Original Article

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Immunohistochemical characterization of the cellular infiltrate in discoid lupus erythematosus

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Summary **Discoid lupus erythematosus (DLE) is a chronic connective tissue disease of unknown etiology, but immunologic factors may play an important role in the pathogenesis. We investigated the features of immunohistochemical characterization of the cellular infiltrate in DLE. Skin samples were obtained from 5 patients using a 6 mm punch biopsy. Samples were stained with monoclonal antibodies against CD1a, CD3, CD4, CD8, CD20, CD25, CD30, and CD57. The number of cells stained with each monoclonal antibody was calculated. The number of cells stained with each monoclonal antibody in the dermis infiltration in DLE was calculated and all were higher than those in the normal control. The numbers of $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, $CD25^+$, or $CD57^+$ cells in DLE were statistically higher than those in normal skin ($p < 0.05$). The numbers of $CD1a^+$ and $CD30^+$ cells in DLE were appreciably increased but had no statistical significance compared with normal skin. In conclusion, this study revealed that T lymphocytes, B lymphocytes, and natural killer cells may play some roles in the pathogenesis of DLE.**

Keywords: CD panel antibodies, immunohistochemistry, T cells, B cells, natural killer cells

1. Introduction

In 1882 Kaposi (*1*) distinguished discoid lupus erythmatosus (DLE) from the systemic form (SLE). The term discoid is used to describe a coin-shaped, dark to erythematosus scaly lesion. Keratinous plugs, whitish depressed areas (atrophy), and telangiectases are also commonly seen. Hypo- and hyper-pigmentation are often residual findings (*2*). Discoid lesions are not exclusive for DLE; they are observed in 15-30% of SLE patients (*2,3*). Only 5-10% of adult patients with DLE develop SLE (*3*). Most adult patients with DLE are between the ages of 20 and 60 years (*4*), and the female/male ratio is 2:1 to 3:1 (*5*).

There is evidence for systemic immune disturbance in DLE patients, as judged by the findings of an increased CD4⁄CD8 ratio in the peripheral blood compared with healthy controls, hypergammaglobulinaemia, and the presence of autoantibodies (*6,7*).

In classic DLE lesions, epidermal changes include hyperkeratosis and variable atrophy. Dermal changes include a dense mononuclear cell infiltrate which usually consists of lymphocytes and plasma cells predominantly in the periappendageal and perivascular area. In active lesions, the infiltrate can be found approximating the dermal-epidermal junction associated with hydropic degeneration. A patchy inflammatory infiltrate also may be present in the upper dermis in an interstitial pattern and around eccrine coils. The infiltrate is often quite dense and typically extends well into the deeper reticular dermis and/or subcutis (*8,9*).

The purpose of our experiments is to investigate primarily the immunohistochemical characterization of the cellular infiltrate in patients with DLE.

2. Materials and Methods

2.1. *Patients*

Five patients with DLE were randomly chosen for this study, and the disease was confirmed by clinical and pathological examination. Three patients were

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males, and the rest were females. Mean age was 38.6 years. Three normal specimens as controls were also obtained from age- and sex-matched healthy volunteers. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki.

2.2. *Cutaneous samples*

A skin fragment of the lesion was obtained from each patient by biopsy. The specimen was fixed in 10% formalin for 24 h and processed by routine procedures for embedding in paraffin. Histological sections (4 μm) were stained with hematoxylin/eosin (HE). The remaining material was used for immunohistochemical analysis.

2.3. *Immunohistochemistry*

Serial sections were prepared from formalin-fixed, paraffin-embedded skin samples. Monoclonal antibodies (mAb) specific for CD1a (1590, Immunotech, Marseille, France), CD3 (PS1, Novocastra Laboratories, Newcastle, UK), CD4 (IF6, Novocastra Laboratories), CD8 (C8/144, Dako, Glostrup, Denmark), CD20 (NJ1, Novocastra Laboratories), CD25 (4C9, Novocastra Laboratories), CD30 (182, Novocastra Laboratories), CD57 (NK1, Novocastra Laboratories) were used for primary staining. Secondary staining was performed using the LSAB2 staining kit (Dako) for 30 min at room temperature.

