Antigen-presenting cells in human cutaneous leishmaniasis due to *Leishmania major*

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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

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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 ($5 \mu m$) were cut with a cryostat and air dried overnight, before the immuno-staining. The other half of the tissue was fixed in mercuric chloride–acetic acid mixture for $1.5 h$ and was then transferred to 70% ethyl alcohol. Paraffin sections ($5 \mu m$) 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% H_2O_2/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 anti-bodies were applied for 15 min at 20°C. The sections were rewashed with PBS and exposed to biotinylated, goat anti-mouse 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 distrene 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 diamenobenzidine (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 $0.05 M$, 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 10^7 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 anti-mouse 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 10^{10} 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

Histological classification of the lesion	Dermis		Epidermis	
	Lysozyme	S100	Lysozyme	S100
B	52*	17	Not found	24
(n = 15)	(4–260)	(1–75)		(0–37)
C	53	18	Not found	13
(n = 7)	(15–70)	(0–42)		(2–38)
Unclassified	25	12	Not found	5
(n = 8)	(1–74)	(0–36)		(0–16)

* 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
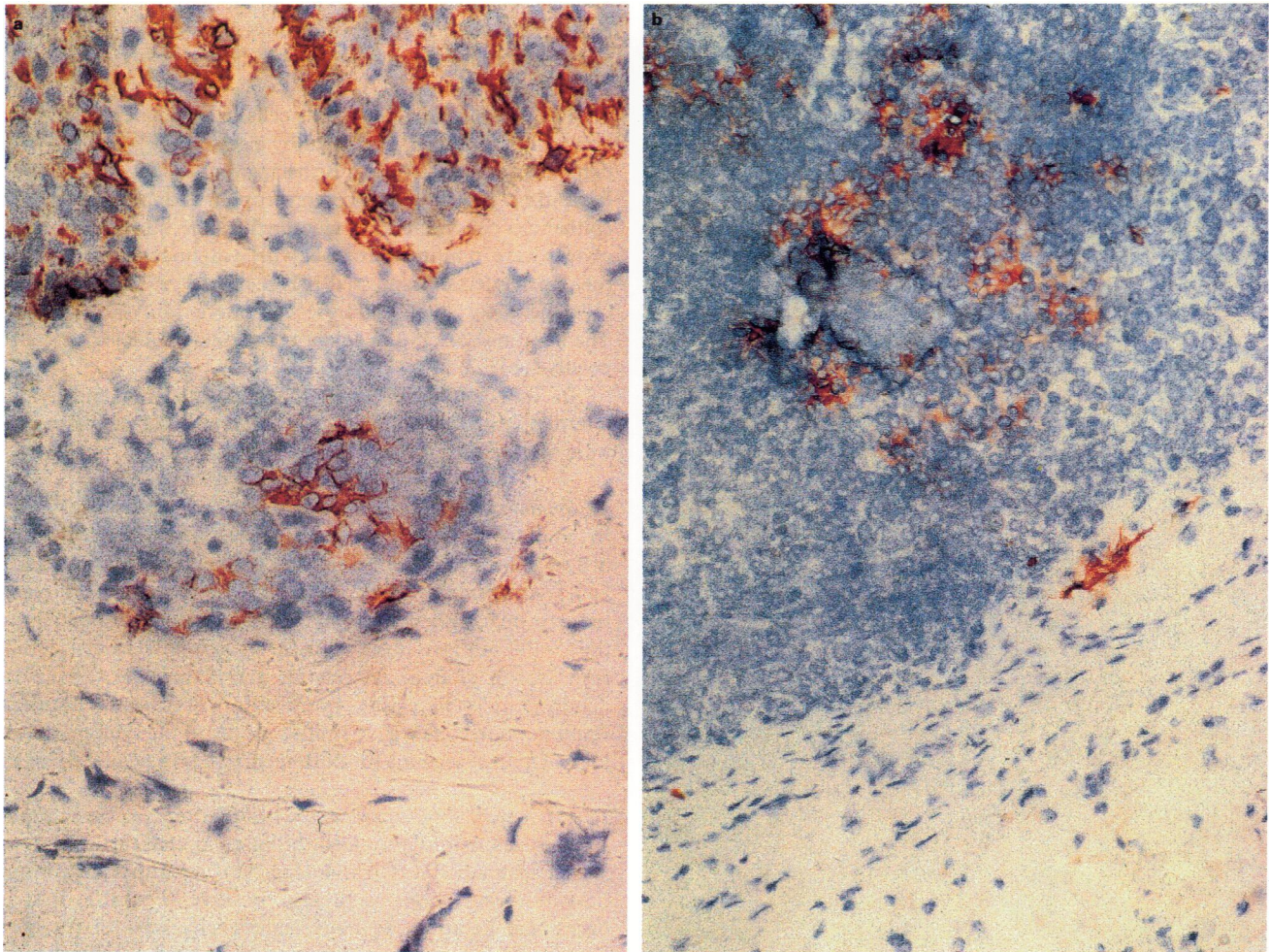

