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IMMUNOPATHOLOGY OF AMERICAN CUTANEOUS LEISHMANIASIS. MODULATION OF MHC CLASS II GENE PRODUCTS BY KERATINOCYTES BEFORE AND AFTER GLUCANTIME THERAPY

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Epidermal changes from 32 cutaneous and 3 mucosal American cutaneous leishmaniasis (ACL) active lesions were studied for HLA-DR, -DQ and -DP expression, Langerhans cells and lymphocyte infiltration. In addition to a DR and DQ positivity at the surface of the cells of the inflammatory infiltrate, a strong reaction for DR antigens was detected on keratinocytes. Hyperplasia of Langerhans cells was present in all cutaneous lesions and epidermis was infiltrated by T lymphocytes. When healed lesions of 14 of these subjects were re-biopsied 1 to 12 months after the end of pentavalent antimonial therapy, MHC class II antigens could no longer be seen on keratinocytes.

Our data represent evidence for the reversibility of the abnormal HLA-DR expression by keratinocytes in ACL after Glucantime therapy or spontaneous scar formation, demonstrating that this expression is restricted to the period of active lesions. The present findings can be regarded as an indirect evidence that keratinocytes may be involved in the immunopathology of ACL.

Key words: leishmaniasis – keratinocytes – HLA-DR – glucantime therapy

American cutaneous leishmaniasis (ACL) is a granulomatous disease clinically characterized by ulcerated lesions which can regress either spontaneously or after treatment, and the regression is associated with the development of a specific cell-mediated immune response (CMI) (Castes et al., 1983). Epidermal alterations are varied and noteworthy in ACL lesions, and can include hyperplasia with proliferation of interpapillary cones, or acanthosis, dysplasia and formation of horny pearls similar to those observed in carcinoma (Kerdel-Vegas & Essenfeld-Yahr, 1966). There is a general agreement that the extension of involvement of connective tissue is associated with infiltration and severe hyperplasia of the epidermis (Magalhães et al., 1986; Ridley et al., 1980).

The epidermis is currently considered as an immunomodulating tissue as indicated by the number and function of the factors produced by keratinocytes such as epidermal cell-derived thymocyte activating factor substance (Luger et al., 1981), epidermal cell-derived natural killer cell activating factor (Luger et al., 1985a), interleukin 3-like (Luger et al., 1985b), epidermal cell-derived lymphocyte differentiating factor (Nicolas et al., 1987) and the expression of Ia by epidermal keratinocytes (Lampert et al., 1981; Volc-Platzer et al., 1984), presentation of antigen by Langerhans cells (Stingl et al., 1980) and secretion of IL-1 by both cell lines (Sauder et al., 1984).

Concerning class II antigens, it has been suggested that Ia expression on keratinocytes represents an utterance of a CMI response (Suitters & Lampert, 1982). In a recent immunohistochemical study of ACL lesions it was briefly reported an abnormal HLA-DR expression by keratinocytes that, as suggested for other skin diseases, might be related to the *in situ* immune response (Modlin et al., 1985). In order to investigate whether this keratinocyte DR expression was restricted to the active disease period, we studied skin lesions from ACL patients before treatment as well as scar fragments obtained after pentavalent antimonium therapy.

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In this communication we show evidence that during the course of active ACL, HLA-DR expression by keratinocytes is restricted to the skin lesions, and that such abnormal expression is reversed after treatment. In addition, we observed that this phenomenon occurs independently of the *Leishmania* subspecies found in the lesion.

MATERIALS AND METHODS

Patients – Thirty-two patients, 29 with cutaneous and 3 with mucosal active lesions, were studied. Twenty-seven cases were from endemic areas of Rio de Janeiro, Brazil; 4 cases were from Amazonas and one from Mato-Grosso (Pantanal). The diagnosis was established by means of clinical, parasitological and/or immunological (Montenegro skin test and indirect immunofluorescence test) criteria. Lesions had an average of 3 months of evolution and all were ulcerated.

Biopsies – Scapel skin biopsy specimens were obtained at the border of the ulcer and were divided for parasitological examination using the following procedures; i) Giemsa-stained smears or imprints; ii) cultivation in an enriched blood-agar medium (NNN); iii) haematoxylin and eosin-stained paraffin sections; and iv) immunohistological examination. In this last case, specimens were rapidly frozen and stored in liquid nitrogen until sectioning. Cryostat sections were cut to 4 μm in thickness and allowed to air dry. Biopsies of normal skin were performed in two ACL patients and were submitted as described above.

