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Expression of NRAMP1 and iNOS in *Mycobacterium avium* subsp. *paratuberculosis* naturally infected cattle

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Abstract

Paratuberculosis (PTB) is a chronic disease caused by *M. avium* subsp. *paratuberculosis* (MAP) that affects several animal species, and some studies have suggested that there may be a relationship between Crohn's disease and PTB. Significant aspects of PTB pathogenesis are not yet completely understood, such as the role of macrophages. Natural resistance-associated macrophage protein 1 (NRAMP1) and the inducible nitric oxide synthase (iNOS) molecules have shown nonspecific effects against several intracellular pathogens residing within macrophages. However, these molecules have been scarcely studied during natural infection with MAP. In this work, changes in NRAMP1 and iNOS expression were surveyed by immunohistochemistry in tissue samples from MAP-infected cattle and healthy controls. Our findings show strong specific immunolabeling against both NRAMP1 and iNOS molecules, throughout granulomatous PTB-compatible lesions in ileum and ileocaecal

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lymph nodes from paratuberculous cattle compared with uninfected controls, suggesting a relationship between the expression of these molecules and the pathogenesis of PTB disease.

Keywords: Johne's disease; Cattle; Paratuberculosis; MAP; NRAMP1; Slc11a1 and iNOS

Résumé

La Paratuberculosis (PTB) est une maladie chronique causée par *M. avium subsp. paratuberculosis* (MAP) laquelle peut affecter plusieurs espèces animales, certaines études suggèrent une relation avec la maladie de Crohn. La pathogénie PTB n'est pas totalement comprise, certains aspects restant inconnus comme par exemple le rôle joué par les macrophages. La protéine 1 du macrophage associée à la résistance naturelle (NRAMP1) et l'oxyde nitrique synthase inducible (iNOS) ont démontré avoir des effets non spécifiques contre plusieurs pathogènes intracellulaires résidant dans les macrophages. Cependant peu d'études ont été réalisées pendant l'infection naturelle du MAP. Au cours de ce travail, en utilisant l'immunohistochimie, des changements dans l'expression de NRAMP1 et de l'iNOS dans des échantillons tissulaires de MAP de bétails infectés et de contrôles sains ont été mis en évidence. Ce travail démontre la présence d'un immunomarquage spécifique contre les deux molécules (NRAMP1 et iNOS), dans toutes les lésions granulomateuses compatibles avec la PTB, dans les ganglions lymphatiques de l'iléon et de l'ileocaecal du bétail ayant la paratuberculose en comparaison avec les contrôles non infectés, suggérant une relation entre l'expression de ces molécules et la pathogénie de la PTB.

Mots clés : La maladie de Johne ; Bétail ; Paratuberculosis ; CARTE ; NRAMP1 ; Slc11a1 et iNOS

1. Introduction

Paratuberculosis (PTB), also known as Johne's disease, is a chronic ailment caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in various animal species and is characterized by clinical signs such as diarrhea, weight loss, decreased milk production, and finally, death. PTB may affect cattle, sheep, goats, deer and muflons, as well as non-ruminant species, including rabbits, pigs and equines [6,7,9,12,21]. MAP infection was recently identified in one patient with HIV [23], and it has been implicated as a possible cause of Crohn's disease, a human intestinal disease of unknown etiology [13,20]. In cattle, advanced PTB produces granulomatous enteritis, characterized by epithelioid cells, giant cells and areas of necrosis; however, many aspects of PTB pathogenesis have not been elucidated. Animals become infected during the first months of life by consumption of contaminated milk or feed; but, in many cases, the disease remains latent without clinical manifestation [4]. The mechanisms responsible for this heterogeneous response are not well understood. It has been suggested that immunological and nutritional conditions, as well as ingested bacterial load may be important for PTB development. The microorganism penetrates the intestinal mucosa through the M cells, is engulfed by resident macrophage, proliferates slowly, and induces the development of early cell-mediated immunity (CMI) characterized by epithelioid and giant cells [19,24]. After the intestinal infection, bacilli may spread to the regional lymph nodes forming new granulomatous foci. Following the

development of CMI, the humoral immune response emerges, frequently associated with the appearance of clinical disease [8,16]. The period of incubation is usually long, from months to years, and older animals are less susceptible to infection than the young [4].