Fig. 1. (a) Langerhans cells in the skin stained for CD1a by immunoperoxidase. Most of the positive cells are in the epidermis, but some are also seen in the dermal infiltrate. (Immunoperoxidase × 400.) (b) CD1a-positive Langerhans cells in the marginal sinus, interfollicular zone, and paracortex of 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

Fig. 2. HLA-DR-positive keratinocytes in the epidermis. (Immunoperoxidase × 1000.)

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 cell, 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 inflammatoty cells
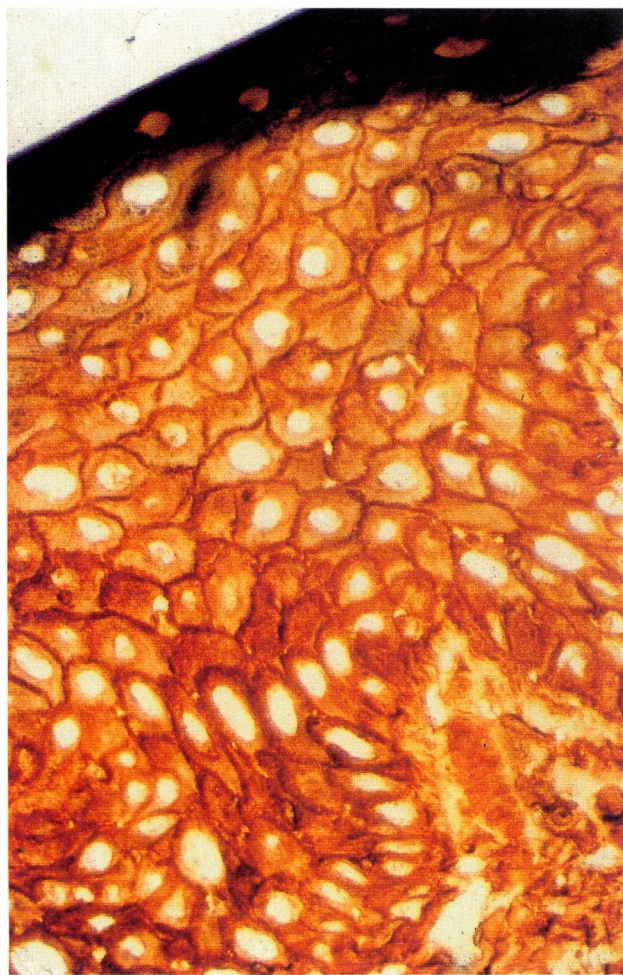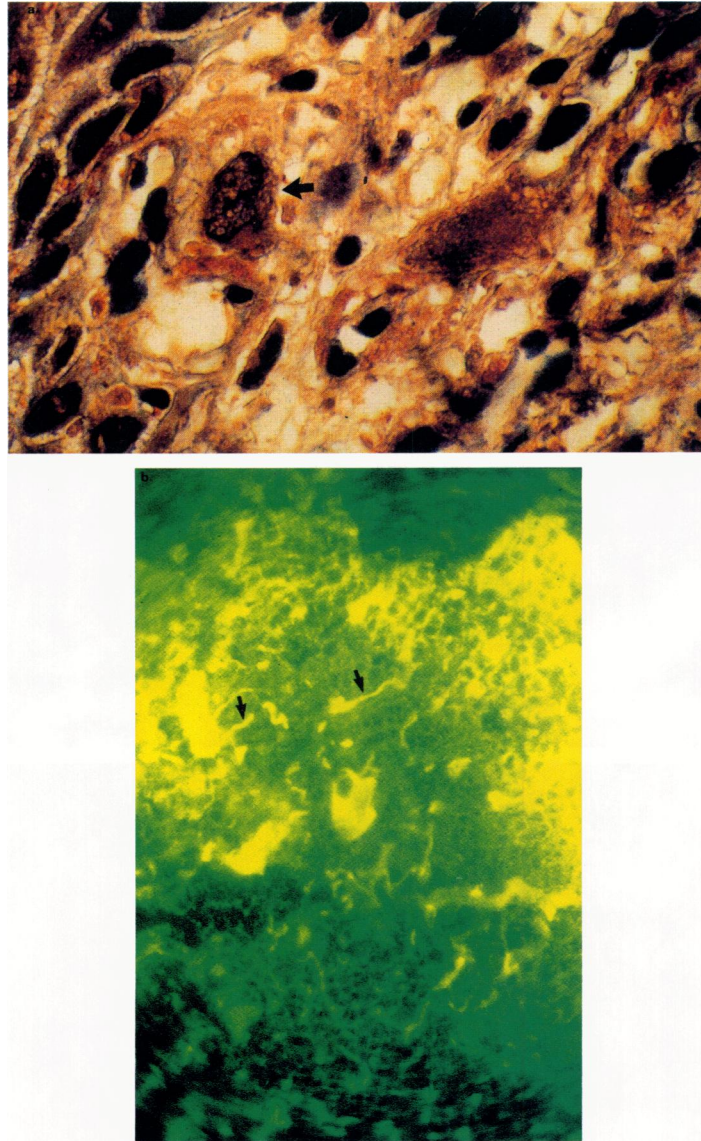