Monoclonal antibodies (moAb) – Murine moAb were used at concentrations predetermined by checkerboard titrations. The expression of class II antigens of the major histocompatibility complex (MHC) were investigated by direct immunofluorescence using fluoresceinated anti-human HLA-DR and HLA-DQ moAb (Becton & Dickinson, New Jersey, USA). An indirect immunoperoxidase technique was used to identify anti-human HLA-DR, -DQ and -DP antigens (Becton & Dickinson), T lymphocyte infiltration in the epidermis (Leu 4, Becton & Dickinson) and Langerhans cells (OKT6, Ortho Immune). DR staining on keratinocytes could be distinguished from Ia on Langerhans cells by morphological criteria. The immunoperoxidade technique was described in detail elsewhere (Modlin et al., 1984). Briefly, sections were sequentially stained with primary moAb, peroxidase conjugated goat anti-mouse and the reaction was developed with aminoethyl carbazol and counterstained with Mayer's haematoxylin. Controls consisted of omission of the primary antibody or the use of a moAb of irrelevant specificity of the same isotype.

Isolated *Leishmania* stocks were immunologically characterized and identified by means of an indirect radioimmune binding assay or immunofluorescence technique (Grimaldi et al., 1987). The moAb specific for members of the *L. mexicana*, *L. braziliensis*, and *L. donovani* complexes have already been described (Jaffe et al., 1984; McMahon-Pratt et al., 1985; Grimaldi et al., 1987).

Therapy – Patients were submitted to pentavalent antimonium therapy with N-methyl-glucamine (Glucantime – Rhodia, São Paulo, Brazil). Patients were treated with daily intramuscular injections containing 60 mg of the salt/kg for 30 consecutive days. Healed lesions of 14 subjects were re-biopsied 1 to 12 months after the end of the therapy and biopsy specimens were submitted as described above. In addition, a biopsy was carried out in a spontaneous cultaneous healed lesion from a patient with mucosal active form.

RESULTS

Histopathology of lesions – The predominant histopathological picture of cutaneous lesions was a diffuse inflammatory reaction in the dermis or submucosae composed by lymphocytes, plasma cells and scattered macrophages. Unorganized granulomata were found in 80% of the cases. In addition, fibrinoid necrosis of the connective tissue was seen in almost all cases, often accompanied by vasculitis. Epidermis or mucosal lining epithelia exhibited severe acanthosis and exocytosis in the areas adjacent to the ulcer, occasionally characterizing a pseudo-carcinoma. Parasites were observed in 36% of the cases, always in the vicinity of the necrotic area.

Parasites – Fourteen parasite isolates were characterized by serodeme analysis and nine of them (all of patients from Rio de Janeiro), were identified as *L. b. braziliensis* based on their characteristic reactivities with *L. braziliensis* species- and subspecies-specific moAb. All cases from Amazonas were identified as *L. b.*

guyanensis and the case from Mato Grosso as *L. mexicana amazonensis*. Histopathological picture was similar in all cases, independent of the causative subspecies.

Expression of MHC class II antigens in epidermis – As expected, normal skin fragments from ACL patients showed only dendritic positive cells in the epidermis and dermis, keratinocytes being negative. In contrast, a strong reaction for DR antigens was consistently observed in skin frozen sections from the ACL patients (Fig. 1). This DR positivity was detected in the granulomas and on the keratinocytes. Mucosal cases also showed a positive reaction on lining epithelia and on cells of the infiltrate. However, HLA-DQ and DP antigens were never seen on keratinocytes but were only expressed on macrophages scattered in the infiltrate (Fig. 2).