Serial 4 μm thick sections were mounted on silane slides (Dako) and submitted to fixation, deparaffinization in xylene and rehydration through graded alcohols. The antigen was retrieved in 0.1 M Tris-HCl buffer (pH 9.5, containing 5% urea) using a microwave oven for 15 min, each for antigen recovery of the molecules. After cooling to room temperature for 50 min, the slides were treated with 0.3% hydrogen peroxide in methanol (Merck, Darmstadt, Germany) for 30 min to block endogenous peroxidases. Nonspecific staining was blocked with 5% normal horse serum for 30 min. Subsequently the slides were incubated with the primary antibodies diluted in horse serum albumin at 4°C overnight in a humidified chamber. The samples were treated with biotinylated secondary antibody (horse anti-mouse) for 30 min. After washing with PBS, the slides were incubated with ABC reagent for 60 min at room temperature. The slides were then stained with DAB solution for 2-4 min under a microscope at room temperature. Finally the reaction was terminated by washing in distilled water. The slides were washed with PBS between each reaction step. Samples in which the primary antibody was omitted were used as a negative control. Positive labeling was identified by a brown staining around the cell membrane or by a brown color of the cytoplasm according to the marker employed.

All sections were examined in an Olympus BX50 light microscope (Tokyo, Japan) and results were expressed as the mean count of cells per 10 high-power fields (400×). Ten microscopic fields, representing the densest cellular inflammatory infiltrate, were selected per specimen and positive cell numbers were estimated as a proportion of lesion area, using a point counting method.

The scale corresponded to the percentage of stained cells with specific antibody each time, compared to the total cellular infiltration and counting was scored as following: –, no cells (negative); +, few cells (0~10%, weak); ++, some cells (10~25%, moderate); +++, many cells (25~50%, intense); ++++, plenty of cells (more than 50%, very intense).

2.4. *Statistical analysis*

All the data were collected, classified and entered into a spreadsheet for statistical analysis, using the *t*-test. The dermal infiltration of DLE and normal skin were compared for the following cell counts (per HPF): CD1a, CD3, CD4, CD8, CD4/CD8, CD20, CD25, CD30, and CD57. Results are given as mean ± S.D. $p < 0.05$ was considered significant.

3. Results

3.1. *Evaluation of CD4/CD8 ratio*

The CD4/CD8 cell ratio is known to be an indicator of the host immunoregulatory status. The ratio of CD4/CD8 ranged from 0.7 to 1.7 in the five DLE patients. The mean was 1.0 (Table 1), and there was no statistical significance compared to that in normal skin (1.1).

3.2. *The percentage of each positive cell in the infiltrates*

Examining serial sections, light microscopy revealed that the inflammatory response was concentrated perivascular and periappendageal. The infiltrates consisted mostly of lymphocytes and plasma cells. The percentages of positive cells for each CD antibody are listed in Table 2.

The CD1a mAb stained all five lesions. Most of them (4/5) showed weak infiltration with a mean percentage of 6.7%. Besides the infiltrate in the dermis, there were also many $CD1a^+$ cells in the epidermis (not calculated). Representative pictures are shown in Figures 1A and 1B.

The CD3 mAb stained all five sections to a very intense degree. The mean percentage was 69.0%. The pictures are given in Figures 1C and 1D.

The CD4 and CD8 mAb stained all five lesions to an intense degree and the mean percentage of CD4 was

Table 1. CD4/CD8 ratio in the lesions examined

Patient No.	1	2	3	4	5	Mean	Control	*p* value
CD4/CD8 ratio*	1.7	0.8	0.9	0.7	0.9	1.0	1.1	> 0.05

* The number of $CD4^+$ cells divided by the number of $CD8^+$ cells.

Table 2. The percentage of each positive cells in the infiltrates

Cell counts	CD1a	CD3	CD4	CD8	CD20	CD25	CD30	CD57
–	0	0	0	0	0	0	1	0
+	4	0	0	0	2	4	4	2
++	1	0	0	0	3	1	0	3
+++	0	0	5	5	0	0	0	0
++++	0	5	0	0	0	0	0	0
Mean (%)	6.7	69.0	34.5	37.8	11.6	7.5	3.5	11.3

–, negative; +, weak (0~10%); ++, moderate (10~25%); +++, intense (25~50%); ++++, very intense (> 50%).

