Expression of Inducible Nitric Oxide Synthase in Skin Lesions of Patients with American Cutaneous Leishmaniasis

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Cytokine-inducible (or type 2) nitric oxide synthase (iNOS) is indispensable for the resolution of Leishmania major or Leishmania donovani infections in mice. In contrast, little is known about the expression and function of iNOS in human leishmaniasis. Here, we show by immunohistological analysis of skin biopsies from Mexican patients with local (LCL) or diffuse (DCL) cutaneous leishmaniasis that the expression of iNOS was most prominent in LCL lesions with small numbers of parasites whereas lesions with a high parasite burden (LCL or DCL) contained considerably fewer iNOS-positive cells. This is the first study to suggest an antileishmanial function of iNOS in human Leishmania infections in vivo.

The production of nitric oxide (NO) from the amino acid l-arginine by the cytokine-inducible (or type 2) NO synthase (iNOS) is one of the key defense mechanisms of mammalian phagocytes (reviewed in reference 15). A large number of studies showed that in rodents, the control of many different infectious pathogens (including viruses, bacteria, protozoa, fungi, and helminths) is strictly dependent on the expression of iNOS activity. Most convincing in this respect were the in vivo analyses of mice treated with inhibitors of iNOS or carrying a deletion of the iNOS gene (reviewed in reference 2). Several reports demonstrated that the NO radical or NO derivatives not only act as direct antimicrobial effector molecules but also exert host-protective immunoregulatory effects (2). A particularly well-studied model is murine cutaneous leishmaniasis, in which iNOS was shown to be indispensable for the innate natural killer cell response to Leishmania major (7), for the resolution of acute skin lesions (6, 17), and for the long-term control of small numbers of parasites persisting in the clinically cured host (3, 22). iNOS-dependent parasite control was also observed in the livers of Leishmania donovani-infected mice (14). However, it is important to bear in mind that in some infections (e.g., influenza virus A pneumonitis), iNOS-derived NO is clearly counterprotective, i.e., it causes tissue damage and accounts for disease progression (reviewed in reference 4).

Unlike their rodent counterparts, human peripheral blood monocytes and monocyte-derived macrophages are more difficult to activate for the production of NO by conventional stimuli such as gamma interferon (IFN-γ) and lipopolysaccharide, which initially caused a dispute about the existence of an NO-based defense mechanism in human phagocytes. More recently, a number of studies revealed that the production of NO is induced in human monocytes and/or macrophages in vitro when stimuli other than IFN-γ and lipopolysaccharide are applied, for example, type I interferon or interleukin 4 (IL-4) plus anti-CD23 (21, 25). Today, there is no doubt that iNOS is expressed in humans in vivo during a wide variety of disease states, including those of infectious diseases, although in most cases its relevance and function remains unclear (reviewed in references 11 and 26). For example, iNOS expression was detected in skin lesions of patients with psoriasis (11), tuberculoïd leprosy (10), or borderline leprosy during type 1 or reversal reactions (12). Furthermore, bronchoalveolar lavage macrophages from patients with pulmonary tuberculosis (16), peripheral blood monocytes and/or macrophages from IFN-α-treated patients with hepatitis C (21), and blood monocytes from children with subclinical cerebral malaria were found to express iNOS mRNA and protein (1). In two recent reports, the presence of iNOS in the spleens or bone marrow of patients with visceral leishmaniasis (infection with L. donovani or Leishmania chagasi) was shown by immunohistochemistry (8, 9). Whether iNOS is also expressed during the immune response to other species of Leishmania parasites in humans is unknown to date. Here, we investigated whether iNOS is present in skin lesions of patients infected with Leishmania mexicana and whether the level of expression correlates with the course of infection.

