Antigen-presenting cells in human cutaneous leishmaniasis due to

*Leishmania major*

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(Accepted for publication 16 November 1994)

**SUMMARY**

In this study biopsies from skin lesions and draining lymph nodes of patients suffering from cutaneous leishmaniasis caused by *Leishmania major* were examined by immunohistochemistry, and by light and electron microscopy to identify the types of antigen-presenting cells (APC) and their location. APC, identified morphologically and by their expression of specific cell markers, included Langerhans cells, macrophages, follicular dendritic cells, and interdigitating reticulum cells of the paracortex of lymph nodes. These cells expressed MHC class II antigens and contained *Leishmania* antigen. Since some keratinocytes and endothelial cells also showed these characteristics, they may also act as APC. By examining tissue samples from skin lesions and draining lymph nodes it was possible to follow the probable route of trafficking of various inflammatory cells between the skin lesion and lymph nodes. *Leishmania* antigen containing Langerhans cells were found in the epidermis, dermis and the regional lymph nodes. We believe these cells translocate from the epidermis to the dermis, where they take up antigen and migrate to the paracortex of the regional lymph nodes. There they are intimately associated with cells of the paracortex, and could be involved in the generation of *Leishmania*-specific T memory cells. LFA-1-positive T cells of the CD45RO phenotype were found in the skin lesion. Venular endothelium in the skin lesions expressed intercellular adhesion molecule-1 (ICAM-1), which is the ligand for LFA-1. The migration of lymphocytes from the vascular lumen to the site of inflammation is possibly a result of the interaction of these two adhesion molecules.

**Keywords** cutaneous leishmaniasis antigen-presenting cells

**INTRODUCTION**

Cutaneous leishmaniasis, due to *Leishmania major*, is endemic in many countries of the Middle East and Africa, including Saudi Arabia [1] and the Sudan [2].

In most of those infected the disease is usually self-limiting, the lesions healing spontaneously, but some patients require treatment. Patients with healed lesions are usually immune to further infection by the same parasite. After healing they develop delayed hypersensitivity [3]. *In vitro*, this cell-mediated immune response is characterized by the production of interferon-gamma (IFN-γ), and it can be described as a Th1-like response [4]. The amastigote form of the parasite infects primarily the tissue macrophage where it multiplies until the macrophage is immunologically activated to kill the intracellular parasites. Activation of the macrophages is by the generation of T lymphocytes which secrete appropriate cytokines, particularly IFN-γ, which enhance the killing capacity of the macrophage [5]. Another mechanism of parasite elimination is a necrotizing process in which the macrophage and the parasite it contains are destroyed, a poorly understood process in which immune complexes [6] or cytotoxic T cells may be involved [7]. Thus the generation of an appropriate immune response is crucial for parasite elimination. The afferent pathway in this response is dependent on the interaction between antigen-presenting cells (APC) and T lymphocytes. It is therefore worthwhile to define precisely the cells involved in this process and to monitor their trafficking in the lesion and draining lymph nodes. In this study we identify the APC as the epidermal Langerhans cells, macrophages, the interdigitating cells of the paracortex of lymph nodes and the follicular dendritic cells. The possible role of keratinocytes and endothelial cells in antigen presentation is discussed.

**PATIENTS AND METHODS**

*Biopsy material for light microscopy and immunohistochemistry*

Patients with cutaneous leishmaniasis were from an endemic...
focus in the Eastern Province of Saudi Arabia (n = 30) or the Sudan (n = 7). The disease is caused by *Leishmania major* in both countries [1,8], and the clinical and pathological features are the same in the two countries [9]. The diagnosis of cutaneous leishmaniasis was made by the demonstration of the parasites in skin biopsies and/or slit smears of the cutaneous lesion. The samples were collected after obtaining informed consent from the patients. The primary skin lesion was biopsied under local anaesthesia using a 4-mm disposable punch. Enlarged lymph nodes draining the skin lesions were removed surgically from two and eight patients from the Sudan and Saudi Arabia, respectively. Half of the material was embedded in OCT and snap frozen in liquid nitrogen. Frozen sections (5 μm) were cut with a cryostat and air dried overnight, before the immuno-staining. The other half of the tissue was fixed in mercuric chloride–acetate acid mixture for 1.5 h and was then transferred to 70% ethyl alcohol. Paraffin sections (5 μm) were stained with haematoxylin and eosin. The histopathology of the lesion was classified according to Ridley [10].