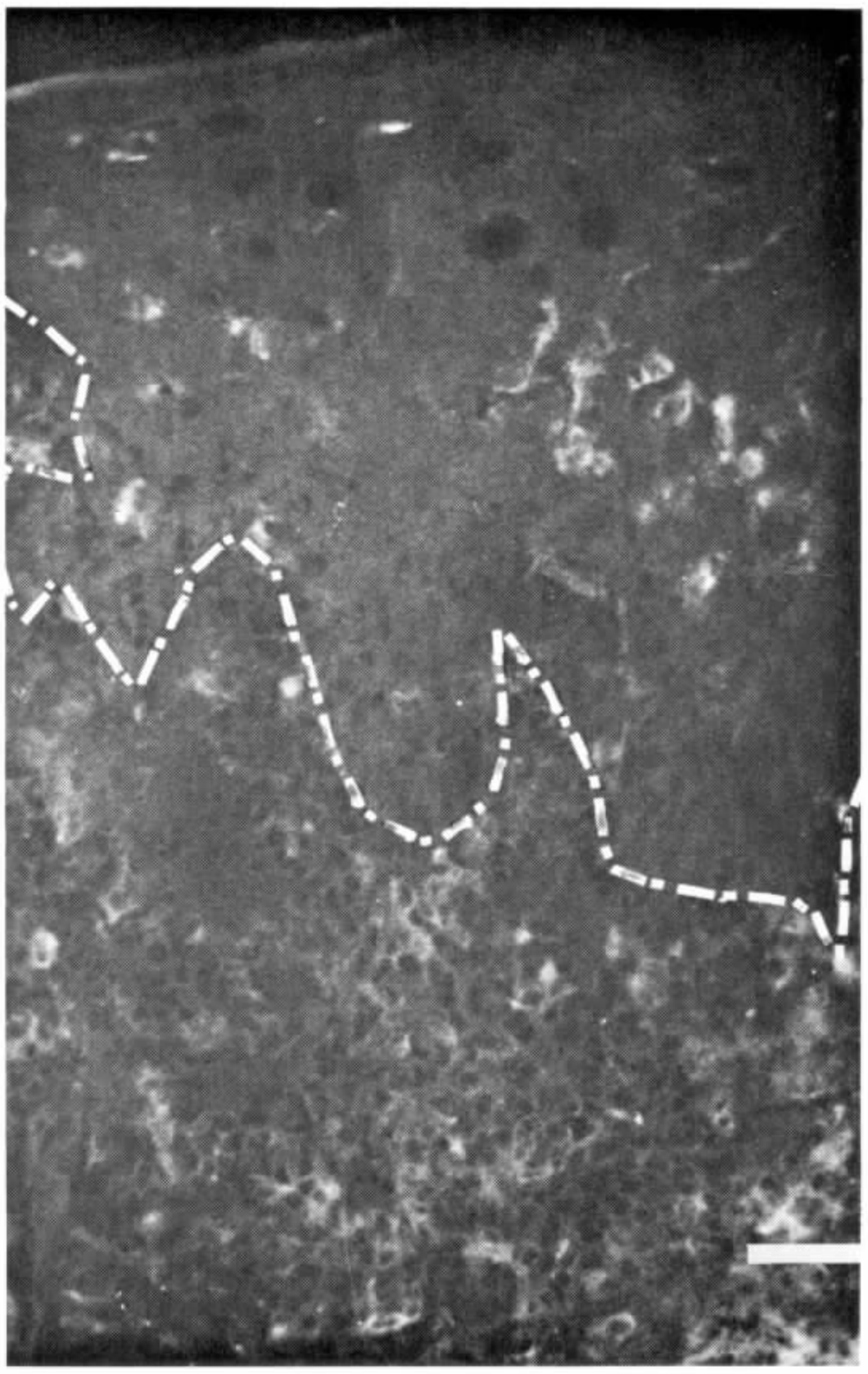

Fig. 2: absence of HLA-DQ expression on keratinocytes and positive expression on cells of the inflammatory infiltrate from an active lesion (scale bar = 20 μm).