Macrophages possess effector mechanisms against intracellular MAP infection which have not been completely elucidated. After MAP infection, the expression of some molecules that might hinder the proliferation of intracellular bacteria may be modified, as occurs in other infections by microorganisms such as *Listeria*, *Salmonella*, *Leishmania* or *Mycobacterium bovis* [10,17,29]. Solute carrier family 11a member 1 (Slc11a1), formerly natural resistance-associated macrophage protein 1 (NRAMP1), displays pleiotropic antimicrobial effects, including up-regulation of iNOS expression. In mice, NRAMP1 may transport Fe^{2+} from the cytosol to phagolysosomes to generate hydroxyl radicals with bactericidal activity and deprive bacteria of Fe^{2+}, limiting growth [3]. In mice with mutated NRAMP1, iNOS production is drastically diminished [2]. In phagolysosomes, iNOS (inducible nitric oxide synthase) catalyzes production of nitric oxide (NO), a potent molecule able to kill *M. tuberculosis* during early infection [14].

To date, few studies exist regarding macrophage activation and the expression of molecules that intervene in the control of MAP infection in cattle. In tuberculous granulomas from *M. bovis* naturally-infected cattle, high expression of NRAMP1 was found by Estrada-Chávez et al. [10], and Pereira-Suarez et al. [22], showed the coexpression of NRAMP1 and iNOS in the cytoplasm of many epithelioid macrophage and multinucleated giant cells in tuberculous granulomas from cattle lymph nodes and lungs. In addition, a striking accumulation of nitrotyrosine, an indicator of iNOS activity and local NO production, has been described [14]. Recently, Hostetter et al. [15] described minimal iNOS immunoreactivity in heavy bacterial burden and poorly delineated granulomas of intestinal specimens from field cases of PTB. To the best of our knowledge, no information has been published yet on the role of NRAMP1 in PTB disease. The precise relationship between NRAMP1 and iNOS remains unknown, but it has been suggested that NRAMP1 may upregulate iNOS expression [11]. The aim of the present work was to evaluate the differences in expression of NRAMP1 and iNOS in specimens from PTB naturally infected cattle and healthy controls using immunohistochemistry.

2. Materials and methods

2.1. Cases studied

Samples of ileum or ileocaecal lymph node tissues were obtained from eight adult cattle with clinical signs of PTB, including weight loss with chronic or intermittent diarrhea (Table 1). Also as negative controls, samples from two adult healthy cattle from PTB-free herds without clinical signs were included (Table 1). Histology findings and lesions associated with paratuberculosis infection were classified, as proposed by Pérez et al. (1996) for sheep and Corpa et al. [7]) for goats, according to the following parameters: presence of granulomatous lesions; location of granulomas in the different gut-associated lymphoid tissue compartments; intensity and distribution of lesions; cell types present in

Table 1
Results of culture, histopathology and immunohistochemistry in different tissues of *Mycobacterium avium* subsp. *paratuberculosis* infected cattle.

ID no.	Organ	Culture	Histopathology	Immunolabeling		
				MAP[a]	NRAMP	iNOS2
1	Ileum	MAP isolation	DM	+++	+++	+++
2	Ileum	N/D	DM	+++	+++	+++
3	Ileum	N/D	DM	+++	+	+++
4	Ileum	N/D	DM	+++	+	+
5	Ileocaecal LN	N/D	M	+++	+++	+++
6	Ileocaecal LN	MAP isolation	DM	+++	+++	+++
7	Ileocaecal LN	MAP isolation	DM	+++	+++	+++
8	Ileocaecal LN	MAP isolation	M	+++	+++	+++
9	Ileum[b]	N/D	NL	−	+/−	+/−
10	Ileocaecal LN[b]	N/D	NL	−	+/−	+/−

LN: Lymph node; DM: diffuse multibacillary; M: multifocal; NL: no lesion; N/D: no data; +/−: scanty; +: low; ++: medium; +++: high.