MATERIALS AND METHODS

Patients. Seventeen patients from Mexico with local (LCL) or diffuse (DCL) cutaneous leishmaniasis who live in rural areas in the states of Tabasco, Campeche, and Veracruz where leishmaniasis is endemic and only L. mexicana mexicana has been isolated in the past were studied (19). Informed consent for taking the biopsies was obtained from the patients. The human experimentation guidelines at the authors’ institutions in Mexico and Germany were followed during the course of this research. The patients were grouped into three different categories according to clinical, immunological, histopathological, and parasitological criteria (Table 1). In accordance with previous studies (5, 13, 19), LCL patients (groups I and II) had only a single or a few circumscribed skin lesions and showed a positive response to leishmanin in the Montenegro skin test. By hematoxylin-eosin (H&E) staining, LCL lesions (groups I and II) were characterized by focal, dermal infiltrates consisting primarily of small lymphocytes and...
a few monocots, conservation of the skin annexae, and the absence of epithelial atrophy. DCL patients, in contrast, had multiple, disseminated lesions and were leishmanin unresponsive. By H&E staining, DCL lesions (group III) showed massive, diffuse dermal infiltrates mainly of large mononuclear cells, pronounced surface epithelial atrophy, and complete obliteration of the skin annexae.

Skin biopsies were embedded in Tissue Tek OCT compound (Sakura, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at −70°C until analysis by (immuno)histology.

Antibodies. Rabbit anti-L. mexicana antiserum was generated as described previously (19, 20) and used at 1:50,000. It showed the same specificity as a monoclonal anti-L. mexicana reference antibody provided by F. Modabber (Leishmania Vaccine Steering Committee, World Health Organization, Geneva, Switzerland), i.e., reactivity with L. mexicana but not with Leishmania braziliensis or L. major (I. Becker, unpublished observations). In contrast, rabbit anti-L. major immunoglobulin G (IgG) used at a dilution of 1:1,000 reacted with L. major, L. donovani, and Leishmania infantum but not with L. mexicana (3, 22).

A rabbit anti-human iNOS-peptide antiserum (directed against an N-terminal peptide of human iNOS, puriﬁed by peptide chromatography, and used at a dilution ratio between 1:250 and 1:500. A rabbit antiserum against the C terminus of human iNOS, puriﬁed by peptide chromatography, and used at 1:250. We also generated a rabbit antiserum raised against the seven last amino acids of the C terminus of human iNOS, puriﬁed by N-terminal chromatography, and used at 1:250 and 1:500.

For the detection of cells in the lesions, mouse monoclonal antibodies against human CD68 (macrophages, clone EBM11, 1:1,000 dilution; DAKO, Hamburg, Germany) and human CD3 (T lymphocytes, clone UCHT1, 1:100 dilution; Becton Dickinson/PharMingen, Hamburg, Germany) were used.

TABLE 1. Clinical and immunohistochemical data for the analyzed LCL and DCL skin biopsies

