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Characterisation of the Inflammatory Infiltrate in Deep Lesions of Localized Scleroderma Compared to Erythema Nodosum

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Abstract

Background: Involvement of deep structures, predominantly the subcutis, may occur in many subtypes of localized scleroderma (LS).

Objectives: The purpose of the present study was to characterize the inflammatory infiltrate in deep lesions of LS (DLLS) with affection of the subcutaneous fat tissue, and to compare the findings with erythema nodosum (EN).

Methods: We assessed 22 patients with DLLS lesions who predominantly suffered from limited (n=8), generalized (n=8), linear (n=4), or deep (n=2) LS. Moreover, we studied 20 patients with EN. In each patient, a spindle-shaped full thickness skin excision was performed before the initiation of any antiphlogistic or immunosuppressive treatment. Biopsy specimens were processed for routine histology and immunohistochemistry for CD3, CD4, CD8, CD20, CD15, and CD68.

Results: Immunohistology of DLLS did not reveal CD4+ infiltrating cells. Immunolabelling for CD3, CD8, and CD68 was significantly higher in lesional skin of EN patients as compared to patients with DLLS. CD20 protein expression did not significantly differ between DLLS and EN. CD15 was negative in DLLS as well as EN. An analysis of LS subtypes did not reveal significant differences with regard to immunolabelling for the markers assessed.

Conclusions: DLLS may occur in many LS subtypes and appears to be immunohistologically characterized by an inflammatory infiltrate consisting of CD3+, CD8+, and CD68+ cells and the absence of CD4+ T lymphocytes. DLLS can be differentiated from EN showing significantly more infiltrating CD3+, CD8+, and CD68+ cells.

Introduction

Localized scleroderma (LS) comprises a heterogeneous spectrum of connective tissue diseases that primarily affected the skin, but may also involve other underlying structures such as the subcutis, fascia, muscle, or bones. Accumulating evidence indicates that LS is an autoimmune disorder. According to a recent classification, [1] LS is divided into four basic subtypes: limited, generalized, linear, and deep. The deep type of LS is by far the most rare variant (<5% of patients). In the deep type, fibrosis mainly affects the deeper layers of the connective tissue, i.e., fat tissue, fascia, and underlying muscle. Nonetheless, all types of LS with extracutaneous involvement may show more or less subcutaneous involvement [1-7]. So far, inflammatory cells of deep lesions of LS (DLLS) with affection of the subcutaneous fat tissue have never been immunohistologically characterised and compared to other types of septal panniculitis such as erythema nodosum (EN). The latter is the most frequent clinicopathologic variant of panniculitis. The process is a cutaneous reaction that may be associated with a wide variety of disorders, including infections, sarcoidosis, rheumatologic diseases, inflammatory bowel diseases, medications, autoimmune disorders, pregnancy, and malignancies. Histopathologically, erythema nodosum is the stereotypical example of a mostly septal panniculitis without vasculitis [8-10]. The purpose of the present study was to characterize the inflammatory infiltrates in DLLS, and to compare the findings with EN.

Materials and Methods

We assessed 22 patients (8 males, 14 females; 54.9 ± 14.6 years) with DLLS lesions who predominantly suffered from limited (n=8), generalized (n=8), linear (n=4), or deep (n=2) LS [1]. The lesions in which biopsies were taken were located at the abdomen (n=7), the chest (n=5), the legs (n=4), the back (n=2), the buttocks (n=1), the arms (n=1), the face (n=1), and the axillar region (n=1). As inclusion criterias for the study, duration of disease had to be shorter than 1 year, and patients had to be naïve to any kind of topical or systemic anti-inflammatory therapy. Exclusion criteria were any concomitant malignant diseases or other types of panniculitis. According to IgM and IgG immunoblot assays, patients with DLLS had negative *Borrelia burgdorferi* serology. Moreover, as a comparative type of panniculitis, we studied 20 patients (5 males, 15 females; 40.4 ± 12.6 years) with EN. In 17 of the 20 EN patients, lesions were located at the lower legs and

