Expression of Inducible Nitric Oxide Synthase in Human Granulomas and Histiocytic Reactions

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Inducible nitric oxide synthase (iNOS) is required in immune response against infections and is involved in granuloma formation in animals; in murine macrophages, iNOS is induced by lipopolysaccharide and interferon- γ . In contrast, the role of iNOS in human immune response against infections is still questioned, and its expression in granulomas is poorly investigated. Using Western blotting and immunohistochemistry, we investigated iNOS expression in human lymph nodes with nonspecific reactions and in tissues containing granulomas caused by mycobacteria, *Toxoplasma*, *Cryptococcus neoformans*, *Leishmania*, *Bartonella*, noninfectious granulomas (sarcoidosis, foreign body), and other histiocytic reactions (Kikuchi's disease, Omenn syndrome). iNOS was undetectable in non-specific reactive lymphadenitis, foreign-body granulomas, and Omenn syndrome, whereas it was strongly expressed in infectious granulomas, sarcoidosis, and Kikuchi's diseases. Immunohistochemistry demonstrated that iNOS was selectively expressed by the epithelioid and multinucleated giant cells within the granulomas. Use of an anti-nitrotyrosine antibody, recognizing nitrosilated amino acid residues derived from nitric oxide production, revealed a consistent positivity within the cells expressing iNOS, thus suggesting that iNOS is functionally active. Detection of cytokines by reverse transcriptase-polymerase chain reaction demonstrated that tissues that were positive for iNOS, also expressed the Th1-type cytokine interferon- γ mRNA, but not the Th2-type cytokine interleukin-4. Taken together, these results indicate that iNOS is involved in different human immune reactions characterized by histiocytic/granulomatous inflammation and associated with Th1-type cytokine secretion. (Am J Pathol 1999, 154:145-152)

Nitric oxide (NO) is one of the smallest endogenous biological mediators synthesized in different cell types by a family of related enzymes called nitric oxide synthase (NOS).¹ At least three distinct isoforms of NOS, encoded by separate genes, are expressed in mammalian cells. Two of them are constitutively expressed in brain neurons (NOS1 or nNOS) or in endothelial cells (NOS3 or eNOS).^{2,3} The third isoform, termed inducible NOS (iNOS or NOS2) is expressed only on stimulation in macrophages and hepatocytes. *In vitro*, expression of the enzyme is associated with NO production and thereby extracellular release of NO metabolites (eg, nitrites, nitrates, peroxynitrites).^{4,5} NO release results into tyrosine residues nitrosation, and nitrotyrosine detection has been used as an indicator of iNOS expression and NO function.⁶⁻⁹

Inducible nitric oxide synthase is expressed *in vitro* as well as *in vivo* by murine macrophages on infection with intracellular pathogens such as mycobacteria, *C. neoformans*, and *Toxoplasma gondii*.¹⁰⁻¹² Expression of iNOS is regulated primarily at the transcriptional level and is modulated by various cytokines and microbial products. Stimulation of murine macrophages with lipopolysaccharide or with other bacterial derivatives induce iNOS expression. In addition, cytokines, such as of interferon- γ (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor- α potentially enhance the extent of iNOS expression.¹³⁻¹⁷ The expression of iNOS induced by these agents is associated with the activation of transcriptional factors, including NF- κ B, STAT-1, and IRF-1.^{13,15,18}

During infection by intracellular pathogens, T cells infiltrating the granulomatous tissue produce large amounts of Th1-type cytokines such as IL-2 and IFN- γ . In contrast, IL-4 and IL-5, cytokines secreted by Th2 lymphocytes, are not detectable.^{8,11,19,20} Genetic deletion of IFN- γ or of IFN- γ receptor genes in mice leads to susceptibility to mycobacteria infections and greatly reduces the degree of NO production.²¹⁻²⁵ Similar features are observed in iNOS^{-/-} mice,²⁶⁻²⁹ suggesting that both iNOS and IFN- γ play a central role in murine immune response against intracellular pathogens.