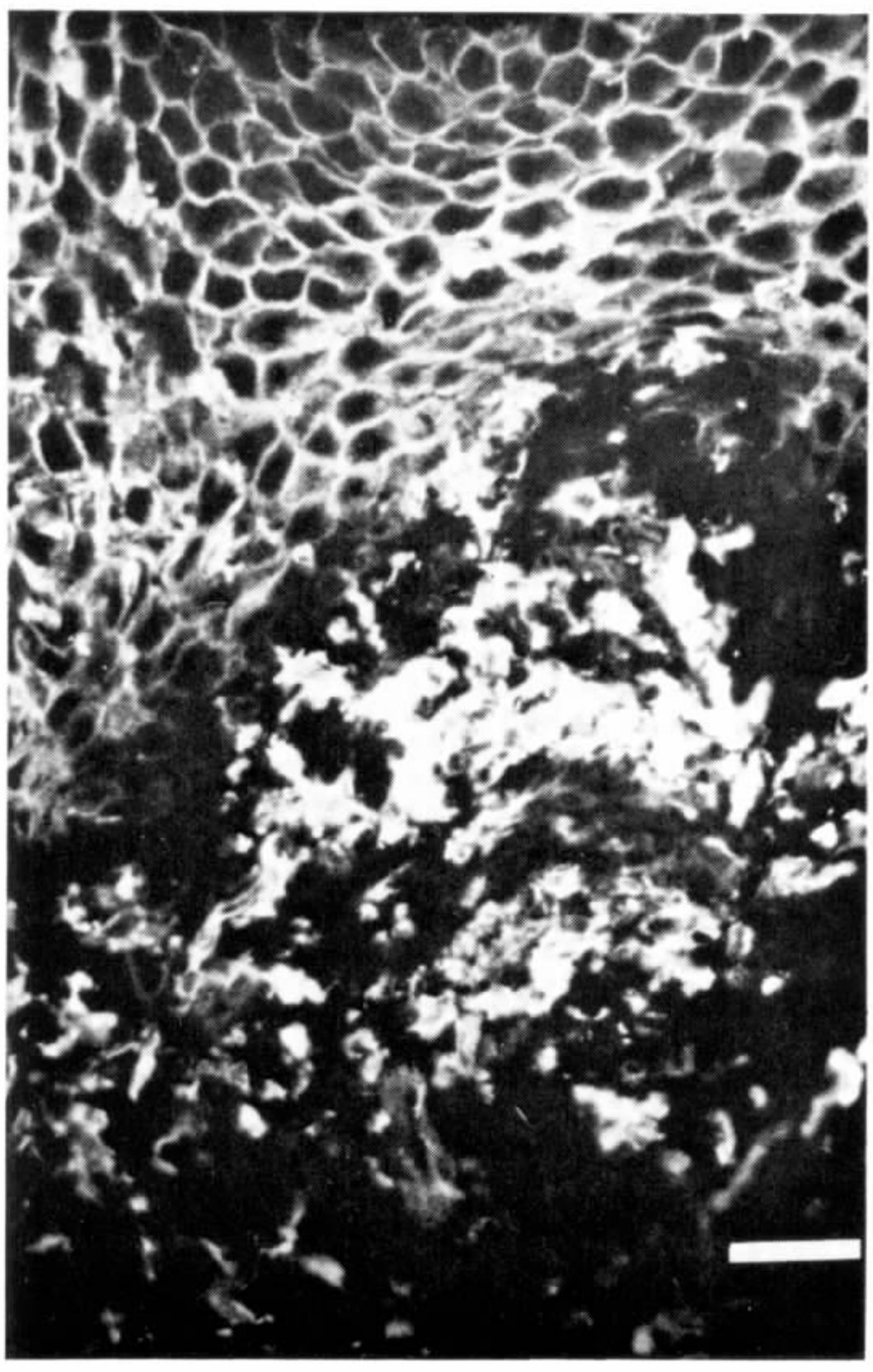

Fig. 1: positive HLA-DR expression on keratinocytes and cells of the inflammatory infiltrate from an active lesion (scale bar = 20 μm).

Epidermal Langerhans cells were increased, as compared to normal skin, in all localized cutaneous forms of ACL. A few number of these cells were also observed in the dermal granulomas (Fig. 3). Interestingly, OKT6 antibody could not detect Langerhans cells in any of the mucosal cases. Exocytosis observed in HE preparations were found to correlate with the T-cell infiltration found by immunoperoxidase staining with Leu 4, and was proportional with Langerhans cell hyperplasia.

Therapy and follow-up – Treatment was successfully obtained in all cases. Scar biopsies were performed in 14 cases: cultures and imprints were negative for leishmania parasites in all of these; histopathological examination showed reepithelization of the ulcer, fibroblastic proliferation and fibrosis of the dermis associated with scattered foci of mononuclear infiltrate (Fig. 4).