**Immunohistochemistry**

Biopsies from donors in Saudi Arabia were stained with anti-CD3, -CD4, -CD8, anti-HLA-DR antibodies (all from Becton Dickinson, Mountain View, CA). The staining procedure was according to the instructions provided by the manufacturer. Briefly, cryostat-cut sections were fixed in cold acetone (4°C) for 10 min, air dried and transferred to PBS 0.5 M, pH 7.4. Sections were incubated with 0.3% H2O2/methanol for 30 min to block endogenous peroxidase, followed by rinsing in water. The sections were then incubated with 1:2 normal goat serum for 30 min to reduce non-specific staining. The primary antibodies were applied for 15 min at 20°C. The sections were re-washed with PBS and exposed to biotinylated, goat antimouse IgG for 15 min at 20°C, and washed again in PBS. The sections were then exposed to avidin peroxidase for 15 min, washed in PBS and exposed to 3,3-diaminobenzidine–hydrogen peroxide mixture for 5 min. The sections were immersed in copper sulphate to enhance the colour, washed in PBS, lightly counter-stained with haematoxylin, dehydrated and mounted in dimetere plasticizer xylene (DPX). The positive cells showed a distinct dark brown rim at the periphery of the cell and/or brown cytoplasmic staining. The number of T cells and subsets of T cells were estimated in these biopsies. At least five fields of the infiltrate with more than 40 cells in each focus were counted, as there was some variation in the cellular composition of individual infiltrates in different areas of the section. The number of CD3+ cells in a section was expressed as a percentage of the total number of cells in the infiltrate, and the number of CD4+ and CD8+ cells as a percentage of the total number of CD3 cells [11,12]. Positive controls for the staining of T cells included non-neoplastic human tonsil and/or lymph node. Negative controls of method specificity were performed by omission of primary antibody or its replacement with normal BALB/c mouse serum. A skin biopsy from one normal individual was investigated in parallel to the biopsies from the patients. Few inflammatory cells were identified in the dermis from the control. Langerhans cells were identified in the epidermis, but these did not stain with HLA-DR. The keratinocytes of the normal tissue did not stain with the antibody against HLA-DR. The anti-*Leishmania* antibody did not stain the normal skin sections.

The biopsies from the Sudanese donors were stained with anti-CD1a, -CD4, -CD11a (LFA-1), and -CD54 (intercellular adhesion molecule-1 (ICAM)) antibodies (all from Dako, Glostrup, Denmark). The biotin avidin immunoperoxidase method with appropriate controls was used as described above. The immunoperoxidase-labelled primary antibodies against CD4 and CD1a were visualized by 3-amino-9 ethyl carbazole, which gives a red colour, and those against LFA-1 and ICAM-1 markers were demonstrated by diaminobenzidine (DAB), which stains positive cells a dark brown colour. These biopsies were also double stained with anti-CD4, and anti-CD45RA or CD45RO [13]. The primary antibody against the CD4 molecule was visualized by DAB and the anti-CD45RO (memory T cells) or CD45RA (naive T cells) antibody was visualized by 3-amino-9 ethyl carbazole. Cells showing a brown reddish colour were considered double positive [13].

Paraffin sections of formalin-fixed tissues from donors in Saudi Arabia were stained for lysozyme and an antibody against S-100 protein antigen using an immunoperoxidase kit (Ortho Diagnostic System Inc., Raritan, NJ) as previously described [14]. The total number of positive cells per five high power fields in a standard section were counted.

**Demonstration of Leishmania antigen in the tissues**

*Leishmania* antigen was demonstrated by an indirect immunoperoxidase sandwich technique in tissue from donors in Saudi Arabia. The method was based on that of Sells & Burton [15]. Sections were deparaffinized, brought to water and mounted on glass slides. Tris saline 0.5 M, pH 7.6 was used throughout the procedure as diluent and unless otherwise stated for washing. Staining was carried out in humidified chambers at room temperature. Anti-*Leishmania* sera were collected from BALB/c mice, which had been infected by 105 *L. major* stationary phase promastigotes 30–45 days previously. Sera were pooled from different mice and stored at −20°C. Serum was used in a dilution of 1:160. Normal mouse serum was similarly stored and diluted. Peroxidase-conjugated goat antimouse and normal goat serum were obtained from Dako. Endogenous peroxidase and non-specific binding of the anti-*Leishmania* serum were blocked as described above. The anti-*Leishmania* serum was applied for 30 min. After washing with buffer for 30 min the sections were incubated with peroxidase-conjugated goat anti-mouse serum at a dilution of 1:2 for 30 min. This was followed by washing in buffer for 30 min. DAB solution (Dako) was prepared according to the instructions of the manufacturer and the sections were incubated for 10 min in the dark. Sections were then washed in tap water, lightly counter-stained with haematoxylin, dehydrated in alcohol and mounted in DPX. Controls included: (i) Tris buffer substituted for the peroxidase-conjugated serum; (ii) normal goat and absorbed anti-*Leishmania* serum. The absorbed serum was prepared by absorbing 0.5 ml of anti-*Leishmania* serum with 105 live washed *L. major* promastigotes for 30 min, after which the material was centrifuged at 500 g for 10 min and the supernatant was absorbed twice more with fresh promastigotes.