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Human immune response against intracellular pathogens is less well characterized; recent findings indicate that IFN- γ has an essential function in immune response against mycobacteria because children that lack IFN- γ receptor α -chain or the IFN- γ receptor β -chain develop severe infections by bacillus Calmette-Guerin or mycobacteria.^{30–33} However, the role of iNOS in human immune defense against intracellular pathogens is still poorly characterized. Several authors have reported that iNOS protein is expressed in monocytes and bronchoalveolar macrophages obtained from patients with mycobacteria infections as well as in hepatocytes from individuals suffering from malaria or chronic viral hepatitis.^{34–36} However, there is no final evidence that iNOS is expressed in human monocyte-derived cells infected by intracellular pathogens. In addition, human monocytes or monocyte-derived macrophages, activated *in vitro* by a variety of stimuli, release only small amounts of NO derivatives, and it is unclear whether the enzyme is indeed active in human cells.^{37–39}

In this study, we investigated the expression of iNOS on human tissues that displayed various forms of infectious granulomas and other pathological conditions predominantly characterized by histiocytic reactions. In addition, the same tissues were assessed for the extent of protein nitrosation as a measure of iNOS activation. Finally, the cytokine expression and NF- κ B cell translocation were evaluated.

Materials and Methods

Normal and pathological tissue samples were used for this study. The former included four lymph nodes showing nonspecific reactive change and two thymuses removed from young adults during heart surgery. Pathological samples were represented by 10 cases of infectious epithelioid cell granulomas, which included lymph nodes with toxoplasmosis (two cases), *Mycobacterium tuberculosis* (three cases), *C. neoformans* (one case), and cat-scratch disease (three cases), and one spleen involved by visceral leishmaniasis. Moreover, six cases of sarcoidosis (four lymph nodes and two skin biopsies), two cases of histiocytic necrotizing lymphadenitis (Kikuchi's disease), and two lymph nodes from patients with Omenn syndrome were studied.⁴⁰ Finally, a vascular plastic prostheses removed because of thrombosis and associated with a foreign-body giant-cell reaction was analyzed. All tissues were fresh frozen in liquid nitrogen-precooled isopentane and stored at -80°C .

Immunoblotting

For Western blot (WB) analysis, small portions of lymph nodes were lysed in buffer containing 300 mmol/L NaCl, 50 mmol/L Tris-HCl, 2 mmol/L EDTA, 0.5% Triton X-100, 2.5 mmol/L *p*-nitrophenyl *p*-guanidinobenzoate, 10 mg/ml aprotinin, and 10 mg/ml leupeptin and centrifuged at $12,000 \times g$. Fifty micrograms of protein was then boiled, subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrotransferred onto polyvi-

nylidene fluoride membranes (Immobilon; Millipore, Bradford, MA). Membranes were probed with a mouse IgG2a monoclonal antibody directed against a 21-kd protein fragment corresponding to residues 961–1144 murine iNOS (Transduction Laboratories, Lexington, KY). This monoclonal antibody was shown to react also with the human counterpart of iNOS.⁴¹ Expression of iNOS was then detected by enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, IL). Filters were then reblotted by a rabbit anti- β -actin polyclonal serum to allow comparability of samples. Cell lysates from the murine lipopolysaccharide and IFN- γ activated macrophage cell line RAW 264.7 provided by the manufacturer (Transduction Laboratories) were used as a positive control.

Immunohistochemistry

Immunostaining for iNOS, performed applying the same antibody used for WB, was applied on frozen sections at the concentration of 20 $\mu\text{g/ml}$; an isotype (IgG2a)-matched antibody was used as negative control. Anti-CD3 (UCHT1, Dako, Milan), anti-CD20 (L26, Dako), and anti-CD68 (KP1, Dako) antibodies were also applied to identify T cells, B cells, and macrophages, respectively. Tissue immunostaining for nitrotyrosine was performed on frozen sections using a monoclonal antibody at the concentration of 15 $\mu\text{g/ml}$ (Dr. Joseph Beckman, University of Alabama, USA). Finally, the p65 component of the transcription factor NF- κ B was analyzed using the polyclonal antibody raised against the p65 component of NF- κ B (Santa Cruz Biotechnology, Heidelberg, Germany), applied at the dilution of 1:100 on both frozen and paraffin sections.