[a] All cases were confirmed using Ziehl–Neelsen (ZN) stain for acid-fast bacilli and immunohistochemistry with anti-MAP monoclonal antibody, as well as, by IS900-PCR (data no shown).

[b] Healthy controls.

the inflammatory infiltrate; and presence of mycobacteria and subjective assessment of their number in lesions.

2.2. Immunohistochemistry

For immunohistochemistry assays, primary anti-MAP polyclonal antibodies raised in rabbit were kindly provided by Queen's University, Belfast, Northern Ireland, and diluted 1:100 in 50 mM Tris–HCl, 300 mM NaCl, 0.1% Tween 20 buffer (TBST); anti-NRAMP1 (Santa Cruz Biotechnology Inc., reg. num. sc-20113) diluted 1:100 in TBST buffer; and anti-iNOS2 (BD Transduction Lab., Cat num. 610332), diluted 1:100 in TBS buffer were used. Immunolabeling was revealed with the LSAB2^{TM} kit, using AEC as chromogenic substrate (Dako, Japan). Briefly, sections were deparaffinized by successive immersion in 100% xylene, 100% ethanol, 96% ethanol and 70% ethanol for 10, 10, 5 and 5 min, respectively. Endogenous peroxidase activity was inactivated with 10% hydrogen peroxide in methanol. Antigens were exposed with 10 mM citrate buffer (pH 6) and autoclaving to 121 °C, 1 atm. for 15 min. After blockade with 50 μl of 1% bovine serum albumin (Sigma, USA) in TBST for 5 min at room temperature, sections were incubated overnight with 40 μl of primary antibodies (anti-MAP, anti-NRAMP1 or anti-iNOS) at 4 °C in a humid chamber. Sections were then washed with TBST, then incubated with one drop of biotinylated secondary antibody (DAKO No. K0675) for 20 min at room temperature. After washing, the sections were incubated 10 min with streptavidin-peroxidase conjugated (DAKO P039701) at room temperature. One drop of chromogenic amino-ethyl-carbazol substrate in TBST was applied (DAKO AEC, K3464, Japan) for 20 min at room temperature. Sections were counterstained with Mayer's hematoxylin and mounted on a hydrosoluble medium (Vectamount AQ). Double immunolabeling was performed on sections from three PTB cases and negative controls. Co-expression of MAP and

NRAMP1, MAP and iNOS, and NRAMP1 and iNOS was assessed in two sections from the same animal, using the double immunolabeling system EnVisionTM Doublestain Kit (Dako Corp).

2.3. Microscope analysis

Specimens were analyzed with an optical microscope (Leitz, Dialux), with the 5×, 10×, 20×, 40× and 100× objectives. In Ziehl–Nielsen stained sections, red colored acid–alcohol resistant bacilli and blue cells were observed. To record the results, 40× fields were photographed with a digital camera mounted on the microscope (Moticam 1000). Cells were counted when immunolabeling was clearly evident and the average of the total of photographed fields was classified as follows: negative (no immunolabeling), very low (<5 marked cells), low (5–10 marked cells), moderate (10–15 marked cells) and intense (>15 marked cells). When immunolabeling was clearly observed, with the 10× objective, even if only in one field, it was classified as intense. Characteristics such as shine and contrast of the images were optimized with software by Vendor (Moticam^{TM}).

3. Results

3.1. Histopathology

Cases 1, 2, 3 and 4 corresponded to animals with severe granulomatous enteritis that showed marked lesions consisting of many macrophages and giant cells spread throughout the mucosa, submucosa, muscle tunic and serosa (Table 1). Macrophages, with foamy cytoplasm and also epithelioid cells, formed a diffuse infiltrate in the intestinal wall, producing severe thickening of the mucosa, with glands widely separated due to the infiltration. Often, fused granulomas were seen mainly in the villi bodies. Lymphocytes and Langhans giant cells were commonly seen in the epithelioid infiltrate. In most of the sections, intestinal glands were dilated and filled with necrotic debris. The submucosa was severely affected; an infiltrate formed almost exclusively of macrophages with some giant cells was present with edema and thrombus formation. Multifocal granulomas with lymphoid follicles were located in the interfollicular zone. Mononuclear cells infiltrated the muscular layer. The serosa was also affected by the presence of multifocal granulomatous infiltrates. Lesions were found in the ileocaecal valve in all cases.