in 3 patients, the upper legs were affected. Mostly, EN was associated with infections (n=9), medication (n=2), and sarcoidosis (n=1). In eight patients, no underlying trigger for EN was found. In each patient, a spindle-shaped full thickness skin excision (15 mm×10 mm) was performed before the initiation of any antiphlogistic or immunosuppressive treatment such as glucocorticosteroids. Only clearly palpable and indurated lesions were selected for biopsy. Biopsy specimens were fixed in 10% formalin, step-sectioned, and stained with hematoxylin-eosin for routine histology. Immunohistochemical staining for CD3, CD4, CD8, CD20, CD15, and CD68 was performed according to standard procedures [alkaline phosphatase anti-alkaline phosphatase (APAAP) technique using the labelled streptavidin-biotin (LSAB) method] (Table 1). Specificity testing was performed by blocking of the primary antibody and negative control staining was performed by omitting the primary antibody. More details on immunohistochemistry performed in the present study are described in Table 2. All immunohistochemical slides were separately evaluated by two observers for patterns of immunohistochemical labeling. Five randomly chosen fields of view under a light microscope at 100xmagnification were assessed in the deep dermis as well as entire subcutis. Quantitative results were expressed as the percentage of positively stained infiltrate cells per field on total infiltrate cell count. Data analysis was performed using the statistical package MedCalc Software (Mariakerke, Belgium). Distribution of quantitative data of immunohistochemistry was assessed by the D`Agostino-Pearson test. For paired data, the 2-sided independent Student-test was used. No adjustment for multiple testing was carried out since this was an explorative study. A P-value<0.05 was regarded as statistically significant.

Cluster of Differentiation (CD) markers	Type of cells
CD3	T lymphocytes
CD4	T helper cells
CD8	Cytotoxic T cells
CD20	B cells
CD15	Eosinophils, neutrophils, monocytes
CD68	Macrophages, monocytes

Table 1: Immunohistochemical markers used for immunophenotyping of inflammatory cells.

Results

On routine histology, DLLS predominantly revealed inflammatory infiltrates localised to the fibrous trabeculae, composed of lymphocytes and plasma cells resulting more or less in the obliteration of the fat lobules. The "center of gravity" for the disease activity of DLLS was the interface of the reticular dermis and subcutis. Tissue eosinophilc infiltrates ranged from none to sparse. Edema and variable mucin deposits were more frequently observed. EN frequently showed changes predominantly confined to the fibrous trabeculae including edema, hemorrhage, and inflammatory infiltrates composed of neutrophils and lymphocytes. Older lesions often exhibited some thickening of the septa and a predominance of lymphocytes. Miescher's granulomas were also observed at this stage. CD3, CD8, and CD68 were moderately expressed in DLLS. However, immunolabelling for CD3, CD8, and CD68 was significantly higher in lesional skin of EN patients as compared to patients with DLLS (Table 2, Figures 1-3). Notably, DLLS did not reveal CD4+ infiltrating cells. CD15 was neither expressed in DLLS nor in EN. CD20 protein expression was moderately expressed in DLLS but did not significantly differ from EN. A subanalysis of LS subtypes did not reveal significant differences with regard to the immunolabelling for CD4, CD8, CD68, and CD20.

Antibody	Source	Pretreatment*	Dilution	DLLS* Median (range)	EN* Median (range)	P-value	Specificity/Effect
CD3	Dako, Hamburg, Germany	Microwave-3-step-technique; 30 min	1:250	15 ± 16.7	30.6 ± 17.6	<0.002	T lymphocytes
CD4	Novocastra Loxo, Dossenheim, Germany	Microwave-3-step-technique; 30 min	1:60	0	5.4 ± 10.37	=0.08	T cell subset (helper/inducer)
CD8	Novocastra Loxo, Dossenheim, Germany	Microwave-3-step-technique; 30 min	1:60	10 ± 12.5	18.3 ± 10.9	=0.0012	T cell subset (suppressor/cytotoxic)
CD20	Novocastra Loxo, Dossenheim, Germany	Microwave-3-step-technique; 30 min	1:50	15.7 ± 25.7	5.6 ± 6.5	=0.77	B lymphocytes

CD15	Novocastra Loxo, Dossenheim, Germany	Microwave-3-step-technique; 30 min	1:1	0	0	-	Neutrophil granulocytes
CD68	Dako, Hamburg, Germany	Protease digestion; 30 min	1:25	14 ± 15.8	38.7 ± 13.4	<0.0001	Macrophages, monocytes

Table 2: Overview on immunohistochemistry performed in patients with deep lesions of localized scleroderma (DLLS) and erythe ma nodosum (EN).