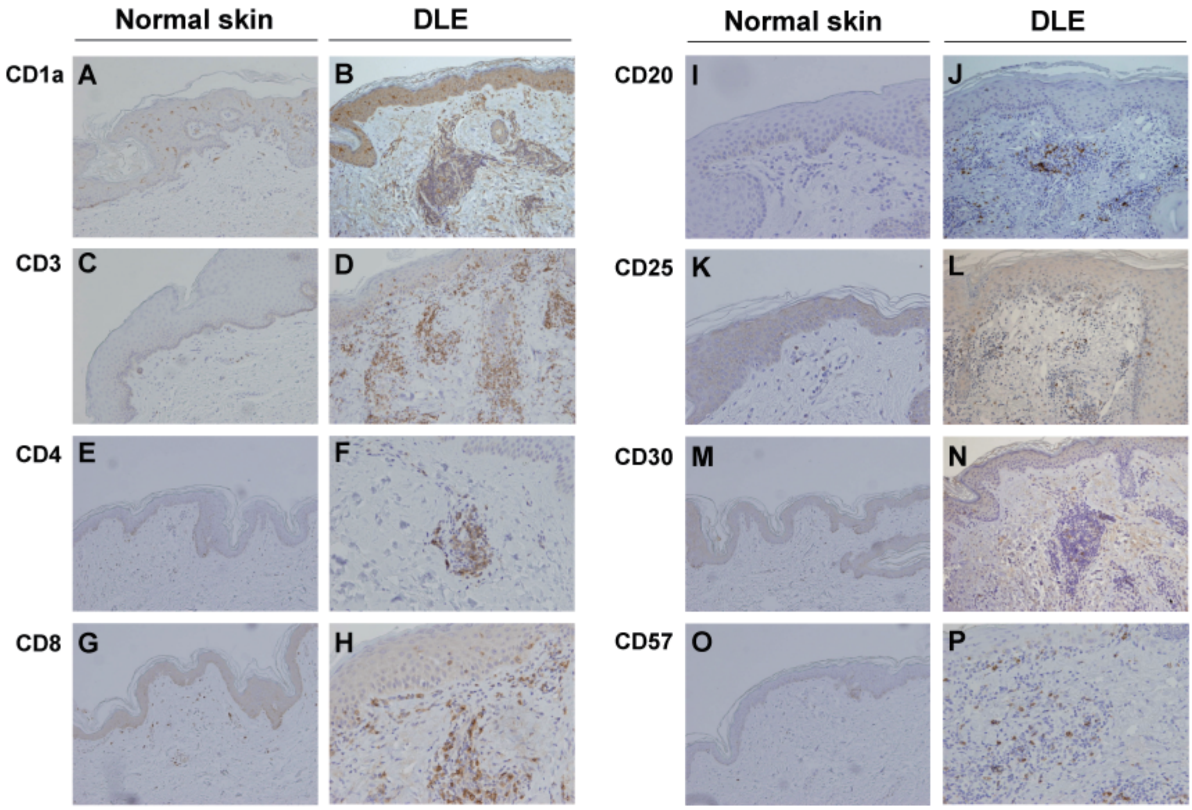

Figure 1. Immunohistochemical staining of CD panel antibodies. The anti-CD1a mAb stained a few infiltrate cells around many lymphocytes in DLE (**A**, normal skin; **B**, DLE). The anti-CD3 mAb stained many infiltrate cells in the dermis (**C**, normal skin; **D**, DLE). The anti-CD4 mAb stained many infiltrate cells around vessels (**E**, normal skin; **F**, DLE). The anti-CD8 mAb stained many infiltrate cells around vessels (**G**, normal skin; **H**, DLE). The anti-CD20 mAb stained some infiltrate cells among the lymphocytes (**I**, normal skin; **J**, DLE). The anti-CD25 mAb stained a few infiltrate cells around vessels (**K**, normal skin; **L**, DLE). The anti-CD30 mAb stained only a few infiltrate cells around many lymphocytes (**M**, normal skin; **N**, DLE). The anti-CD57 mAb stained some infiltrate cells among many lymphocytes (**O**, normal skin; **P**, DLE). Original magnification, ×100.

34.5%, while that of CD8 was 37.8%. The expressions of the two mAbs are exhibited in Figures 1E, 1F, 1G, and 1H.

CD20 positive cells were observed in all five skin lesions. Two of them were weak, the other three were moderate. The mean percentage was 11.6%. The pictures are shown in Figures 1I and 1J.

$CD25^+$ cell infiltrates were weak in four sections and moderate in one with a mean percentage of 9.4%. Representative pictures are shown in Figures 1K and 1L.

The CD30 mAb stained four lesions to a weak degree, while the other was negative. The mean percentage was less than 3.5%. The expression of CD30 is exhibited in Figures 1M and 1N.