On both cryostat and paraffin sections, immunostaining followed the streptavidin-biotin immunoperoxidase technique. Inhibition of endogenous peroxidase was performed on paraffin sections only. Chromogen reaction was developed with 3-amino-9-ethylcarbazole or with 3–3'-diaminobenzidine (DAB) solution, and nuclei were counterstained with Mayer's hematoxylin.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Extraction of total RNA and RT-PCR analysis were performed as previously described.⁴² Briefly, total RNA was purified using RNeasy B (Tel-test, Friendswood, TX) following the manufacturer's instructions in liquid nitrogen to prevent RNA degradation. cDNA synthesis was performed with 1 μg of RNA in a total volume of 20 μl containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 5 mmol/L MgCl_2 , 1 mol/L dNTPs, 20 U of ribonuclease inhibitor (Ambion Inc., Austin, TX), 2.5 $\mu\text{mol/L}$ of oligo (dT), and 50 U of reverse transcriptase (Perkin Elmer Corp., Norwalk, CT). The reaction mixture was incubated at 42°C for 40 minutes and stopped at 94°C for 5 minutes. A 10- μl aliquot of the cDNA obtained was amplified in a 20- μl reaction containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 1.5 mmol/L MgCl_2 , 0.2 mmol/L each dNTP,

Figure 1. Expression of iNOS by Western blot analysis in a reactive lymph node (a) and in lymph nodes with mycobacterial granuloma (b and d), *Bartonella*/cat scratch granuloma (c), Kikuchi's disease (e), and cryptococcal granuloma (f). The same filter was stripped and reblotted with an anti- β -actin antibody to allow comparability of the samples. Results are representative of four independent experiments.

200 nmol/L each primer, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The following previously described oligonucleotides were used in PCR reaction: IFN- γ sense 5' AGTTATATCTTGGCTTTTCA 3', IFN- γ antisense 5' ACCGAATAATTAGTCAGCTT 3', with cycling conditions of 1 minute at 94°C, 1 minute at 45°C, and 2 minutes at 72°C for 40 cycles; IL-4 sense 5' CTTCCCCCTCTGTTCTTCCT 3', IL-4 antisense 5' TTCCTGTCGAGCCGTTTCAG 3', with cycling conditions of 1 minute at 94°C, 1 minute at 50°C, and 2 minutes at 72°C for 40 cycles; β -actin sense 5' GTGGGGCGC-CCCAGGCACCA 3', β -actin antisense 5' CTCCTTAATGTCACGCACGATTTC 3', with cycling conditions of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C for 35 cycles.⁴³ A sample (15 μ l) of each PCR reaction was electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide. β -actin PCR product was used as external standard and the mRNA expression for each cytokine was evaluated as comparison with the PCR product over β -actin PCR product.

Results

Detection of iNOS Expression by Western Blotting in Tissue Lysates

Human lymph nodes showing nonspecific reactive changes or containing infectious and noninfectious granulomas were subjected to WB analysis by an anti-iNOS monoclonal antibody. As shown in Figure 1, a 130-kD protein corresponding to the molecular weight of iNOS was detected at variable extent in human lymph nodes. Low amounts of iNOS protein were found in reactive nonspecific lymphadenitis, whereas high contents of the enzyme were detected in granulomatous lymphadenitis

and in Kikuchi's disease. The same 130-kD band was detected in lysates from murine activated macrophages, whereas human thymus was negative for iNOS.