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient no. (age [yr])</th>
<th>Clinical diagnosis</th>
<th>No. of lesions (age of biopsied lesion[s])</th>
<th>Histological and parasitological diagnosisa</th>
<th>Parasites/field (×100)b</th>
<th>No. of iNOS-positive cells (mean ± SD)/field (×100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1 (34)</td>
<td>LCL</td>
<td>1 (5 yr)</td>
<td>LCL</td>
<td>4.0 ± 2.0</td>
<td>81 ± 17</td>
</tr>
<tr>
<td></td>
<td>2 (30)</td>
<td>LCL</td>
<td>2 (4 mo)</td>
<td>LCL</td>
<td>2.3 ± 0.7</td>
<td>20 ± 2</td>
</tr>
<tr>
<td></td>
<td>3 (15)</td>
<td>LCL</td>
<td>6 (1 mo)</td>
<td>LCL</td>
<td>21.0 ± 9.3</td>
<td>25 ± 8</td>
</tr>
<tr>
<td></td>
<td>4 (30)</td>
<td>LCL</td>
<td>1 (2 mo)</td>
<td>LCL</td>
<td>26.0 ± 1.7</td>
<td>32 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>5 (34)</td>
<td>LCL</td>
<td>1 (?)</td>
<td>LCL</td>
<td>30.0 ± 9.3</td>
<td>8 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>6 (41)</td>
<td>LCL</td>
<td>1 (?)</td>
<td>LCL</td>
<td>28.0 ± 8.3</td>
<td>36 ± 11</td>
</tr>
<tr>
<td></td>
<td>7 (28)</td>
<td>LCL</td>
<td>1 (2 mo)</td>
<td>LCL</td>
<td>1.2 ± 1.1</td>
<td>56 ± 14</td>
</tr>
<tr>
<td></td>
<td>8 (28)</td>
<td>LCL</td>
<td>2 (2 mo)</td>
<td>LCL</td>
<td>5.0 ± 1.6</td>
<td>11 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>9 (91)</td>
<td>LCL</td>
<td>1 (?)</td>
<td>LCL</td>
<td>38 ± 8.5</td>
<td>13 ± 6.5</td>
</tr>
<tr>
<td>II</td>
<td>10 (25)</td>
<td>LCL</td>
<td>1 (1 mo)</td>
<td>Similar to LCL</td>
<td>ca. 2–3%</td>
<td>19 ± 11</td>
</tr>
<tr>
<td></td>
<td>11 (16)</td>
<td>LCL</td>
<td>1 (5 mo)</td>
<td>Similar to LCL</td>
<td>ca. 30%</td>
<td>14 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>12 (43)</td>
<td>LCL</td>
<td>2 (1.5 mo)</td>
<td>Similar to LCL</td>
<td>ca. 50–70%</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>13 (68)</td>
<td>LCL</td>
<td>1 (5 mo)</td>
<td>Similar to LCL</td>
<td>ca. 60%</td>
<td>4 ± 4.2</td>
</tr>
<tr>
<td>III</td>
<td>14 (40)</td>
<td>DCL</td>
<td>Multiple (2 mo)</td>
<td>DCL</td>
<td>ca. 100%</td>
<td>6 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>15 (40)</td>
<td>DCL</td>
<td>Multiple (2 mo)</td>
<td>DCL</td>
<td>ca. 100%</td>
<td>7 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>16 (53)</td>
<td>DCL</td>
<td>Multiple (3 mo)</td>
<td>DCL</td>
<td>ca. 100%</td>
<td>7 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>17 (18)</td>
<td>DCL</td>
<td>Multiple (5 mo)</td>
<td>DCL</td>
<td>ca. 50%</td>
<td>18 ± 5.4</td>
</tr>
</tbody>
</table>

a Clinically and histopathologically (as assessed by H&E staining; see Material and Methods), the lesions of group II patients did not differ from those of group I patients except for the presence of a high number of parasites (which was otherwise seen only in DCL lesions).

b For group I patients, clusters of Leishmania parasites per section were determined because individual parasites could not be counted. Numbers of parasites per field are given as means ± standard deviations. For group II and III patients, neither individual parasites nor clusters of parasites could be determined due to the extremely high parasite burden. Therefore, parasite burden is given as the estimated percentage of the L. mexicana-immunostained area per section.

Question marks denote unknown ages.

NADPH diaphorase staining was combined with the immunoperoxidase staining of cells because immunoperoxidase technique with 3-amino-9-ethyl-carbazole as a substrate (3, 22). Omitting the first-step antibody revealed negative results confirming the specificity of the staining. For some tissue samples, the specificity of the iNOS staining obtained with the N-terminal antibody was further demonstrated (i) by blocking with the respective peptide immunogen and (ii) by the comparative use of another antibody directed against the C terminus of human iNOS (see above). All sections were counterstained with hematoxylin and mounted in aqueous mounting medium (Faramount; DAKO).

As an alternative method for the detection of iNOS, we also used NADPH diaphorase staining, which has been previously shown to be a reliable marker of iNOS activity (reference 22 and references therein). For the simultaneous detection of iNOS and cell markers, NADPH diaphorase staining was combined with the immunoperoxidase staining of cells because immunofluorescent double-labeling techniques previously successfully applied for the confocal detection of iNOS and cell markers in mouse tissues [6]) were hampered by high background staining in the case of human skin samples.

