Lichen sclerosus et atrophicus is a chronic inflammatory skin disease of unknown aetiology. Recent studies have indicated that autoimmune mechanisms might be involved in its pathogenesis and have suggested a role for autoreactive cytotoxic T lymphocytes. Based on recent observations we now hypothesize that a type I interferon-driven inflammation might be involved in the pathogenesis of this disease. Lesional skin biopsies were analysed by immunohistochemistry (CD3, CD4, CD8, CD68, CD123, Tia1, Granzyme B, Myxovirus resistance A, IP10/CXCL10 and CXCR3). Sequential double staining was performed to analyse co-expression of Tia1 and CXCR3. Significant expression of Myxovirus resistance A was found, indicating type I interferon production. This expression was closely associated with the expression of the interferon-inducible protein IP10 and the recruitment of CXCR3+ cytotoxic T lymphocytes. Plasmacytoid dendritic cells appeared to be a major source of type I interferon in lichen sclerosus et atrophicus. Interestingly, several infiltrating lymphocytes contained IP10 in their granules. This is the first study providing evidence that a type I interferon-associated recruitment of CXCR3+ cytotoxic T lymphocytes is involved in the pathogenesis of several cell-mediated autoimmune skin disorders, such as discoid lupus erythematosus and lichen planus (11–13). Since LSA shares some common features, such as lymphocytic inflammation and interface dermatitis with these disorders, we hypothesized that similar recruitment mechanisms might be involved in LSA.

LSA skin lesions are characterized by a lymphocytic infiltrate, which is dominated by T lymphocytes. Numerous cytotoxic T lymphocytes (CTL) are detectable, along with a hydropic degeneration of basal keratinocytes and loss of elastic fibres (9, 10). We have recently shown that the type I interferon (IFN) associated recruitment of CTL via IP10/CXCR3 interaction is involved in the pathogenesis of several cell-mediated autoimmune skin disorders, such as discoid lupus erythematosus and lichen planus (11–13). Since LSA shares some common features, such as lymphocytic inflammation and interface dermatitis with these disorders, we hypothesized that similar recruitment mechanisms might be involved in LSA.

**Patients and Methods**

**Patients and healthy donors**

Lesional skin biopsies were taken from 9 patients with LSA (5 men and 4 women). All specimens were taken for diagnostic or therapeutic purposes. Detailed patient data are given in Table I. Control biopsies were taken from the unaffected skin of 5 patients undergoing surgery for skin tumours. Additionally, we analysed 7 biopsies from lesional skin of patients with chronic discoid lupus erythematosus (CDLE) as positive controls. All patients gave written informed consent before the biopsies. The studies were performed in accordance with the local ethical guidelines.
Histology and immunohistochemistry

Sections were prepared from formalin-fixed, paraffin-embedded skin biopsies. Standard haematoxylin and eosin (H&E), periodic acid Schiff and elastica reactions were performed for diagnostic purposes. The inflammatory infiltrate was characterized by immunohistochemistry. Monoclonal antibodies specific for CD4 (IF6, Novocastra™, Newcastle, UK), CD3 (F7238), CD8 (C8/144B), CD20 (L26) and CD68 (PGM1; all from DAKO™, Hamburg, Germany) were used as primary antibodies following the manufacturers protocol. Myxovirus resistance A (MxA) (M143, Prof. Haller, University of Freiburg, Germany, dilution 1:100), IP10/CXCL10 (Clone 33036, R&D Systems, Minneapolis, USA), CXCR3 (1C6, PharMingen™, San Diego, USA, dilution 1:100) labelling was performed on paraffin-embedded tissue sections (4 µm) as described previously (14). The cytotoxic capacity of infiltrating lymphocytes was determined using monoclonal antibodies targeting Tia1 (Clone 26gA10F5, Immunotech, Marseille, France) and Granzyme B (GrB7, DAKO). Anti-CD123 (7G3; PharMingen™, dilution 1:150) monoclonal antibodies were used to identify plasmacytoid dendritic cells. Appropriate isotype-matched controls were included.

Visualization was performed using the LSAB2™ staining kit (DAKO) with Fast Red as chromogen, for sequential double staining in combination with the Envision System™ (DAKO) with DAB as chromogen. Results were evaluated independently on blinded specimens by 2 experienced dermatopathologists (JW and TT). Cells were counted per 3 high power fields (>200) and the mean number was calculated. The expression of IP10 and MxA in epidermis and inflammatory infiltrate was scored semi-quantitatively (0 = no expression; + = weak expression; ++ = fair expression; +++ = strong expression).

Statistical analysis

Statistical analysis was performed using SPSS™ software (version 12). The non-parametrical Mann-Whitney U test was employed to compare the expression of CD3, CD4, CD8, CD20, CD68, Tia1, GrB, CXCR3, IP10 and MxA in different disease subsets and healthy controls in the skin. Correlation analyses were performed using by Spearman’s rho (ρ). Probabilities <0.05 were considered to be significant (*), p-values <0.01 as highly significant (**).