In cases 6 and 7, ileal lymph nodes showed a severe and diffuse granulomatous lymphadenitis, with macrophages and a moderate number of giant cells located in the cortex and paracortex, altering the normal lymph node architecture (Table 1). Acid-fast bacilli were demonstrated by Ziehl–Neelsen staining in large numbers, in all sections. Both macrophages and giant cells present in lymph nodes were always immunohistochemically positive.

Cases 5 and 8 showed focal lesions, consisting of a few small groups of macrophages and giant cells surrounded by a slight infiltrate of lymphocytes in the subcapsular sinus and paracortex of lymph nodes (Table 1).

Cases 1–4 and 6–7 were considered as diffuse multibacillary lesions, while cases 5 and 8 as multifocal lesions (Table 1). Necrosis was not observed in tissues of any case. In healthy controls, pathological changes were not seen. Sporadically, in some lymph node samples, including the healthy control, macrophages which contained a yellow granular pigment were encountered in the paracortex, but were unrelated to MAP presence or paratuberculosis infection.

3.2. MAP detection

All cases were confirmed using Ziehl–Neelsen stain for acid-fast bacilli and immunohistochemistry with anti-MAP monoclonal antibody, as well as, by IS900-PCR (data no shown). No signal was seen in samples from healthy controls.

3.3. NRAMP1 immunostaining

NRAMP1 inmunolabeling was detected in all analyzed preparations of MAP-positive animals. Labeling was high in almost all cases, and low in two samples (Table 1). Representative immunohistochemical findings are shown. Immunolabeling was detected in macrophage, epithelioid and Langhan's cells (Fig. 1E–F). In the tissue from MAP-negative animals, few NRAMP1 cytoplasmic granules were observed in macrophages from the paracortex of lymph nodes (Fig. 1I).

3.4. iNOS immunostaining

Similar intense iNOS immunolabeling was observed in all MAP positive specimens analyzed in macrophage, epithelioid and Langhans cells of the ileocaecal lymph nodes and ileal mucosa (Fig. 1G and H). Representative immunohistochemical findings are shown. In one sample of ileum, iNOS expression was low, similar to that observed with NRAMP1 expression (Table 1). In samples from control animals, a garbage macrophage showed minimal immunolabeling (Fig. 1J).

3.5. Double immunolabeling

Using the double immunolabeling technique, MAP and NRAMP1, MAP and iNOS, and NRAMP1 and iNOS were simultaneously detected in sections from positive animals. Immunolabeling was observed in granulomatous areas. Multinucleated giant cells of the Langhans type showed immunoreactive cytoplasmic granules for both MAP and iNOS as observed by brown and pink label, respectively (Fig. 2A). This pattern was also observed in macrophage and epithelioid cells (data not shown). Similar results were obtained with double immunohistochemistry by MAP and NRAMP1 (not shown).

Using anti-NRAMP1 and anti-iNOS serum in the same slide, immunolabeling indicated the presence of both in the same tissue area, and in the same cell group (Fig. 2B). The immunoreactivity of iNOS is shown by pink (one arrow) and the NRAMP1 by brown color (two arrows). Also, the others cells showed low expression of iNOS (Fig. 2B).