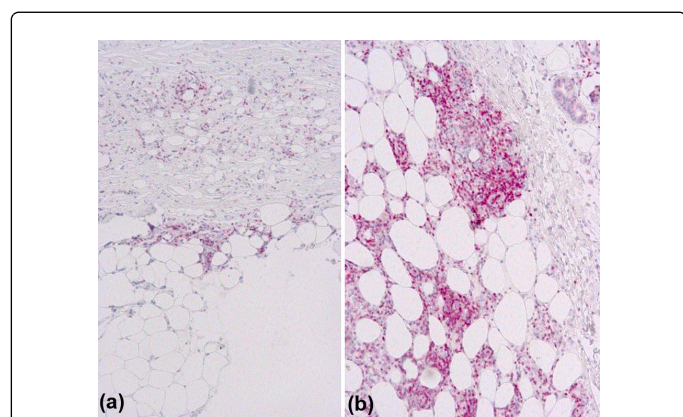*Percentage of positively stained cells.

Figure 1: Immunohistochemical staining for CD3 of subcutaneous fat tissue of patients with localized scleroderma (A) and erythema nodosum (B). The CD3 expression is significantly higher in erythema nodosum as compared to localized scleroderma. These pictures are representative for the whole immunohistochemical study (CD3-staining, original magnification×100).
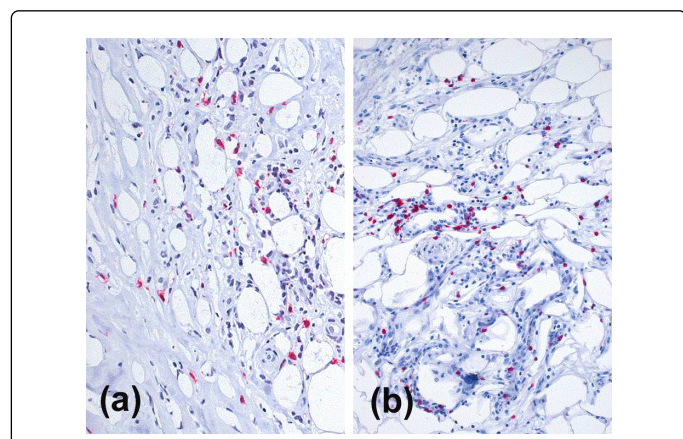

Figure 2: Immunohistochemical staining for CD8 of subcutaneous fat tissue of patients in localized scleroderma (A) and erythema nodosum (B). The CD8 expression is significantly higher in erythema nodosum as compared to localized scleroderma. These pictures are representative for the whole immunohistochemical study (CD8-staining, original magnification×100).

Figure 3: Immunohistochemical staining for CD68 of subcutaneous fat tissue of patients in localized scleroderma (A) and erythema nodosum (B). The CD68 expression is significantly higher in erythema nodosum as compared to localized scleroderma. These pictures are representative for the whole immunohistochemical study (CD68-staining, original magnification×100).
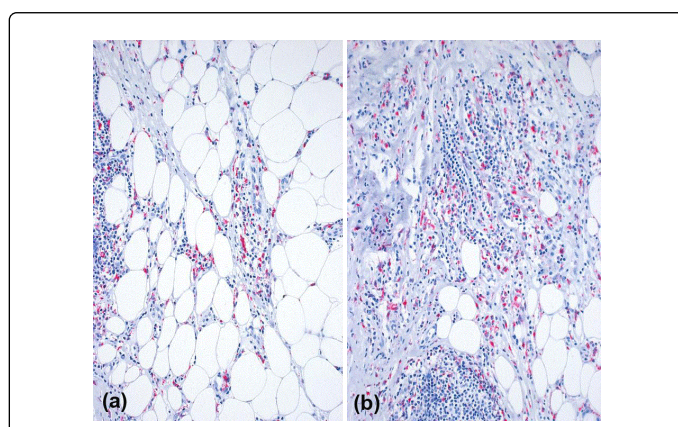
Discussion