Cell Localization of iNOS Expression by Immunohistochemistry

The localization of iNOS reactivity in lymph nodes used for WB analysis as well as on the other tissue samples was evaluated by immunohistochemistry. In nonspecific reactive lymphadenitis and in normal thymuses, most monocyte-derived cells (including sinus macrophages, tingible body macrophages, interfollicular macrophages, and plasmacytoid monocytes) and dendritic cells (interdigitating reticulum cells and follicular dendritic cells) were consistently negative for iNOS (Figure 2, a and b). In contrast, in epithelioid cell granulomas, the epithelioid and the multinucleated giant cells revealed obvious cytoplasmic reactivity for iNOS (Figure 2, d, e, g, and 3a). The reactivity was particularly intense in small epithelioid cells clusters and small necrotizing granulomas, whereas larger granulomas associated with extensive necrosis were formed by epithelioid cells that were either weakly positive or negative for iNOS. In infectious granulomas in which microorganisms could be easily identified on frozen sections (*C. neoformans* and *Leishmania donovani*-associated granulomas), the parasitized macrophages were clearly positive for iNOS (Figures 2, d and g). In Kikuchi's lymphadenitis, the collections of histiocytes associated or not with central necrosis showed strong reactivity for iNOS, which in contrast was completely negative in the plasmacytoid monocytes at the periphery of the lesions (Figure 3b). Noteworthy, in all cases showing iNOS positive granulomas, the reactivity was strictly confined to the cells within the granulomas and was absent from the surrounding tissues as well as from the remaining lymphoid parenchyma. Serial sections stained with CD68, CD3, and CD20 revealed that iNOS positive cells were represented by CD68⁺ macrophages, whereas T and B lymphocytes did not stain for iNOS. Interestingly, in patients with Omenn syndrome, a pathological condition characterized by hypereosinophilia, T-cell activation, and production of Th2 cytokines,⁴⁰ lymph nodes did not display iNOS staining, although macrophages were detectable in large numbers and focally formed small epithelioid cell granulomas (Figure 3, c and d). In the foreign-body granuloma associated with vascular plastic prosthesis, the palisade of CD68 positive macrophages and multinucleated giant cells surrounding the foreign material did not show any reactivity for iNOS (Figure 2c). Practically no lymphoid cells were found associated with this granulomatous reaction (data not shown).

Study of Nitrotyrosine Staining in Tissues

Immunostaining for anti-nitrotyrosine revealed a weak granular reactivity that was consistently detectable in the cytoplasm of the macrophages forming the granulomas (Figure 2, f and h); no obvious differences were found between infectious and noninfectious granulomas. In ad-

Figure 3. Expression of iNOS in lymph nodes from patients with sarcoidosis (a), Kikuchi's disease (b), and Omenn syndrome (d). Whereas iNOS is strongly expressed by the epithelioid and multinucleated giant cells within the sarcoid granuloma and by the aggregates of histiocytes in Kikuchi's disease, no reactivity is observed by the numerous histiocytes in the lymph node from Omenn syndrome, which are labeled by CD68 (c). Eosinophils, which are typically numerous in Omenn syndrome, are recognized in (d) because of their endogenous peroxidase, which was not inhibited during immunostain. Immunohistochemistry for iNOS (a to c) and CD68 (c), 3-amino-9-ethylcarbazole development, and hematoxylin counterstain. Magnification, $\times 60$ (c), $\times 160$ (a, b, and d).

dition to macrophages, endothelial cells also displayed nitrotyrosine expression. Lymph nodes showing nonspecific reactive changes, as well the foreign body reaction that did not express iNOS were negative for anti-nitrotyrosine staining (data not shown).

Expression of Interferon- γ and IL-4 mRNAs

We extracted total RNA from lymph nodes obtained from patients affected with mycobacteria, cryptococcal, and cat scratch/*Bartonella* infections, Kikuchi's lymphadenitis, or from nonspecific reactive lymphadenitis (Figure 4). By reverse transcription followed by cDNA amplification, we found that IFN- γ mRNA was detectable in lymph nodes that displayed a granulomatous reaction; in particular higher levels were detected in one out of two mycobacteria lymph nodes, in the *Cryptococcus* granuloma and in the Kikuchi's lymphadenitis. A lower extent of IFN- γ mRNA expression was observed in the other lymph node with mycobacteria and *Bartonella* granuloma. In contrast, reactive lymph nodes did not express IFN- γ mRNA (Fig-

ure 4). IL-4 mRNA was not detectable in any of the tissue samples analyzed but was expressed in activated peripheral blood lymphocytes that were used as positive control. These observations suggest that iNOS expression in macrophages from granulomatous tissues could be related to local production of IFN- γ .