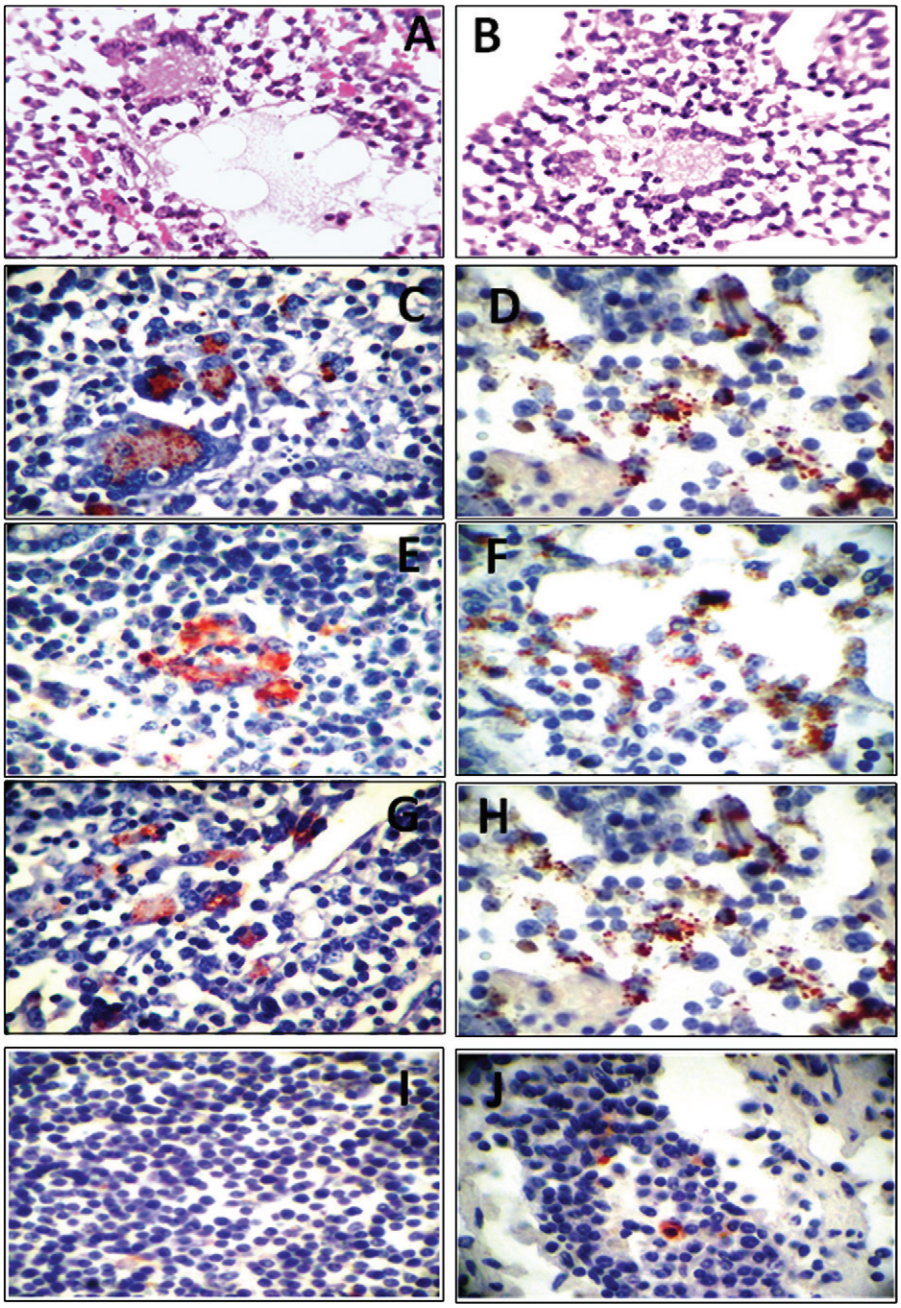

Fig. 1. Immunohistochemistry with anti-MAP, NRAMP1 and iNOS in ileal mucosa (A, C, E, G) and in ileocaecal lymph nodes (B, D, F, H, I, J). (A and B) H&E staining showing pathological changes. (C and D) anti-MAP, (E and F) anti-NRAMP1; (G and H) anti-iNOS (I) negative control omitting anti-NRAMP1; (J) omitting anti-iNOS. Slides were counterstained with Mayer's hematoxylin.

