine and caprine microabscesses. Conversely, there was overt expression *L. monocytogenes* in the latter lesions, whereas bacteria were often hard to find in bovine microabscesses. This finding is consistent with, but does not prove, a role of NO in the elimination of the pathogen. The availability of Mϕ from closely related species that are either high or low iNOS responders may now facilitate an assessment of the relative importance of arginine/NO-dependent and -independent antibacterial pathways in listeriosis.

These studies are possibly of relevance for the intensely debated question of iNOS expression by human Mϕ in two ways. (i) The ex vivo differences in iNOS expression by Mϕ of various species are less pronounced than differences in iNOS expression by Mϕ in culture. (ii) iNOS is expressed in vivo and in vitro by a restricted subset of Mϕ only. The species difference observed in vitro may therefore be due to differences in the proportion of Mϕ which are competent for iNOS induction by conventional stimuli. Mϕ from putative low-responder species may be incompetent because the in vitro system adopted did not favor the acquisition of a competent state in vitro by a large proportion of cells. The critical requirements for promoting competence remain to be determined. The availability of closely related species behaving as either high responders or low responders and the present proof that Mϕ of low-responder species may be induced to express iNOS by a defined bacterial infection in vivo may help to identify the hypothesized factor(s).

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