IMMUNOHISTOCHEMICAL ANALYSIS OF THE MUCOSAL LESION IN MUCOCUTANEOUS LEISHMANIASIS

ESTERRE P.*, GUERRET S.**, RAVISSE P.***, DIMIER-DAVID L.****, DEDET J.P.**** and GRIMAUD J. A.**

Summary :

Using antibodies against surface antigens of inflammatory cells and against extra-cellular matrix components, immunocytochemical studies permit the investigation of the *in situ* cellular immune response and the associated fibrosis, in mucosal lesions. 57 paraffin embedded skin biopsies of mucocutaneous leishmaniasis originating from Bolivia, and due to *Leishmania (Viannia) braziliensis*, were studied. Memory T cells predominate within a non-organized granulomatous reaction, in which were observed proliferating macrophages. At the top of the lesion, epithelial cells expressed HLA-DR. Some characteristics of this lesion, particularly its chronic destructive pattern, presently lack any clear explanation.

KEY WORDS : immunology. immunopathology. pathology. mucocutaneous leishmaniasis. *Leishmania braziliensis*.

Résumé : ANALYSE IMMUNOHISTOCHIMIQUE DE LA LÉSION MUQUEUSE AU COURS DE LA LEISHMANIOSE CUTANÉO-MUQUEUSE

Cinquante sept biopsies de lésions muqueuses de leishmaniose cutanéomuqueuse à *Leishmania (Viannia) braziliensis*, originaires de Bolivie, ont été incluses en paraffine. Ces biopsies ont fait l'objet d'une analyse immunohistochimique, les objectifs étant de caractériser l'infiltrat cellulaire et le tissu conjonctif. Dans l'infiltrat inflammatoire muqueux, on a observé la présence de lymphocytes T "mémoire" distribués au sein d'un granulôme riche en macrophages qui prolifèrent. En superficie de la lésion, les cellules épithéliales sont positives pour HLA-DR. Certaines particularités de cette lésion, plus particulièrement la destruction tissulaire, ne sont pas clairement explicables à l'heure actuelle.

MOTS CLES : immunologie. immunopathologie. anatomo-pathologie. leishmaniose cutanéomuqueuse. *Leishmania braziliensis*.

INTRODUCTION

The estimated number of leishmaniasis worldwide is now considered as surpassing 12 millions of people (Wijeyaratne, 1992). In South America, the localized cutaneous (LCL form) and the mucocutaneous leishmaniasis (MCL or "espundia") represent a severe public health problem. The MCL lesions, metastasizing from primary healed cutaneous lesions, are particularly destructive for the oral and/or nasopharyngeal cavities. Furthermore, the MCL lesions are often refractory to antimonial therapies.

During the last decade, the evolution of LCL (Ridley, 1979; Ridley, 1983; Magalhaes, 1986; Sotto, 1989; Gutierrez, 1991) and MCL (Ridley, 1980; Marsden, 1986; Ridley, 1989; Huaman, 1990) lesions has been particularly studied. The *in situ* analysis of cell populations and lymphokine production has been well

documented in LCL (Castes, 1983; Carvalho, 1985; Magalhaes, 1986; Modlin, 1989; Pirmez, 1990; Esterre, 1992), but less in MCL. In this last pathology, the immune response has been characterized in the peripheral blood (Carvalho, 1985) and at the T lymphocyte level (Pirmez, 1990). The tissular and peripheral T cell populations are significantly different (Modlin, 1985; Pirmez, 1990). But the histopathological approach revealed other cellular events, such as *in situ* macrophage differentiation, plasma cell infiltration, extra-cellular matrix remodeling and specific vascular changes (Sotto, 1989; W.H.O., 1990). This study examines *in situ* the macrophage phenotype and its topography within the lesions, and the associated tissue remodeling, in order to obtain new findings on the pathogenesis of this mucosal lesion. We analysed in more detail, by immunohistochemistry, the well-documented collection of material previously studied with an histopathological approach (Dimier-David, 1994).

MATERIAL AND METHODS

Fifty-seven biopsies of MCL lesions were taken from Bolivian patients, with leishmaniasis confirmed by the laboratories of the IBBA, La Paz. The disease was related to *Leishmania (Viannia) braziliensis*, by isoenzyme electrophoresis of parasite culture isolates. The histological analysis

* Immunology Unit, Institut Pasteur de Madagascar, BP1274, Antananarivo, Madagascar.

** Molecular and Cellular Pathology Unit for Tissue Fibrosis, CNRS URA 1459, Institut Pasteur de Lyon, F-69365 Lyon Cédex 07, France.