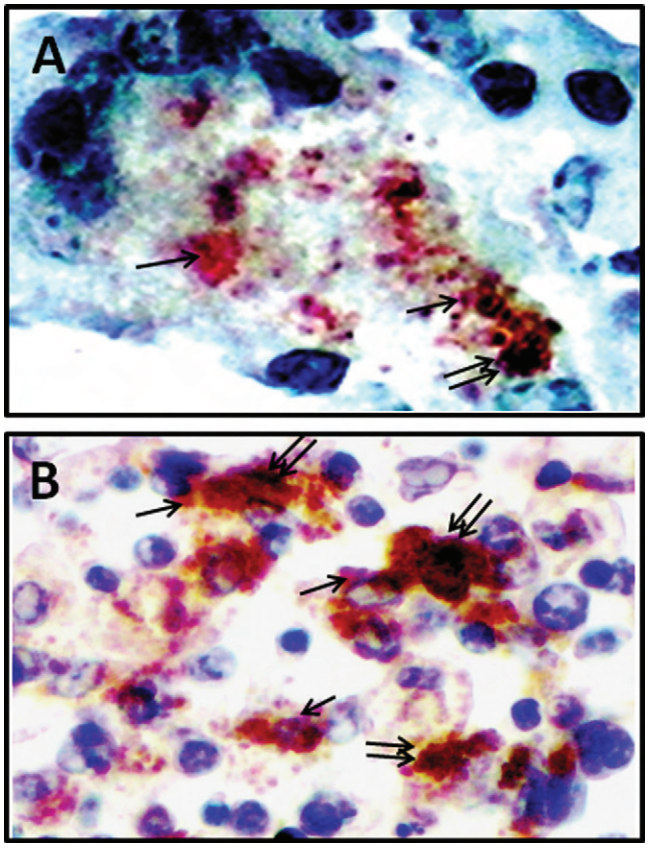

Fig. 2. Double immunohistochemistry labeling (A) ileal mucosa, double immunostaining, indicating presence of MAP and iNOS, brown label (DAB) indicates presence of MAP (two arrows) and pink label (AEC) of iNOS (one arrow), counterstained with hematoxylin, (B) ileocaecal LN double immunostaining, pink indicates iNOS (one arrow) and brown NRAMP1 (two arrows).

4. Discussion

In the present work, we aimed to determine the possible correlation between infection with *M. avium* subsp. *paratuberculosis* and the incidence of expression of the immune markers NRAMP-1 and iNOS, during natural cattle infection. In the majority of the tissues analyzed in this study, histopathological findings indicated development of multibacillary lepromatous-like lesions. However, tuberculoid-like and lepromatous-like granuloma lesions in different portions of the intestines have been reported in cattle infected with MAP and the diffuse intermediate forms between both borderline types are not uncommon [12]. Tuberculoid granulomas seem to be associated with control of the mycobacterial infection and a more favorable outcome than with lepromatous types [5]. The spectrum of lesions of paratuberculosis is likely to be a consequence of the strength of the host CMI, considered to be the principal mechanism for clearing infection. An intense CMI response, with high concentrations of IFN-γ and TNF-α cytokines might be related to encapsulated

tubercles [25,16,27]. The progression of such cases, over a long incubation period, to multibacillary, clinical forms of paratuberculosis is not understood but may be triggered by suppressed CMI leading to mycobacterial proliferation and faecal shedding, as in all the cases here described. The progression of paratuberculosis to clinical stages has been associated with reduced expression of INF-α, since gene expression was significantly higher in ileum and ileocaecal lymph node samples from subclinically infected cows than from clinically infected cows. Also inhibition of pro-inflamatory cytokine IL-18 gene expression in lepromatous-type lesions has been associated with the shift from the Th1-dominant state to Th2-predominant state in PTB [26]. CMI may wane over the protracted period of a persistent infection, leading to mycobacterial proliferation and disease associated with multibacillary lepromatous lesions.