Indirect immunofluorescence for the detection of *Leishmania* antigen, in the skin and lymph nodes, was performed according to the method of Bray modified to detect *Leishmania* antigen in frozen sections [16]. Briefly, frozen sections were exposed to anti-*Leishmania* serum obtained from BALB/c mice.
Table 1. Number of lysozyme and S100 protein-positive cells in skin lesions of cutaneous leishmaniasis patients classified according to histological type

<table>
<thead>
<tr>
<th>Histological classification of the lesion</th>
<th>Dermis</th>
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<td></td>
<td>Lysozyme</td>
<td>S100</td>
<td>Lysozyme</td>
<td>S100</td>
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<tr>
<td>B (n = 15)</td>
<td>52*</td>
<td>17</td>
<td>Not found</td>
<td>24</td>
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<td></td>
<td></td>
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<td></td>
<td>(4–260)</td>
<td>(1–75)</td>
<td>(0–37)</td>
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<td></td>
</tr>
<tr>
<td>C (n = 7)</td>
<td>53</td>
<td>18</td>
<td>Not found</td>
<td>13</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>(15–70)</td>
<td>(0–42)</td>
<td>(2–38)</td>
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<tr>
<td>Unclassified (n = 8)</td>
<td>25</td>
<td>12</td>
<td>Not found</td>
<td>5</td>
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<td></td>
<td>(1–74)</td>
<td>(0–36)</td>
<td>(0–16)</td>
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* Total number of positive cells in the section (mean and range).

as described above. After 30 min the slides were washed in three changes of Tris buffer of 5 min each, followed by the application of fluorescence-labelled goat anti-mouse serum (Institute Pasteur, Paris, France). After thorough washing the sections were mounted in glycerine-buffered saline and examined in a Leitz epifluorescence microscope. Control sections included omission of anti-Leishmania serum and its replacement with normal BALB/c serum.

Electron microscopy
Electron microscopy was performed on tissues from five Sudanese patients as described in detail previously [17]. Briefly, fresh material was fixed in 4% glutaraldehyde, at 4°C for 1·5 h, washed in phosphate buffer pH 7-2, post fixed in 1% osmium tetroxide and embedded in epon resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss-10 transmission electron microscope.

RESULTS
Histological classification of cutaneous lesions and the pathology of lymph nodes
Out of the 30 biopsies from donors in Saudi Arabia 15 were of type B response, in which there is necrosis of individual or small clusters of parasitized macrophages. Type C, which is

Fig. 1. (a) Langerhans cells in the skin stained for CD1a by immunoperoxidase. Most of the positive cells are in the epidermis, but some are also seen in the dermal infiltrate. (Immunoperoxidase × 400.) (b) CD1a-positive Langerhans cells in the marginal sinus, interfollicular zone, and paracortex of a lymph node. (Immunoperoxidase × 400.)
characterized by focalized necrosis involving large numbers of parasitized macrophages, was found in seven biopsies. Eight lesions could not be fitted in the classification [10]. The pathology in this unclassified group showed mononuclear cells consisting of lymphocytes, plasma cells and macrophages. Irrespective of the histological type the venules had hypertrophied endothelium.

The lymph nodes showed follicular hyperplasia with prominent germinal centres, the paracortex was expanded and the medulla contained many plasma cells.

Epithelioid granulomas were seen in five lymph nodes. In four of the nodes the granulomas showed central necrosis. These were negative for mycobacteria. Leishmania parasites were seen in four of the nodes and antigen was demonstrated by immunoperoxidase or immunofluorescence in all.

**Distribution of APC**

Lysozyme-positive cells were found in the infiltrate of all histological types with a wide range of variation (Table 1). They were most frequently seen in type B and C responses.