*** Histopathology Unit, Institut Pasteur, F-75235 Paris cédex 15, France.

**** Parasitology Lab., Instituto Boliviano de Biología de Altura, La Paz, Bolivia.

Corresponding author : Dr. J.P. Dedet, Laboratoire d'Écologie Médicale et de Pathologie Parasitaire, Faculté de Médecine, 163 rue A. Broussonet, F-34059 Montpellier cédex, France. Tél. 67 33 69 60 – 67 63 27 51 – Fax 67 63 00 49.

Antibody	Cluster*	Isotype	Specificity	Source**
PD7/LB11	CD45	IgG1	Leukocyte Common Ag	Dako
L26	CD20	IgG2a	B Lymphocytes	Dako
A452	CD3	(Rabbit)	mature T lymphocytes	Dako
DFT1	CD43	IgG1	T lymphocytes, some myeloid cells	Dako
TAL1B5	/	IgG1	HLA-DR	Dako
OPD4	CD4	IgG1	helper T subset	Dako
Mac387	/	IgG1	Histiocytes	Dako
KP1	CD68	IgG1	Monocyte/macrophages	Dako
PGM1	CD68	IgG3	Monocyte/macrophages	Dako
NP57	/	IgG1	Neutrophil elastase	Dako
JC/70A	CD31	IgG1	Endothelial cells, platelets	Dako
BNH9	/	IgG1	Endothelial cells	Immunotech
F8/86	/	IgG1	Endothelial cells, Megakaryocytes	Dako
S100	/	(Rabbit)	Dendritic cells	Dako
PCNA	/	IgG2a	Proliferating cells	Dako

Table I. – Characteristics and specificity of the antibodies used in this study

* Clusters of differentiation (CD) established at the 4th Workshop on Human Leukocytes Antigens, Vienna, Austria, 1989.

** Dako, Dako France SA, Trappes, France.

Immunotech, Marseille-Luminy, France.

	Normal epithelium	Perilesional fibrotic matrix	Inflammatory granuloma
Collagen isotype I	++	++	+
Isotype III	++	++	++
Isotype IV	++	++	+
Fibronectin	+	++	+
Laminin	++	++	+
Elastin	++	+	-

Table II. – Extra-cellular matrix proteins expressed in the lesion of mucocutaneous leishmaniasis (indirect immunofluorescence technique)

of each biopsy, fixed in formaldehyde solution, was performed with haematoxylin and eosin (H&E) staining in a reference laboratory (Histopathology unit, Institut Pasteur). Immunohistochemistry was made on 5 µm section with the labelled streptavidin-biotin method (LSAB kits, Dako France, Trappes) and a semi-automatized staining station (Sequenza workstation, Shandon Scientific, Runcorn, UK). A panel of 14 antibodies, described in table I with their specificity and origin, was used in this study.

Morphometric data on fibrosis extent were obtained on Picrosirius red stained sections, using an automatic image analysis system (Biocom 200, Biocom, France). The ratio fibrous area/section area was automatically calculated in each section, and the results are expressed as the mean value of 10 sections. The different extra-cellular matrix components were identified by indirect immunofluorescence, using the following chromatography-purified antibodies developed in our

laboratory (Esterre, 1991) : they bind to collagen isotypes I, pro-III, III and IV, elastin, laminin and fibronectin.

Two biopsies of individuals undergoing surgical excision of a nasal polyp were used as control biopsies. Their histological examination reveals weak inflammation without any epithelium alteration.

RESULTS

T lymphocytes, most of them expressing CD4, greatly predominate over B lymphocytes in the lesion. As expected (Pirmez, 1990), an increase of memory (CD45RO+) T cells has been noticed with the UCHL1 marker (Dako lab.), but this preliminary result on paraffin material must be confirmed on a larger sample of frozen biopsies. Resident macrophages (Mac 387+, HLA DR-), described as histiocytes

on conventional pathological examination, were observed admixed with activated macrophages (Mac 387-, HLA DR+, fig. 4), lymphocytes and fibroblasts. No special topography suggesting an epithelioid pattern could be observed in our sample (fig. 2), but some of the macrophages were immunostained by the PCNA marker (fig. 3). The macrophages were rarely infected, but we observed some of them with many intracellular amastigotes (fig. 1). No extracellular amastigotes could be identified. The immunostaining with the anti-HLA DR antibody enabled the detection of positive epithelial cells at the top of the inflammatory reaction (fig. 4).