RESULTS

T lymphocytes dominate the inflammatory infiltrate of LSA

The inflammatory infiltrate was characterized using antibodies against CD3, CD4, CD8, CD20 and CD68. The analyses revealed a clear majority of CD3+ T cells among the infiltrating cells, with a slight predominance of the CD8+ over the CD4+ phenotype. T cells were accompanied by several CD68 macrophages, while only a minor number of CD20+ B cells were found in the investigated LSA skin biopsies. This cell type distribution pattern of LSA was comparable to that seen in CDLE, but the number of infiltrating immune cells was about 2–3 times lower (Fig. 1). Fig. 1b shows a representative picture of CD8+ T cells, which dominated the junctional inflammation.

High number of cytotoxic lymphocytes in LSA skin lesions

Earlier studies indicated that potentially autoreactive, cytotoxic lymphocytes might be involved in the pathogenesis of LSA. We therefore analysed the cytotoxic capacity of the infiltrating immune cells by staining for the cytotoxic markers Tia1 and Granzyme B (GrB). We found a fair-to-strong expression of these cytotoxic molecules in all investigated LSA skin lesions. The highest number was seen in early lesions, which were characterized by a dense inflammation accompanied by a loss of elastic fibres, but less sclerosis, as shown in Fig. 3a. In general, the number of Tia1+ cells was twice as high as the number of GrB+ cells (Fig. 1c).

Again, this distribution pattern seen in LSA was similar to that in CDLE. Fig. 1d shows a typical finding of Tia1 expression in an early LSA lesion.

Strong expression of MxA indicates type I IFN production in LSA

To investigate the involvement of type I IFNs in lesional skin inflammation in LSA we used a monoclonal antibody against MxA. MxA is an antiviral protein which is specifically induced by type I IFNs. Other cytokines, including IFN gamma, are poor inducers. Therefore it is an ideal and widely used marker for type I IFNs. Our analyses revealed MxA expression in all investigated LSA skin specimens. The expression was significantly higher than in healthy controls, but not as strong as seen in CDLE (Fig. 2a).

The level of MxA expression was closely associated with the number of infiltrating lymphocytes (Fig. 2b). The expression was significantly higher than in healthy controls, but not as strong as seen in CDLE (Fig. 2a).

Fig. 2c depicts typical findings of MxA expression in LSA. Strong expression of MxA indicates type I IFN production in LSA.

Plasmacytoid dendritic cells (pDC) are known to be a major source of type I IFNs in skin inflammation (16). We therefore investigated our specimens for the presence of pDCs using immunohistology. As expected, we were able to identify numerous CD123+ cells with a plasmacytoid morphology in the inflamed skin areas of LSA, in particular in the junctional zone, but also in the dermis and epidermis (Fig. 2d).
The majority of infiltrating lymphocytes in LSA express the chemokine receptor CXCR3

Several earlier studies have shown that the chemokine receptor CXCR3 plays an important role for lymphocyte recruitment in type I IFN driven skin inflammation, such as lupus erythematosus (11, 17, 18). Therefore, we hypothesized that CXCR3-mediated lymphocyte migration might also be involved in LSA. We found a significant number of CXCR3+ lymphocytes in all investigated LSA skin biopsies, similar to findings in CDLE (Fig. 3a). The CXCR3 receptor was expressed in the lesional inflammatory infiltrate of lichen sclerosus et atrophicus (LSA) was characterized by immunohistochemistry. Skin biopsies taken from patients with chronic discoid lupus erythematosus (CDLE) and healthy skin (HC) were included for control purposes. The inflammation in LSA was dominated by CD3+CD8+ T lymphocytes (a). Labelling for Tia1 and GrB (Granzyme B) confirmed the high cytotoxic capacity of these cells (c). (a) and (c) show the mean number of infiltrating cells per high power field (HPF, ×200) ± SEM. (b) and (d) depict representative findings of CD8 and Tia1 expression (original magnification ×100 and ×200, with digital magnification).

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on the majority (80–95%) of all infiltrating cells. To investigate if these CXCR3+ lymphocytes might be the potential cytotoxic effector cells, we performed sequential double stainings for CXCR3 and Tia1. Fig. 3b shows a representative finding in a patient with early LSA. The picture clearly demonstrates co-expression of CXCR3 (brown, cell surface) and Tia1 (red, typical intracellular granula) of infiltrating lymphocytes.

Expression of IP10/CXCL10 links type I IFN production and recruitment of CXCR3+ lymphocytes

Type I IFNs induce lesional expression of several cytokines in the skin, including the IFN-inducible protein IP10/CXCL10. Since this chemokine is a ligand for CXCR3 we hypothesized a role for IP10/CXCL10 for lymphocyte skin recruitment in LSA. Immunohistochemical analyses revealed a significant expression of IP10 in the investigated LSA skin lesions (Fig. 3c). This expression was predominantly seen in the basal layers of the epidermis, in particular in areas with pronounced junctional inflammation (Fig. 3d), but sometimes also in a homogenous pattern in the whole epidermis. Interestingly, at higher magnifications we identified infiltrating lymphocytes, which carried IP10+ perinuclear granules, similar to the expression pattern of the cytotoxic markers Tia1 and GrB (Fig. 3d, arrows).