Fig. 3. (a) *Leishmania* antigen in dendritic cells in the upper dermis. The dendritic cells can be identified by their ramifying dendrites and the lobulated nucleus (arrow) in one cell. In serial sections the same cells were S-100 protein-positive. There are no identifiable parasites in the figure, only *Leishmania* antigen. The keratinocytes are also *Leishmania* antigen-positive. (Immunoperoxidase staining × 1000.) (b) *Leishmania* antigen in dendritic cells in the paracortex of a lymph node. Some of the dendrites are indicated by arrows (Immunoflouresence × 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
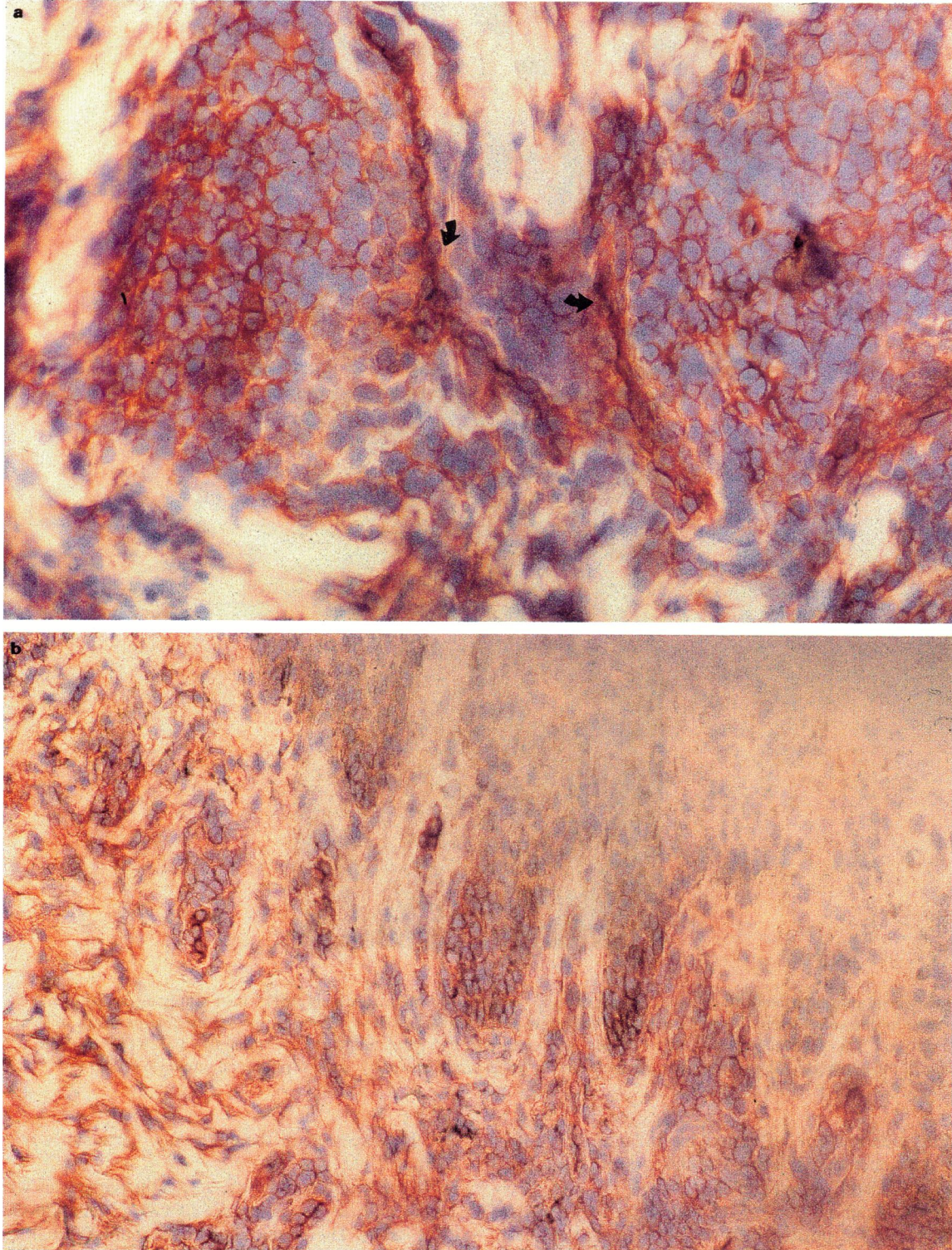