The morphological data obtained with the endothelial cell markers (fig. 5) were quantified by image analysis, in which the vessels morphometric size variables were statistically different between mucosal and skin lesions. Furthermore we noticed a frequent periarteritic fibrosis in some MCL lesions. In the inflammatory areas, the mean values of fibrosis extent in the mucosal lesion of MCL (13.2 ± 4.6 p. cent of the area) are in line with that calculated in the cutaneous lesion of MCL (20.1 ± 11.3 %) and LCL (19.1 ± 10.1 %). In the fibrotic periinflammatory areas, this proportion is increased in the mucosal lesion (49.3 ± 14.6 %) as compared with the cutaneous lesions of MCL and LCL (26.1 ± 11.1 % and 27.3 ± 5.1 %, respectively).

By immunofluorescence (table II), the extra-cellular matrix components were found to include collagen type I and III, fibronectin and laminin. No elastin was detected in this sample, as with the LCL lesion (Esterre, 1991).

DISCUSSION

The local accumulation of effector molecules and immunocompetent cells (Allardyce, 1984; Gutierrez, 1991; Brandtzaeg, 1992; Kuper, 1992; McGhee, 1992) is important in the resistance to mucosal infections. But we have, actually, only speculative explanations for the chronic destructive features occurring in the MCL lesion. *In vitro* studies of the immunologic responsiveness in MCL patients suggest a status of hyperactive immune response (Castes, 1983; Pirmez, 1990). A preliminary *in vivo* analysis revealed a predominant $\gamma\delta$ T memory (CD4+ CD45RO+) delayed type hypersensitivity reaction (Pirmez, 1990), with no increase of $\gamma\delta$ T lymphocytes by opposition with the results obtained on LCL lesions (Modlin, 1989; Esterre, 1992). In our opinion, T cells have a potential role in the early LCL lesions (Esterre, 1991), where a non-specific inflammatory reaction occurs, but not in more chronic stage such as old LCL and MCL lesions (Pirmez, 1990). As far as this hypothesis is true, the

MCL lesion can be considered as a "delayed" (Ridley, 1989) or "disorganized" (Pirmez, 1990) granulomatous reaction. Although tissue macrophages are one of the most important cell population in the human airways (Juliussen, 1991), their potential role has not been studied in detail in the MCL lesion. In the present study the immunophenotyping and the topography of macrophages was analysed in the mucosal lesion. No epithelioid organisation could be clearly demonstrated, supporting the previous hypothesis, as the presence of a non negligible *in situ* proliferation (probably revealing recently recruited monocytes). The nature of chemokines/adhesive molecules leading to the recruitment of T lymphocytes within MCL lesions remains poorly defined. S100 + dendritic cells, alone or in addition to tissue macrophages, might play such a role. The topography of vessels within MCL lesion seems to indicate that a strong neo-angiogenesis occurs in MCL lesion by comparison with the LCL lesion. Future characterization of site-specific epitopes expressed on inflammatory lymphocytes (such as HECA-452 and MLA for skin-homing and mucosal-homing lymphocytes, respectively) and their corresponding vascular addressins might be highly informative. The periarteritic fibrosis process which develops in MCL lesion remains unexplained.

In contrast to the observations made in New World LCL (Huaman, 1990; Esterre, 1991) and MCL (Ridley, 1980; Ridley, 1983; Ridley, 1989), the involvement of a connective tissue reaction is considered to be insignificant in Old World LCL (Ridley, 1979). The fibrotic process has been linked to a good prognosis (Ridley, 1980; Ridley, 1989; Huaman, 1990; Gutierrez, 1991), in contrast to the macrophage lysis and the associated tissue necrosis (Ridley, 1989). We must remember that the alterations observed are identical in the different mucosae concerned by this disease (Dimier-David, 1994). More investigations on the *in situ* cytokine network and the nature of enzymes are needed before understanding the evolution of the MCL lesion as well as the nature of factors predisposing to mucosal metastasis of Leishmania in the early phase of MCL development (Gutierrez, 1991).

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 1. – *Leishmania* amastigotes (arrowheads) are sometimes in intracellular localization within macrophages, here immunostained with a CD 68 marker. LSAB (x 1000).

Fig. 2. – A dense inflammatory infiltrate mainly composed of lymphocytes (not stained) and macrophages (KP1 +), in a sub-epithelial (E : epithelium) zone. LSAB (x 250).

Fig. 3. – In the same zone as in Fig. 2, numerous proliferating macrophages (PCNA +) can be observed. E : epithelium. LSAB (x 250).

Fig. 4. – In addition to activated lymphocytes and macrophages (in the inflammatory infiltrate on the right), a positive HLA-DR immunostaining can be observed on epithelial cells (on the left of the section). LSAB (x 250).

Fig. 5. – The inflammatory foci are highly vascularized, as indicated on this section with the BNH9 marker. E : epithelium. LSAB (x 250).

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