S-100 protein-positive cells were found in variable numbers within the infiltrate and the overlying epidermis irrespective of the histological type. In individual cases there was an inverse relation between the number of S-100-positive cells in the epidermis and that in the dermis. In one lesion suprabasal S-100-positive cells were dividing. An occasional positive cell was crossing the epidermo–dermal junction. The cells measured 10–17 μm and had large vesicular nuclei. In parallel sections the S-100-positive cells were always negative for lysozyme. The cells were scattered amongst the inflammatory cells in the dermis, and sometimes formed a rim around epithelioid granulomas.

Both lysozyme and S-100 protein-positive cells were found in the lymph nodes. The numbers varied considerably not only from one node to another but also with marked variation in different parts of the same node. Lysozyme-positive cells were found in the marginal sinus, the paracortex and occasionally in the follicles. S-100 protein-positive cells were seen in the afferent lymphatics, the marginal sinus and paracortex.

In order to substantiate further that cells with similar morphology and location as the S-100-positive cells were indeed Langerhans cells, tissues were stained by CD1a, which is a marker for Langerhans cells in the skin [18]. Anti-CD1a-positive cells were identified in the epidermis and in the dermal infiltrate of all the skin biopsies stained by anti-CD1a (Fig. 1a). Some were crossing the epidermo–dermal function. In the lymph nodes the anti-CD1a-positive cells were found in the afferent lymphatics, the marginal sinus and the paracortex (Fig. 1b).

The presence of Langerhans cells in the epidermis and the dermal infiltrate was confirmed by electron microscopy by the demonstration of cells with Birbeck granules (data not shown). Parasitized and unparasitized macrophages, lymphocytes, and epithelioid cells were also identified in the infiltrate. Macrophages were in intimate contact with lymphocytes. Most of the cells in the dermis expressed MHC class II as 70–90% of the cells including some dendritic cells were HLA-DR-positive. The keratinocytes in the epidermis and hair follicles were also positive (Fig. 2).

In the lymph nodes the high endothelial venules, the interdigitating reticulum cells, and the follicular dendritic cells were HLA-DR-positive.

**Leishmania antigen in skin lesions and lymph nodes**

In immunoperoxidase-stained sections parasites stained a brown colour and both nucleus and kinetoplast were identifiable. Antigen was identified in the cytoplasm and cell membrane of macrophages and in dendritic cells corresponding to the S-100 antigen-positive cells in the epidermis, dermis (Fig 3a, b), afferent lymphatics and paracortex. Follicular dendritic cells were also positive for *Leishmania* antigens. Immunofluorescence gave similar results to the immunoperoxidase. Control sections for both methods gave negative results.

**ICAM-1 and LFA-1 expression on cells in the cutaneous lesion**

In the seven lesions examined the vascular endothelium gave a strong reaction for ICAM-1 (Fig. 4c). Keratinocytes in and near the basal layer were also positive (Fig 4b), and an occasional keratinocyte more superficially was weakly positive. In one biopsy a particularly strong ICAM-1 expression on keratinocytes was associated with an infiltrate of LFA-1-positive mononuclear cells in the rete pegs. In another biopsy, there was a clear zone of dermis devoid of inflammatory cells.

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*Fig. 2. HLA-DR-positive keratinocytes in the epidermis. (Immunoperoxidase × 1000.)*
between the epidermis and an underlying inflammatory infiltrate. The keratinocytes overlaying this clear zone did not react with the ICAM-1 antibodies (Fig. 5a).

In the infiltrate about 50% of the leucocytes were LFA-1-positive. Some LFA-1-positive cells were infiltrating the lower epidermis (Fig. 5b). Scattered LFA-1-positive leucocytes were seen in the epidermis.

**T cell phenotype in skin lesions and lymph nodes**
In skin biopsies from the 11 patients examined the CD3+ T cells formed between 18% and 79% (mean 50%) of the cells in the dermal infiltrate. The mean percentage of CD4+ cells and CD8+ cells was 56-5% (range 30-90%) and 43-5% (range 9-68%) of total T cells, respectively. Small numbers of both types of cells were seen in the epidermis, particularly in the basal layer.

In the lymph nodes most cells in the paracortex were CD3+ T cells with a preponderance of CD4+ cells (70-80%) over CD8+ cells. The germinal centres contained small clusters of CD4+ cells and no CD8+ cells.