Fig. 4. (a) Intercellular adhesion molecule-1 (ICAM-1)-positive endothelial cells in the dermis (arrows). Some of the surrounding inflammatory cells are also ICAM-1-positive. (Immunoperoxidase × 400.) (b) ICAM-1-positive keratinocytes in and near the basal layer of the epidermis. The more superficial 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
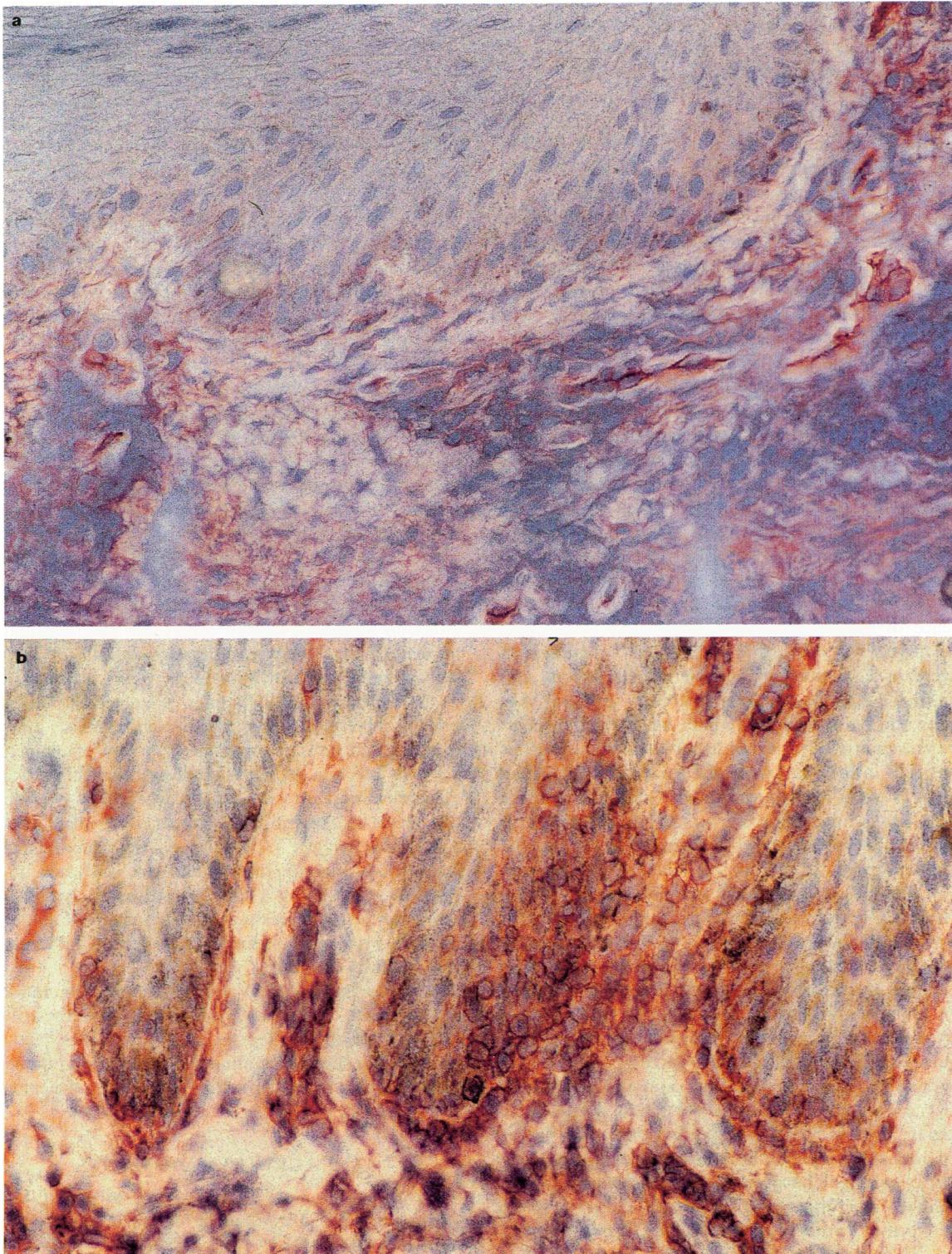
Fig. 5. (a) Skin lesion stained for intercellular adhesion molecule-1 (ICAM-1). The epidermis is separated from the underlying inflammatory infiltrate by a zone devoid of inflammatory cells. The overlying keratinocytes are negative for ICAM-1. The vascular endothelium in the dermis shows a strong expression 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.)