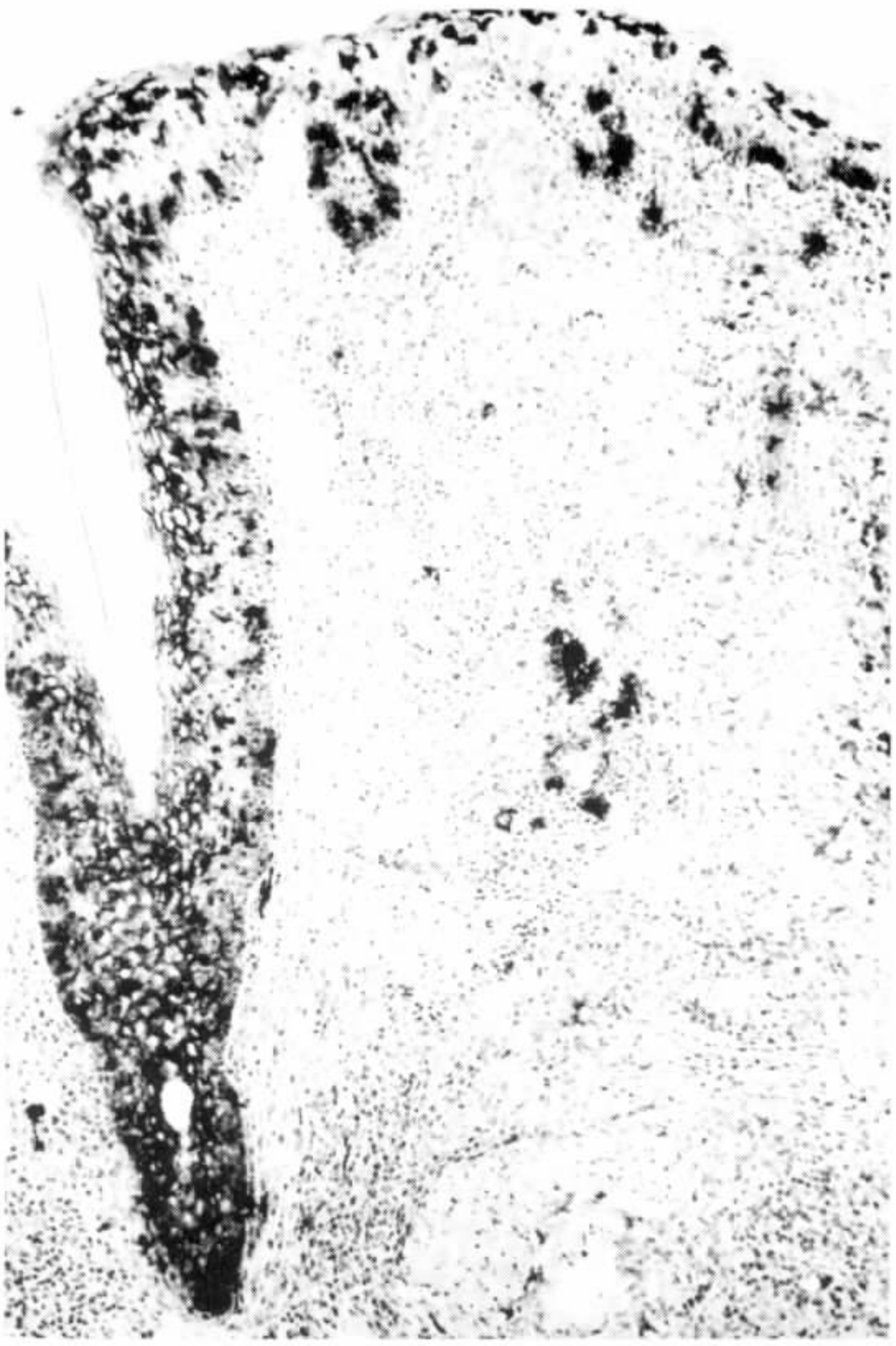

Fig. 3: immunoperoxidase staining with OKT6 moAb in a cutaneous lesion. Langerhans cells are numerous and the dendrites of adjacent cells appear to be touching one another. Note the presence of positive cells within the inflammatory infiltrate of the dermis (scale bar = 30 μm).

Fig. 4: clinically healed lesion one month after the end of the therapy showing reepithelization, fibrosis and moderate chronic inflammatory infiltrate (H & E, scale bar = 50 μm).

Our results showed that in all cases, at least one month after therapy, HLA-DR antigens could no longer be detected on keratinocytes, whereas HLA-DR and DQ positivity was found in residual inflammatory infiltrate (Fig. 5). The positivity on inflammatory cells persisted for at least 6 months, disappearing afterwards as was observed in 2 out of 3 cases biopsied 7 months after therapy. Moreover, in the patient that developed a spontaneous scar, keratinocytes were also HLA-DR negative.