CD57$^+$ cell infiltrates were weak in two and moderate in three with a mean percentage of 11.3%. The pictures are shown in Figures 1O and 1P.

3.3. *Positive cell numbers of immunostaining with anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD25, anti-CD30, and anti-CD57 mAbs*

All lesional skins showed dermal perivascular and periappendageal infiltration. The results of numbers for each positive cell in per HPF are summarized in Table 3. The numbers of CD3$^+$ (94.1 ± 51.8), CD4$^+$ (48.3 ± 18.2), CD8$^+$ (55.9 ± 27.9), CD20$^+$ (21.3 ± 16.4), CD25$^+$ (6.7 ± 1.9), and CD57$^+$ (11.8 ± 4.8) cells in DLE were significantly higher than those in normal skin ($p < 0.05$). Although the numbers of CD1a (2.3 ± 1.3) and CD30$^+$ (4.0 ± 3.3) cells in DLE were also higher than those in normal skin, there was no statistical difference between them.

4. Discussion

DLE is a connective tissue disorder characterized by well-demarcated, erythematous, slightly infiltrated "discoid" plaques that often show adherent thick scales and follicular plugging. The etiology of DLE is still unknown but considered to be multifactorial at this time. Immunologic factors may play an important role in its pathogenesis. In active lesions, the dense inflammatory infiltrate is observed in the dermal-epidermal junction. The infiltrate in dermis is often quite dense in the periappendageal and perivascular area and typically extends well into the deeper reticular dermis and subcutis.

The present immunohistochemical study was conducted to show the features of a panel of CD monoclonal antibodies in dermal infiltrate in the skin of DLE patients.

Analysis of the lymphocyte population by immunohistochemistry revealed a conspicuous infiltrate of T lymphocytes in the dermis of DLE patients which were usually observed around appendices and blood vessels. CD3 positive cells were found in the dermal infiltrate of all biopsies and showed a considerable percentage in the infiltrating area (69.0%). The number of CD3$^+$ T cells per HPF (94.1 ± 51.8) in DLE was significantly higher than that in normal skin (5.8 ± 5.3, $p < 0.05$). Amoura *et al*. (*10*) suggested that chronic discoid lupus erythematosus (CDLE), the most common clinical subtype of cutaneous lupus erythematosus (CLE), was characterized by a dense lymphocytic infiltrate composed of CD3$^+$ lymphocytes with a slight predominance of CD4$^+$ over CD8$^+$.

T lymphocytes are critical as mediators of inflammation immunity through the combined activity of CD8$^+$ and CD4$^+$ cells. Immunohistochemical analysis of different subsets of T cells revealed the host immunoregulatory status. There are many more CD4$^+$ T cells (48.3 ± 18.2) and CD8$^+$ T cells (55.9 ± 27.9) in the dermal infiltration than those of normal controls (2.9 ± 1.0 and 2.5 ± 0.6, respectively). It was detected that the number of CD8$^+$ cells were appreciably higher than CD4$^+$ cells in the DLE lesions. This was appreciably different from other authors (*11,12*), but a similar result was also obtained by Wenzel *et al*. (*13*). The CD4/CD8 ratio, which ranged approximately from 0.7 to 1.7 (mean 1.0), was a slight decrease compared to that in normal skin (1.1), but there was no statistical significance between them. Wouters *et al*. (*14*) studied the circulating lymphocyte profiles in patients with DLE and found that the levels of CD4$^+$ T cells were similar in DLE patients and healthy controls, but that CD8$^+$ T cells were decreased both in absolute numbers and percentages, which resulted in a mild increase of the CD4/CD8 ratio. The decreased number of circulating CD8$^+$ T cells found in their study might result from a shift of these cells to the skin that may be involved in T-cell mediated cytotoxic damage of basal keratinocytes. This may be compatible with the immunohistological finding of CD8$^+$ T cells close to damaged keratinocytes in lesional epidermis (*14*).