the Fc fragment of immunoglobulin [21]. Unlike macrophages it is lysozyme-negative, and expresses S-100 [14] and CD1a [21]. Ultrastructurally it contains specific Birbeck granules and constitutively expresses MHC class II molecules [21]. Langerhans cells are poorly phagocytic but are potent stimulators of T cells in an antigen-specific and MHC-restricted manner [22]. They are recognized as the prominent APC of the skin [21]. It is believed that the cells pick up antigens from the epidermis and migrate from the epidermis to the regional lymph nodes draining the skin [21]. In the paracortex the Langerhans cells transform into interdigitating cells and present antigen to naive T cells [21]. In this study we have shown that Langerhans cells identified by the various markers are found in the infiltrate of the skin lesions of cutaneous leishmaniasis and in the draining lymph nodes. In the material from Saudi Arabia, Langerhans cells were demonstrated in the epidermis by the presence of S100-expressing cells. Since the S100-specific antibody also stains melanocytes, the S100 protein-positive cells in the basal layer may be Langerhans cells or melanocytes, whereas the positive cells in the suprabasal position of the epidermis are Langerhans cells. Using the specific CD1a marker on the material from the Sudan, Langerhans cells were demonstrated in a suprabasal position in the epidermis, in the basal layer, and were found crossing the epidermo–dermal junction. A similar pattern was observed when S100 protein was used as a marker for the cells. In all the sites including the dermal infiltrate, the Langerhans cells contained Leishmania antigen and occasionally single parasites. The cells were traced to the regional lymph nodes, where they also contained Leishmania antigen. In the paracortex the cells were in intimate contact with the T cells. The presence of Leishmania antigen-containing Langerhans cells in the afferent lymphatics and the marginal sinus suggests that Langerhans cells take up antigen in skin lesions and migrate to the lymph nodes. This migratory pathway was recently shown to occur in BALB/c mice infected by L. major [23]. There was an inverse relationship between the numbers of Langerhans cells in the dermis and the epidermis, supporting the finding of Blank et al. [24], who showed that in the murine model of L. major infection there was considerable loss of Langerhans cells in the segment of the epidermis overlying the parasite-containing infiltrate. This was taken to indicate that Langerhans cells migrate from the epidermis and pick up parasites from the dermis. The signals for the translocation of the Langerhans cells from the epidermis to the dermis may be mediated by IL-1β or tumour necrosis factor-alpha [20].

The keratinocytes expressed HLA-DR and also contained Leishmania antigen, and thus may act as APC. Keratinocytes can phagocytose and digest extracellular debris, erythrocytes, Candida albicans [25] and killed whole Mycobacterium leprae [26]. They also produce cytokines that are similar to those released by various lymphoid cells [27]. We did not find whole amastigotes in keratinocytes. This may have been due to the fact that parasites had already been digested, or alternatively the keratinocytes might have picked up Leishmania antigen from parasitized macrophages in the dermal infiltrate or the epidermis. Normal keratinocytes are HLA-DR-negative, but are induced to express this molecule by IFN-γ [28]. It was recently shown that HLA class II-positive human keratinocytes are able to present Myco. leprae antigens to Mycobacterium-specific T cells [26]. Although there is no proof as yet that keratinocytes present Leishmania antigen in vivo, 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.

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