The objective of the present work was to evaluate the expression of both NARMP1 and iNOS molecules in PTB naturally infected cattle. Inmunohistochemical results revealed the high expression level of both NRAMP1 and iNOS, in multibacillary lepromatous-like lesions (diffuse and multifocal) from clinical PTB cases, whereas in the healthy controls both were practically absent. Differences observed between cases and controls may be associated with MAP infection, since induction of high levels of NRAMP1 expression have been previously reported during natural infection by *M. bovis*, mainly in giant cells [10,22]. Characteristic lesions of bovine tuberculosis consist of tuberculoid granulomatous inflammatory changes, primarily in association with a CMI, and an increase in phagocytic and bactericidal activity. iNOS is highly expressed in bovine tuberculous granulomas resulting from effective macrophage activation, which probably contributes to control of mycobacterial proliferation [22]. Our results indicate high levels of iNOS expression in lepromatous-like PTB infection, and similar findings have been obtained using infected macrophage-monocyte derived cell cultures stimulated with rINF-γ [30]. However, it has been proposed that INF-γ mediated protection against MAP infection by means of induction of iNOS and NO production might be efficient only in early stages of the disease [26]. Also the amount of NO produced by in vitro INF-γ activated bovine macrophage appears to be insufficient to kill intracellular MAP [25].

The fundamental changes in PTB occur primarily within the intestine. Intestinal tissues have balanced cytokine profiles of active immunity and tolerance [4]. In this work, ileum or ileocaecal lymph nodes tissues from eight clinical cases of PTB were studied, based on the consideration of their being the anatomical portion most affected by MAP during PTB infection [5,12]. iNOS immunolabeling was abundantly detected in macrophage, epithelioid and Langhans cells of the tissues analyzed. High concentrations of TNF-α and IL-1 pro-inflammatory cytokines generated in response to mycobacterial lipoarabinomannans, peptidoglycans or heat-shock proteins may encourage inflammation by the production of toxic amounts of nitric oxide and contribute to the lesions of PTB [1].

In contrast to our results, a recent report describes low expression levels for iNOS in samples of granulomatous enteritis from PTB infected cows, with different degree of severity [15]. Using tissues samples, Hostetter et al. [15] showed low expression of iNOS in PTB granulomas, suggesting that the lack of iNOS expression could affect the CMI against MAP. However in this work, no specificity of the iNOS antibody, origins of the portion of analyzed intestinal samples or lesions types were detailed.

In all cases, in our analyzed samples, acid-fast bacilli were demonstrated in large numbers, in macrophages and giant cells, by Ziehl–Neelsen staining or immunohisto-

chemestry. The heavy bacterial load circulating from the intestinal mucosa (lamina propria) to Peyer's patch and ileocaecal lymph nodes, may be caused by the switch of a Th1- to Th2-like suppressor immune response commonly observed in clinical PTB [8,16,27]. Discrimination between this type of anergy and other possible mechanisms of tolerance responding to high bacterial traffic has not been well documented in MAP infection [28]. Inhibitory or anergic influences might skew the balance, increasing mycobacterial loads and prompting the production of the multibacillary lepromatous-like lesions of paratuberculosis. Reduced apoptosis and prolonged survival of recruited macrophages have been related with the failure to express or to sense enough amounts of TNF-α for efficient granuloma formation, regarded as the mechanism responsible for appearance of the diffuse granulomatous lesions during PTB [18,1].

Minimal immunolabeling of NRAMP1 and iNOS was found in the paracortex of lymph nodes, in spite of the absence of granulomas. Likewise, negative control tissues did not stain for these markers, suggesting basal expression levels of these proteins in garbage macrophages, which may be induced by other environmental infections (e.g. *M. avium* subspecies other than *M. avium paratuberculosis*) since they were taken from two adult healthy cattle from tuberculosis and paratuberculosis free herds without clinical signs, lesions, Ziehl–Neelsen or immunohistochemistry MAP positive labeling [10,22].

In this work NRAMP1 immunolabeling was abundantly detected in macrophage, epithelioid and Langhan's cells in ileocaecal lymph nodes and in about half of ileal tissues analyzed. To the best of our knowledge, no data have been reported about NRAMP1 production in response to natural infection by MAP. Moreover, simultaneous detection of MAP and iNOS2, and MAP and NRAMP1 strengthen the notion of NRAMP and iNOS induction by MAP, and the detection of both molecules in the same area and cell group support the hypothesis of iNOS expression is regulated by NRAMP1 [11]. Further studies using more samples and different tissues from PTB naturally infected cattle, including measurements of CMI and humoral response parameters will contribute to determine the role of NRAMP1 and iNOS in PTB pathogenesis.

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