Localization of the Transcriptional Factor NF- κ B in Granulomatous Reactions

In normal lymphoid tissue sections, occasional nuclear reactivity is found in rare lymphocytes and endothelial cells, whereas NF- κ B is localized in the cytoplasm of the majority of lymphoid and nonlymphoid cells (Figure 5a). In contrast, immunostaining for NF- κ B in granulomas showed that most epithelioid and multinucleated giant cells were positively labeled in their nuclei (Figure 5b). Finally, no nuclear NF- κ B reactivity was detected in foreign-body associated granulomas.

Figure 2. Expression of iNOS and nitrotyrosine in infectious granulomas (d, *C. neoformans* lymphadenitis; e and f, *Mycobacterium tuberculosis* lymphadenitis; g to h, *L. donovani* splenic granuloma), and comparison with a reactive lymph node (a and b) and with a foreign-body granuloma (c). The numerous intra- and interfollicular CD68⁺ macrophages observed in the reactive lymph node parenchyma (a) and in the foreign body granuloma (c1) are totally negative for iNOS (b and c2). In contrast, the epithelioid and the multinucleated giant cells in infectious granulomas are strongly positive for iNOS (d, e, and g), and show granular cytoplasmic positivity for nitrotyrosine (f and h). In d and g, intracellular cryptococci and *Leishmania* bodies are indicated by arrows. Immunohistochemistry for CD68 (a and c1), iNOS (b, c2, d, e, and g), and nitrotyrosine (f and h), 3-amino-9-ethylcarbazole development, and hematoxylin counterstain. Magnification, $\times 60$ (e), $\times 160$ (c1 and c2), $\times 200$ (a and b), $\times 300$ (f), $\times 400$ (d, g, and h).

Figure 4. Detection of IFN- γ , IL-4, and β -actin mRNAs expression by RT-PCR. Total RNA was extracted from activated peripheral blood mononuclear cells (a), from tissue samples represented by a reactive lymph node (b), and lymph nodes with mycobacterial granuloma (c), *Bartonella*/cat scratch granuloma (d), Kikuchi's disease (e), and sarcoidosis (f). Results are representative of two independent experiments.

Discussion

We have shown that human tissues containing different forms of granulomas associated with intracellular pathogens strongly express inducible nitric oxide synthase (iNOS). Expression of iNOS was not restricted to infectious diseases, because sarcoid granulomas and histiocytes in Kikuchi's disease also contained high levels of iNOS protein. In contrast, in lymph nodes showing non-specific reactive changes, low levels of iNOS were detected by Western blot, and no immunoreactivity for iNOS was found on different monocyte-derived cell types; in particular, sinus macrophages, tingible-body, and interstitial macrophages, as well as T-cell-associated dendritic cells were totally devoid of iNOS expression. These results extend previous reports that monocytes and monocyte-derived cells from healthy individuals contain little or no iNOS protein,³⁹ whereas high levels of iNOS are readily detectable in cases of infections or chronic inflammatory diseases.⁴¹ Interestingly, iNOS positivity was selectively found in macrophages forming the granulomas, whereas only a few positive cells were recognizable in the surrounding tissues; this might indicate that induction of iNOS expression depends on mechanisms related to the granuloma microenvironment. In addition, the observation that iNOS is strongly expressed in the cells infected by intracellular pathogens, such as *C. neo-*

formans and *L. donovani*, suggests that microbial products might be responsible for iNOS induction as previously reported for liver cells infected by hepatitis B virus.³⁶

Besides microbial pathogens, cytokines associated with the immune response against infectious agents also play a role in regulating iNOS expression. In particular, the Th1-type cytokines IFN- γ , IL-2, and TNF are potent inducers of iNOS expression,^{13-15,44} whereas Th2 cytokines, such as IL-4, suppress iNOS expression.⁴⁴⁻⁴⁷ The potential role of cytokines in the induction of iNOS is supported by the observation that noninfectious granulomas were also strongly positive for iNOS. *In vitro*, lipopolysaccharide-induced iNOS expression in murine macrophages is enhanced by stimulation with IFN- γ , a Th1-type cytokine. It should be noted that Th1-type cytokines, including IFN- γ are required to direct cell-mediated immune responses leading to the eradication of intracellular pathogens³⁰⁻³² and are also fundamental in the immune mechanisms involved in the formation of granulomas in sarcoidosis.⁴⁸ Tissue samples containing iNOS positive cells in both infectious and noninfectious granulomas were expressing IFN- γ mRNA as detected by RT-PCR. In particular, mycobacterial, cryptococcal granulomas, and Kikuchi's lymphadenitis, which were strongly positive for iNOS protein, also expressed at high levels IFN- γ mRNA, thus suggesting a possible cause-effect relationship between macrophage expression of iNOS and IFN- γ production. The lack of IL-4 mRNA expression in these tissue samples is in agreement with the observation that IL-4 is a potent inhibitor of IFN- γ mediated iNOS expression *in vitro*.^{44-46,49} To further support the role of IFN- γ secreting T cells in the induction of iNOS expression in macrophages, we have shown that lymph nodes derived from patients with Omenn syndrome, a disease characterized by predominant production of the Th2-type cytokines, IL-4 and IL-5,⁴⁰ were totally negative for iNOS although tissues contained a large number of activated T cells.