Different classification schemes for LS have been proposed for the presentations of disease variants that share overlapping features like panniculitis. LS include clinicopathological variants that extend to involve the panniculus, fascia and underlying muscle. These variants are morphea profunda, linear scleroderma "en coup de sabre", eosinophilic fasciitis, and disabling linear morphea of childhood which correspond in accordance with a current classification to the deep type, linear type, and generalized type of LS, respectively [1-3,7]. Hence, the depth of involvement is likely not a proper discriminator for the classifications of LS subtypes-as demonstrated in the present study involvement of the subcutis is a common feature of many subtypes of LS. The latter can be roughly divided into an inflammatory (early-stage disease) and a sclerotic phase (late-stage disease). The characteristic plaque lesions have central sclerosis; in the early phase the "lilac ring" represents inflammatory changes. More specific histological differentiation of the various clinical subtypes is barely possible. In addition, the histological appearances in LS can strongly resemble those seen in systemic scleroderma and thus the two entities cannot be differentiated histologically. In the early stage, typical findings include dense perivascular and periadnexal inflammatory infiltrates in the reticular dermis. The infiltrates may also be detected in the subcutis. Lymphocytes predominate, but plasma cells and histiocytes are also found. The dermal connective tissue often contains thickened collagen fiber bundles with a superficial parallel

arrangement as well as edema in the upper dermis. In the late phase, the dermis is sclerotic and there is marked reduction of skin adnexa. The eccrine sweat glands are atrophic and are "walled in" by recently performed collagen. Because of dermal thickening and the involvement of subcutaneous fat tissue, they are found higher up in the dermis. Thickening of the walls of small blood vessels has also been observed. There is generally only a small amount of inflammatory infiltrates, and the collagen fibers are tightly packed and highly eosinophilic [2,3,6].

In the present study, we have studied DLLS which were found in different subtypes of LS. Our immunohistology data reveal that the lymphocytic infiltrates in DLLS predominantly consist of CD3+ T lymphocytes-mainly CD8+ T suppressor cells-CD20+ B lymphocytes, and CD68+ histiocytes, whereas CD4+ T helper cells have not been observed. By contrast, previous studies on LS have shown that CD4+ cells belong to the infiltrate as well, [11-14] whereas these studies investigated LS lesions not specifically accounted as DLLS. Hence, deeper skin involvement in LS may be accompanied by changes of the composition of infiltrating cells. When compared to DLLS, the infiltrate of EN consists of significantly higher numbers of CD3+, CD8+, and CD68+ cells. In EN, the septa of subcutaneous fat are always thickened and variously infiltrated by inflammatory cells that extend to the periseptal areas of the fat lobules. The composition of the inflammatory infiltrate in the septa varies with age of the lesion. In early lesions edema, hemorrhage, and neutrophils are responsible for the septal thickening, whereas fibrosis, periseptal granulation tissue, lymphocytes, and multinucleated giant cells are the main findings in late stage lesions of EN. The absence of CD15+ cells in EN of the present study may indicate that we predominantly assessed late-stage EN. A histopathologic hallmark of EN is the presence of the so-called Miescher's radial granulomas, which consist of small, well-defined nodular aggregations of small CD68+ histiocytes arranged radially around a central cleft of variable shape. Long-standing septal panniculitis such as EN may result in septal thickening and fibrosis that may suggest DLLS. However, EN usually do not show the degree of septal sclerosis, associated involvement of the dermis or fascia, or the abundance of lymphoplasma cellular infiltrates as observed in DLLS [6,10,15].

In conclusion, DLLS may occur in many LS subtypes and appears to be immunohistologiclly characterized by moderate infiltrates consisting of CD3+, CD8+, and CD68+ cells and the absence of CD4+ T lymphocytes. DLLS can be differentiate from EN showing significantly more infiltrating CD3+, CD8+, and CD68+ cells. The observations reported in this study should now be confirmed in a larger number of subjects, including additional experimental methods.

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