Semiquantitative evaluation of sections and statistical analyses. The numbers of iNOS-positive cells as well as of Leishmania parasite clusters (group I LCL patients) per field were determined by evaluation of six to eight fields of at least two identically stained sections at a magnification of ×100. Results in the text are given as the mean ± the standard error of the mean. For Leishmania parasites in group II LCL sections and DCL sections, the number of parasite clusters could not be determined due to their extremely high prevalence in the tissue and the confluency of the stainings. In these cases, we estimated the percentage of the microscopic field (magnification, ×100) covered by the immunostain.

RESULTS

L. mexicana parasite burden, iNOS protein expression, and cellular infiltration in skin lesions of patients with LCL and DCL were studied by immunohistochemical analysis of consecutive sections. In lesions from patients who suffered from one or two isolated skin ulcers (LCL, group I), Leishmania
parasites formed isolated clusters of elongated cells that were randomly distributed throughout the dermis (Fig. 1A) and could be detected only with the anti-L. mexicana, but not the anti-L. major, antiserum (N. Donhauser and C. Bogdan, unpublished observations). The clustering made it difficult to count the individual parasites. Thus, the parasite burden in these sections was determined as the number of parasite clusters per microscopic field (Table 1). The cellular infiltrate in these lesions was dominated by aggregations of macrophages and CD3+ cells that were concentrated in the upper part of the dermis (stratum papillare [Fig. 1B and C]). Some of these cells were associated with the expression of iNOS protein that was readily detectable in the very same area of the dermis in all lesions (Fig. 1D). The average number of iNOS-positive cells per field in sections from LCL patients (group I) was 31.3 (± 7.9, n = 9). In preliminary experiments, double labeling of skin biopsies from eight LCL patients (NADPH diaphorase-iNOS staining plus anti-cell-type immunoperoxidase staining) revealed that about 70% of the iNOS-positive cells were CD68+ macrophages whereas ca. 15% each were positive for CD3 or a fibroblast marker (data not shown).

In accordance with previous observations (19), sections from patients with DCL (group III) showed a complete loss of the rete ridges and were much more fragile than those from LCL patients (group I). Upon counterstaining with hematoxylin, the majority of cells in DCL lesions showed only poor nuclear staining, indicating some degree of cell damage. DCL lesions were characterized by an extraordinary abundance of Leishmania parasites that appeared to be oval rather than elongated (Fig. 1E). Macrophage and T-cell antigens were evenly distributed across the whole area of inflammation in the dermis (Fig. 1F and G). iNOS protein was also found in DCL lesions, but in general, its expression, i.e., the number of positive cells (9.5 ± 2.8 per field, n = 4; significantly different from group I LCL lesions, P < 0.05), as well as the intensity of the iNOS staining, was considerably weaker than that in group I LCL lesions (Fig. 1H and Table 1).

Based on clinical, immunological, and pathological criteria (one or two ulcers, positive leishmanin test, focal dermal infiltrates, intact rete ridges, no epithelial atrophy), we found four patients within the group of patients who suffered from LCL with lesions that parasitologically resembled those of DCL patients (i.e., extensive numbers of parasites along with macrophages) and who therefore were placed into a separate group (II). Clinically, these patients did not differ from the patients in group I (except for patient 13, whose single cutaneous lesion had a considerably larger diameter [ca. 10 cm]). In particular, none of these patients was infected with human immunodeficiency virus or had an additional bacterial skin infection. The expression of iNOS protein in group II lesions was low (10 ± 3.9 iNOS-positive cells per field, n = 4) and was not significantly different from that in highly parasitized DCL lesions (group III, P < 0.001). Thus, there is an inverse correlation between iNOS expression and parasite burden in American human cutaneous leishmaniasis.

**DISCUSSION**

This study provides the first evidence for a differential expression of iNOS in human cutaneous leishmaniasis. Immuno-histochemical analysis of skin biopsies of 17 patients with LCL or DCL revealed that iNOS protein was strongly expressed in classical LCL lesions with small numbers of parasites whereas in highly parasitized DCL lesions, only a few iNOS-positive cells were detected. The same was true for a subgroup of LCL lesions (group II), in which a low expression of iNOS was accompanied by a drastically increased number of parasites. Similar to cutaneous leishmaniasis in the mouse (3, 22), the vast majority of iNOS-positive cells were macrophages. In addition, iNOS protein was also detected in a few fibroblasts and CD3+ lymphocytes (data not shown). Whereas it is tempting to assign an antimicrobial activity to iNOS in macrophages and fibroblasts (which both function as host cells for Leishmania), the possible function of iNOS in human T cells remains to be investigated.