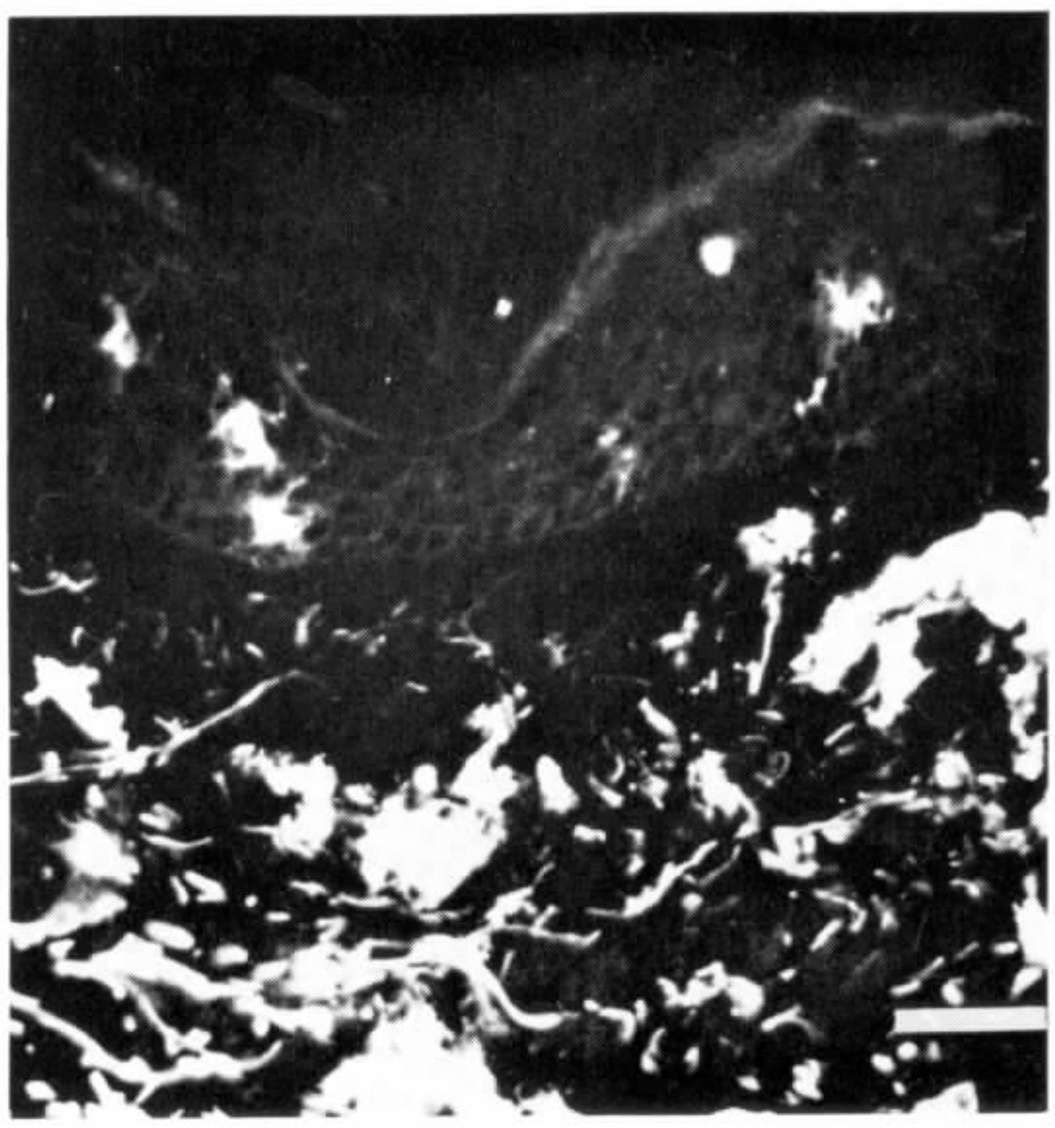

Fig. 5: absence of HLA-DR expression on keratinocytes of a clinically healed lesion. Positivity is restricted to the residual inflammatory cells and Langerhans cells of the epidermis (scale bar = 30 μm).