In this study we determined the presence of B lymphocytes in cutaneous lesions based on the CD20 marker. CD20 is a protein solely expressed on B

Table 3. Results of immunohistochemical staining

CD mAb	Patients (n = 5)	Control (n = 3)	p value
CD1a$^+$ (per HPF dermis)	2.3 ± 1.3	1.1 ± 0.1	> 0.05
CD3$^+$ (per HPF dermis)	94.1 ± 51.8	5.8 ± 5.3	< 0.05
CD4$^+$ (per HPF dermis)	48.3 ± 18.2	2.9 ± 1.0	< 0.05
CD8$^+$ (per HPF dermis)	55.9 ± 27.9	2.5 ± 0.6	< 0.05
CD4/CD8 ratio	1.0 ± 0.4	1.1 ± 0.2	> 0.05
CD20$^+$ (per HPF dermis)	21.3 ± 16.4	0.6 ± 0.9	< 0.05
CD25$^+$ (per HPF dermis)	6.7 ± 1.9	2.5 ± 1.8	< 0.05
CD30$^+$ (per HPF dermis)	4.0 ± 3.3	0.5 ± 0.7	> 0.05
CD57$^+$ (per HPF dermis)	11.8 ± 4.8	2.4 ± 0.6	< 0.05

Data are mean ± S.D. $p < 0.05$ was considered statistical significance.

lymphocytes (*15*). In our sections, B cells were found in perivascular aggregates, surrounded by T lymphocytes. The number of $CD20^+$ cells (21.3 ± 16.4) in the infiltration of DLE lesions was significantly higher than that in normal skin (0.6 ± 0.9) and there was a statistical significance ($p < 0.05$). The mean percentage of $CD20^+$ cells was 11.6% of the inflammatory cells. Previous studies reported that B lymphocytes were rare or absent (< 5%) in DLE lesions (*11,16-18*). However, Akasu *et al*. observed B lymphocytes accounted for > 25% in one of his five DLE cases (*19*). Wolfgang *et al*. found B lymphocytes were over 5% of the inflammatory cells in 31% of their DLE cases and in 8 of 49 cases, and that B lymphocytes accounted for > 20% of the inflammatory cell infiltrate. In short, our study substantiated Akasu and Wolfgang's findings that numerous B lymphocytes may be found in DLE lesions. In a circulating lymphocyte study (*14*), the percentage of $CD19^+$ B cells was increased in DLE patients compared with healthy controls, which was compatible with earlier studies that demonstrated increased percentages of cytoplasmic immunoglobulin-containing and immunoglobulin-secreting cells in the DLE peripheral blood. The role of B lymphocytes in DLE is unknown, but local secretion of specific antibodies is possible, which may act through mechanisms such as opsonization and activation of the complement system.

CD25 is the marker for human interleukin (IL)-2 receptor. In this study the percentage of $CD25^+$ cells in dermal inflammatory cells was relatively low (6.7 ± 1.9), just as previously described by other authors (*11,20,21*), however, it was slightly increased compared to that in normal skin (2.5 ± 1.8) and there was statistical significance. Following the activation of T cells with antigen or mitogen in the presence of the IL-1, IL-2 is rapidly synthesized and secreted. In response to this, a subpopulation of T cells expresses high affinity receptors for IL-2. These cells proliferate and expand the T cell population which is capable of mediating helper, suppressor and cytotoxic functions. The activation of T lymphocytes by IL-2 suggests that non-specific, non-major histocompatibility complex (MHC)-restricted mechanisms of cellular cytotoxicity is involved in the pathogenesis of the skin lesions in LE (*11*).

CD30 or Ki-1 antigen expression was rarely seen in dermal DLE infiltration. Comparably, normal skin controls in this study showed less positive findings. CD30 has been suggested to be a marker for Th2 cells (*22*). Our result indicates that the Th2 cells may not be included in the dermal immunoreaction in DLE.

We found an increased number (11.8 ± 4.8) and percentage (11.3%) of $CD57^+$ NK cells in DLE compared with normal skin and there was statistical significance. NK cells are thought to play an important role in tissue damage as well as in modulation of B cell activity (*23*). Wouters *et al*. (*14*) found a lower number of $CD57^+$ cells in peripheral blood in DLE. They hypothesized that the reduction of these subsets in peripheral blood may be the result of skin recruitment. Our result provided evidence for their hypothesis. Their study proved the systemic activation of the cellular immune system in DLE.

The result of this study revealed that T lymphocytes, B lymphocytes cells, and natural killer cells may play some roles in DLE pathogenesis. The inflammatory effector mechanisms of DLE may be confined to the skin and mediated by T cells, B cells, and NK cells which may migrate from the circulation to the site of inflammation. The number of patients included in this study was not large. Further studies are needed to analyze the *in situ* participation of key cytokines on these cells to better understand the pathogenesis of this disease.

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