Previous in situ analyses of skin biopsies from patients suffering from American cutaneous leishmaniasis revealed a strikingly different pattern of cytokines in LCL and DCL lesions. In LCL, IL-1β, IL-6, tumor necrosis factor alpha (TNF-α), and macrophage chemoattractant protein 1 were strongly expressed and IFN-γ was clearly more dominant than IL-4 whereas only low levels of IL-5 and IL-10 were detected. In contrast, DCL lesions were characterized by type 2 T-helper (Th2) cell cytokines (IL-4, IL-5, and IL-10), a high expression of macrophage inflammatory protein 1α (MIP-1α), and a lack or low-level expression of IL-6, TNF-α, IL-1β, and IFN-γ (5, 13, 19). Considering the fact that IL-1β, TNF-α, and IFN-γ have all been described to upregulate iNOS in mouse and human cells (reviewed in references 4 and 26), it is feasible that the higher expression of iNOS in LCL lesions is mediated by these cytokines. This possibility is also suggested by related findings in human leprosy, where iNOS was detected only in lesions characterized by a Th1 cytokine response (i.e., in the tuberculoid form or in borderline leprosy during type 1 or reversal reactions), whereas lepromatous (diffuse) lesions (characterized by a Th2 response) were found to be largely iNOS negative (10, 12). Based on these correlative data (and on previous immunohistological and functional studies with the mouse model), the more benign course of disease in LCL (and in tuberculoid leprosy) might result from the higher expression of iNOS. Work by Vouldoukis et al. (24, 25) further supports this hypothesis because human monocyte-derived macrophages activated by anti-CD23 or IgE–anti-IgE immune complexes killed intracellular Leishmania parasites in an NO-dependent manner and this NO-mediated leishmanicidal activity was downregulated by IL-10 and IL-4 which were found to inhibit the generation of NO by these cells.

Despite these quite compelling data, there are a couple of caveats as to the role of iNOS in human cutaneous leishmaniasis. First, it is important to bear in mind that human DCL lesions were not devoid of iNOS expression. The high expression of MIP-1α (19) might account for the presence of iNOS in human DCL because in vitro MIP-1α activated human monocyte-derived macrophages for the production of NO and killing of a related intracellular pathogen (23). Second, in the lesions of three LCL patients of group I (Table 1, patients 5, 8, and 9), the expression of iNOS was low without any evidence for disseminated disease or a higher parasite burden. This might reflect successful killing of the parasites with subsequent down-regulation of iNOS but could also indicate an iNOS-indepen-
FIG. 1. Immunoperoxidase staining of skin lesions from patients with LCL (A to D) or DCL (E to H) for *L. mexicana* parasites (panels A and E), macrophages (panels B and F), T cells (panels C and G), and iNOS (panels D and H). Magnifications: panels A to C and E to G, ×200; panels D and H, ×400; insets, ×1,000. Note that in the sections from LCL, there were only a few parasites compared to the DCL sections (cf. panels A and E), whereas the intensity and the frequency of the iNOS staining was much more prominent in the former than in the latter (cf. panels D and H) (panel H shows one of the strongest iNOS stainings obtained in a DCL lesion).
dent control of *Leishmania*. In this respect, it is worth noting that human monocyte-derived macrophages could be activated by macrophage chemotactic protein 1 (which is highly expressed in LCL lesions) for the killing of intracellular *Leishmania* without upregulation of iNOS (18).

In conclusion, although iNOS is unlikely to be the only effector mechanism in human cutaneous leishmaniasis, our data as well as analyses of other infectious diseases suggest that there is a correlation between iNOS expression and disease outcome. These studies should therefore encourage further investigations of the function of human iNOS in infections.

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REFERENCES