DISCUSSION

Our data represent clear-cut evidence for the reversibility of the abnormal HLA-DR expression by keratinocytes in ACL after Glucantime therapy. HLA-DR expression before therapy presented the same pattern in ACL, independently of the causative subspecies, and the reversibility after therapy was similar in all cases. These findings correlate with the clinical cure of the patients and the absence of parasites in culture from biopsies of scarred skin.

The mechanisms for induction of HLA-DR antigens on keratinocytes are unknown. *In vivo* keratinocyte expression of class II MHC (Ia or DR) molecules has been observed in delayed-type hypersensitivity (DTH) or cell-mediated

immune reactions (Suitters & Lampert, 1982; Scheynius & Tjernlund, 1984; Volc-Platzer et al., 1984). In such diseases, there is always a close contact between the lymphocyte infiltrate and the epidermis. It is unlikely that the induction of Ia antigens on keratinocytes is a primary event in triggering in immune response but is rather due to secondary increased levels of interferon-gamma (IFN-gamma). This lymphokine has been found to increment the synthesis and expression of HLA-DR antigens in several cell types including epithelial tissues (Winchester et al., 1978; Steeg et al., 1985). Murine experimental models of leishmaniasis have shown that healing is correlated with the ability to produce IFN-gamma (Murray et al., 1982; Titus et al., 1984; Sadick et al., 1986) and *in vitro* studies with peripheral blood lymphocytes from patients with mucocutaneous leishmaniasis have shown that these patients are able to produce significant amounts of IFN-gamma (Carvalho et al., 1985). In addition, *in vivo* studies using *in situ* hybridization showed the presence of IFN-gamma mRNA either in LCL or MCL lesions (Pirmez et al., 1988). The induction of MHC class II antigens on keratinocytes in ACL lesions might thus be secondary to the activation of infiltrating T lymphocytes followed by the release of lymphokines and particularly IFN-gamma. In this context, the reversibility of HLA-DR expression by keratinocytes after healing could be secondary to a cessation of *in situ* IFN-gamma secretion.

It is largely known that MHC class II molecules play an important role in antigen presentation as well as in various cellular interactions that are required during an immune response (Schwartz, 1985). These antigens are usually expressed only in cells associated with the immune system. In normal skin, solely Langerhans cells have been definitely demonstrated to express these immune response-associated antigens (Tjernlund, 1980). There is, however, indirect evidence that DR^+ keratinocytes could act as antigen presenting cells (APC) since HLA-DR-expressing keratinocytes induce a greater T cell response to PPD than normal epidermal cell suspensions do, in which Langerhans cells are the only DR^+ cells (Tjernlund & Scheynius, 1987). On the other hand, when lymphocytes are depleted of their CD4 subsets they fail to respond in mixed skin lymphocyte reaction, suggesting that helper/inducer play a major role, being the main source of IFN-gamma (Czernielewski & Bagot, 1986).

As another hypothetic vein, DR molecules might act addressing the movement or lymphoid cells into the skin in response to antigenic stimulation. In experimental contact hypersensitivity dermatitis it was strongly suggested that Ia expression by keratinocytes should potentiate the local immune response at least by increasing the number of lymphocytes *in situ* (Roberts et al., 1985). These data correlate with the negative findings on normal skin of ACL patients and also the reversibility of the DR expression of keratinocytes after Glucantime therapy. On the other hand, it should be also pointed out the possibility that secondary increases in classe II MHC antigen expression could worsen autodestructive processes DR^+ keratinocytes being targets for activated cytotoxic T lymphocytes. This is in keeping with the ulceration of the epidermis. Also in this context, immunohistochemical studies have shown that the majority of $CD8^+$ cells present in the lesions have a cytotoxic phenotype and that a high proportion of cells express a serine esterase mRNA (Pirmez et al., 1988), which is an enzymatic marker of T-cytotoxic cells (Gershenfeld et al., 1988).

The reversibility is likely to parallel to the scar formation whenever it occurs, independently of being spontaneous or following antimonial therapy. In any case, the fact that DR expression on keratinocytes is restricted to the period of active lesions, suggests that keratinocytes are somewhat involved in the immunopathology of ACL. If their role is distinct from a classical APC, these cells could act indirectly through changes of soluble factors such as ETAF or other cytokines that are released from DR expressing keratinocytes. Our results, showing that in addition to a strong Ia positivity on keratinocytes, there is a Langerhans cell hyperplasia and epidermal T lymphocyte infiltration, supports the view that a cell-mediated immune mechanism is involved in ACL lesions.

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