DISCUSSION

Recent studies have provided increasing evidence that LSA is an autoimmune disease in which autoreactive CTL play an important role. Gross et al. (10) identified high numbers of Tia1+ CD8+ cytotoxic T lymphocytes in LSA skin lesions. Activated macrophages and lymphocytes indicate persistent antigen-driven inflam-

Fig. 3. CXCR3 and IP10/CXCL10 are involved in the inflammation seen in lichen sclerosus et atrophicus (LSA). The majority (80–95%) of infiltrating lymphocytes in LSA expressed the chemokine receptor CXCR3. These findings were similar to the results in chronic discoid lupus erythematosus (CDLE) (a), given are mean numbers of CXCR3+ cells ± SEM. (b) Co-expression analyses confirmed the cytotoxic phenotype of several lesional lymphocytes; CXCR3: brown, on the surface; Tia1: red, typical intracellular granule, (red arrows). (c) A significant lesional expression of the interferon (IFN) inducible protein IP10 links type I IFN production and recruitment of CXCR3+ lymphocytes. (d) Strong expression of IP10 was seen, in particular in areas with an extensive interface dermatitis. Interestingly, several lesional lymphocytes contained IP10 in their granules (black arrows), which might present a potential self-perpetuating mechanism.
filtration and at the dermo-epidermal interface (10). The authors concluded from their results, that a cytotoxic T-cell immune response directed against keratinocyte-associated antigens may play a major role in the pathogenesis of LSA. Carlson et al. (19) analysed 100 biopsies from patients with LSA using 2-colour immunohistochemistry. The authors identified numerous activated CD8+CD57+ lymphocytes in LSA. These cells were thought to represent terminally differentiated and activated effector cytotoxic T cells (20).

Expansion of CD8+CD57+ lymphocytes is suspected to be the result of chronic excessive antigen exposure. It is associated with dermal sclerosis and also found in viral infections, autoimmune disease (morpha), malignancies, and sclerosing graft versus host reaction (19). Additionally, Lukowsky et al. (6) found, that nearly all CD8-positive cells investigated in LSA expressed cytotoxic granules (Tia1), possibly causing the basal cell destruction.

Our analyses confirmed that cytotoxic T cells are involved in the lesional inflammation of LSA. We found numerous Tia1+ and GrB+ lymphocytes in the upper dermis as well as in the junctional zone. The number of Tia1+ cells was twice as high as that of GrB+ cells. One possible explanation for the lower expression of GrB compared with Tia1 is that most CD8+ T cells had already secreted their cytotoxic granules, as has been demonstrated in skin biopsy samples of lichen planus (21). Interestingly, Vermeer et al. (22) reported that in skin biopsy samples of lichen planus, lupus erythematosus and graft versus host disease, most CD8+ T cells expressed Tia1, whereas GrB was expressed only by a small number of these cells. Epidermal injury and apoptotic keratinocytes were a constant finding in these biopsies, illustrating the presence of functionally active CTLs. These authors concluded that the low expression of GrB compared with Tia1 is that most CD8+ T cells in these CTLs does not exclude that they are functionally active CTLs (22).

The type I IFN associated recruitment via CXCR3/IP10 interaction plays an important role in the migration of CTL into the skin in several conditions, including lupus erythematosus and lichen planus. This has been demonstrated in several recent studies (11, 12, 17, 18, 23). The results presented here show that similar mechanisms are involved in LSA. We found a significant expression of the MxA protein, indicating type I IFN expression, in all investigated LSA skin lesions. This expression was clearly associated with the recruitment of CXCR3+ CTL. Plasmacytoid dendritic cells were detectable within the inflammatory infiltrate and might be an important source of type IFNs in LSA. Lesional expression of the CXCR3 ligand IP10/CXCL10 was demonstrated in LSA lesions, linking the expression of type I IFNs and the recruitment of CXCR3+ lymphocytes. Interestingly, we found several infiltrating lymphocytes which carried IP10/CXCL10 in their granules. These cells might amplify the IP10-induced recruitment of CXCR3+ CTL and take part in a self-perpetuating mechanism in LSA. This might be responsible for the chronic inflammation that is characteristic for this disease. Similar observations were made before by Iijima et al. (23) in oral lichen planus. However, other CXCR3 ligands including CXCL9 and CXCL11 might play an additional role.

In conclusion, this study provides the first evidence that cytotoxic lymphocytes expressing the chemokine receptor CXCR3 participate in the pathogenesis of LSA. The lesional expression of the interferon-inducible protein IP10/CXCL10 seems to play a role in the recruitment of CXCR3+ cells into the skin. Additionally, IP10 detectable in perinuclear granules of infiltrating lymphocytes appears to participate in a pro-inflammatory self-perpetuating mechanism.

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