**DISCUSSION**

The role of macrophages in antigen presentation is well documented. We have found amastigotes and/or *Leishmania* antigen in lysozyme-positive macrophages in the cutaneous lesions and lymph nodes in the present and a previous study [14]. These cells also expressed MHC class II molecules and
Fig. 4. (a) Intercellular adhesion molecule-1 (ICAM-1)-positive endothelial cells in the dermis (arrows). Some of the surrounding inflammatory cells are also ICAM-1-positive. (Immunoperoxidase × 400.) (b) ICAM-1-positive keratinocytes in and near the basal layer of the epidermis. The more superficial are negative for ICAM-1. (Immunoperoxidase staining × 400.)

were in intimate contact with lymphocytes, when viewed under the electron microscope. Appropriately activated macrophages also function as effector cells in the elimination of intracellular parasites [19]. The other important APC in cutaneous leishmaniasis is the epidermal Langerhans cell [20]. This cell is a bone marrow-derived, dendritic cell that shares some features with the macrophage, such as surface expression of ATPase, non-specific esterase, receptors for complement and
Fig. 5. (a) Skin lesion stained for intercellular adhesion molecule-1 (ICAM-1). The epidermis is separated from the underlying inflammatory infiltrate by a zone devoid of inflammatory cells. The overlying keratinocytes are negative for ICAM-1. The vascular endothelium in the dermis shows a strong expression of ICAM-1. (Immunoperoxidase × 400.) (b) LFA-1-positive cells in the rete pegs of the epidermis. The infiltration is particularly heavy in the rete peg in the middle of the illustration. Cells in the upper dermis are LFA-1-positive. (Immunoperoxidase × 400.)
from the migration of Langerhans cells and macrophages. The finding that parasites had migrated to the dermis was supported by the presence of an inflammatory infiltrate and the fact that parasites were able to activate the immune system. Therefore, it is conceivable that keratinocytes bearing Leishmania antigen and expressing MHC class II molecules may present antigen to primed T cells in the epidermis, thus contributing to the amplification of the immune response in the lesion. Similarly, endothelial cells that were reported here to express both MHC class II and Leishmania antigen may also be acting as accessory APC. However, it is likely that the antigen-presenting capability of keratinocytes and endothelial cells is overshadowed by that of Langerhans cells and macrophages. Follicular dendritic cells were found to contain Leishmania antigens and express MHC class II molecules, and may participate in the generation of B memory cells in cutaneous leishmaniasis.

We found that T cells form a substantial component of the inflammatory infiltrate of cutaneous leishmaniasis lesions. Most of the cells in the epidermis and at least half of those in the dermal infiltrate were CD45RO-positive T (memory) cells expressing the LFA-1 adhesion molecule. We have previously shown that Leishmania-specific T cells expand as a result of the infection and circulate in the peripheral blood of patients recovered from cutaneous leishmaniasis [4]. It is plausible that such cells home to the inflammatory site during the infection. In support of this notion, we found that the inflammatory infiltrates showed hypertrophied endothelial cells with a high ICAM-1 expression, thus resembling the high endothelium post-capillary venules in the paracortex of lymph nodes. Normally, capillary and venular endothelium expresses low levels of ICAM-1, but this is up-regulated by various cytokines such as IFN-γ and IL-1 [29]. ICAM-1, which is the ligand for LFA-1, is thought to play an important role for the extravasation of the LFA-1-expressing leucocytes. These two adhesion molecules among others are also essential for the intimate contact between the various cells of the immune response and their activation [30].

In conclusion, the findings presented in this study of patients suffering from cutaneous leishmaniasis are in agreement with previous experiments performed in animal models of L. major infection. It seems that Langerhans cells residing in the epidermis migrate to the dermis, where they take up antigen and travel to the regional lymph nodes, where they present Leishmania antigen to T cells. At the site of infection the endothelial cells of the venules are activated to express ICAM-1. Circulating T cells primed in the lymph node and other leucocytes expressing LFA-1 home to the infection site through venules expressing ICAM-1, and participate in the inflammatory reaction.

Other cells possibly involved in antigen presentation include macrophages, follicular dendritic cells, interdigitating reticulum cells of the paracortex of lymph nodes, and possibly endothelial cells and keratinocytes. The role of the latter may be confined to the inflammatory response in the epidermis.

ACKNOWLEDGMENTS

The work was supported in part by grant 104.Dan.8.I/401 from the Danish International Development Agency (DANIDA). A.M. E.-H. was supported a grant from the King Abd El Aziz City for Science and Technology, Riyadh, Saudi Arabia. The help of Lis Christiansen, Dr Henning Laursen, Dr Henrik Permin and Dr Arsalan Kharazmi, National University Hospital (Rigshospitalet), Denmark, Mr Sayed Osman and Miss Wafa Salih, EM Unit, Faculty of Science, University of Khartoum, Sudan, and Nahla ElHassan is greatly appreciated.
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