In humans, other nongranulomatous inflammatory conditions characterized by a Th1-dependent cell mediated immune response are associated with iNOS expression by mononuclear macrophages, such as rheumatoid arthritis, celiac disease, graft *versus* host reactions, and

Figure 5. Expression of NF- κ B in a reactive lymph node (a) and in cryptococcus granuloma (b). In the reactive lymph node, NF- κ B is mostly localized in the cytoplasm of lymphoid and nonlymphoid cells (a). Whereas in the cryptococcus granuloma, the multinucleated giant cells show evident nuclear translocation of NF- κ B (b). In (b) two intracellular yeast cells stained by periodic acid-Schiff are also recognizable. Immunohistochemistry for NF- κ B, 3'-3'-diaminobenzidine development, periodic acid-Schiff counterstain (b). Magnification, $\times 160$ (a), $\times 400$ (b).

psoriasis.^{9,41,50,51} The present study provides evidence that also in Kikuchi's lymphadenitis the histiocytic reaction is associated with strong iNOS expression by the histiocytes and with high IFN- γ mRNA tissue content, suggesting that iNOS induction by activated T cells might have a relevant role in the pathogenetic mechanisms of this disease.

It has been shown that iNOS expression is primarily regulated at the transcriptional level and depends on NF- κ B activation, as truncation of NF- κ B binding sites in the promoter region of iNOS gene virtually abrogates iNOS inducibility.^{13–15} Interestingly, we have shown that staining for NF- κ B complex primarily occurs on the nucleus of many epithelioid and giant cells within the granulomas, suggesting that activation and translocation of this transcription factor might be involved in iNOS activation. In contrast, foreign-body granulomas neither expressed iNOS in the cytoplasm nor showed NF- κ B positivity in their nuclei.

Up-regulation of iNOS mRNA and protein by murine macrophages is strictly associated with their capacity to produce NO and consequent microbicidal activity *in vitro* and *in vivo*.⁴ In mice, infections by intracellular pathogens are exacerbated by administration of NOS inhibitors, and mice rendered genetically deficient in iNOS show decreased resistance toward intracellular pathogens.^{27–29} In humans, the antimicrobial activity of iNOS expressing cells is not completely elucidated. iNOS has been detected in monocytes and monocyte-derived cells from patients with tuberculosis, chronic and acute pneumonia, *Leishmania*, malaria infection, and in hepatocytes from patients with viral hepatitis.^{34–36,52–54} Nevertheless, despite the fact that human macrophages can express iNOS mRNA and protein, their ability to generate NO has been questioned because nitrates or nitrites are hardly detectable in macrophage-derived supernatants.³⁹ The evidence that iNOS is strongly expressed in the sites of infections sustained by intracellular pathogens seems to indicate that this enzyme is involved in the cellular defense mechanisms elicited against these infectious agents. Moreover, our observation that iNOS expression in infectious granulomas is associated with protein nitrosation suggests that iNOS is functionally active during infections sustained by intracellular pathogens, thus supporting a possible role of the enzyme in human immune responses against infectious agents.

In conclusion, the present study provides evidence that expression of iNOS and nitrotyrosine formation represent a common finding in human granulomas and histiocytic reactions with different etiology, but all characterized by a Th1-type cytokine production.

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