Diminished reactive oxygen intermediates (ROI) production, decreased the T suppressor cells number and increased cytokine production constitute outline of the lichen sclerosus pathogenesis

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Abstract

The involution of lichen sclerosus (LS) affected tissues may be the effect of suppression exerted by T lymphocytes which are involved in the pathogenesis of this disease. Inflammatory infiltrates in the affected regions of the skin consist mainly of CD3+ lymphocytes consist with diminished number of CD3+/CD26+ subsets. We revealed the diminished ROI production by peripheral blood, granulocytes after receptor dependent and receptor independent stimulation. The diminished number both of CD3+/CD25+ and CD3+/CD26+ in peripheral blood baseline increase of IL-12 and stimulated increase of IL-2, IL-5, IL-10 and TNF-α production by peripheral blood lymphocytes were also noted. We postulate that immunereactions are involved in LS pathogenesis.

Key words: *lichen sclerosus, lymphocytes, cytokines*

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Introduction

Lichen sclerosus (LS) is a chronic dermatosis of unknown etiology and pathogenesis. Morphological and immunohistochemical parameters like T lymphocytes subpopulations, complement fractions, immunoglobulins and several autoantibodies, NK and B cells in affected areas according to Scrimin et al do not confirm the autoimmune or immune pathogenesis for LS [1, 2]. However other authors have proved that LS associated skin lesions shown clonal T cell enriched infiltrates detected by T cell receptor-gamma polymerase chain reaction [10]. The clonal T cells could not be assigned to CD4+ or CD8+ phenotype. The presence of clonally expanded infiltrating T cells detected in LS may be a response to an as yet unknown LS associated antigen [3]. Infiltrating T lymphocyte produce a plethora of regulatory molecules – interleukins which till now were not precisely analyzed in LS. Several lines of evidence have suggested that reactive oxygen intermediates (ROI) serve as messengers of NF-κB activation and interleukin-6 gene expression through activation of IL-6 promotor [4]. The evaluation of ROI production in response to various stimuli is a reasonable model for evaluation of cell reaction because H2O2 is associated with NF-κB activation [5].

The involvement of T cells in inflammatory skin disorders on the level cell-to-cell interaction which depend on the suppression exerted by CD4+ CD25+ cells and by CD26 molecules regarded as an anti-inflammatory principle can argue for immune pathogenesis of LS [6]. Observed in the presented paper decreased number of CD25+ and CD26+

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cells, diminished ROI production and high basal secretion of IL-10 by peripheral blood white cells and overproduction of regulatory cytokines by lymphocytes of LS patients may directly be involved in dysregulated immune response and suggest the participation of activated T cells in LS pathogenesis. We also confirmed increased number of CD3/HLADR in the lichen sclerosus specimens [7].

Materials

We have investigated two groups of patients: 19 healthy woman who undergo plastic surgery, aged from 18 to 78 and 22 women with vulvular lichen sclerosus, aged from 20 to 79.

The examinations were carried out on patients, registered at the Gynaecology Outpatients Clinic of Polish Mother's Memorial Institute. The diagnosis was performed, according to the following histopathological criteria: hyperkeratosis, epithelial thinning, flattening of the rete pegs, follicular plugging, presence of the homogenized zone under the epithelium and chronic inflammatory infiltration, consisting mainly of T-lymphocytes.

Methods

We measured the ROI production by peripheral blood neutrophils and cytokines profile in the lymphocytes of peripheral blood and the percentage of lymphocytes bearing CD25+, CD26+, CD69+, CD71+, and HLADR antigens. The IL-2, IL-5, IL-10, IL-12 and TNF-α cytokine baseline production and after PHA stimulation were also evaluated *in vitro.*

The venous blood was withdrawn between 8–9 a.m. on heparin 10 U/ml (Polfa), all tests were performed within 2h after withdrawal. Peripheral blood lymphocytes (PBL) were isolated on Gradisol G gradient, washed and cultured ($1x10^6$ cells/ml) in a CO_2 (5%) incubator in 0.2 Nunc plates in RPMI medium supplement with 10% foetal calf serum (Hungarpol), glutamine and antibiotics. PHA (Sigma) 10 μg was used as a stimulator. After 72 h culture ($1x10^6$ cells/ml), the cultures were terminated by centrifugation and supernatants were stored at -80°C for interleukins evaluation. The concetrations of: IFN-γ, TNF-α, IL-10, IL-5, IL-4, IL-2 in supernatants of non-stimulated and PHA stimulated culture were performed using CBA (Cytometric Bead Array, Pharmingen) kit according to the manufacturer's instruction. Measurements of IL-12 and TGF-β were performed in non-stimulated and PHA stimulated culture supernatants using ELISA Endogen kits according to manufacturer's instruction. The results were expressed as a pg/ml of studied samples.

Biopsies of lichen sclerosus invaded tissues (*ca2g*) were taken surgically from the most characteristic regions. Excised biopsies were mechanically disrupted using Medimachine (Consul 15) equipped with 50 μm filters. Isolated cells were washed twice in PBS buffer. Non-disrupted fragments were discarded. Obtained single cell suspensions were adjusted to $0.5–1x10^6$ cells/ml. Analyzed tissue fragments were controlled morphologically.

The isolated PBL were washed and next submitted to 72 h culture. The cells isolated from tissue sections were labeled with anti- CD3/CD25, CD3/CD26, CD3/CD69, CD3/CD71, CD3/HLADR antibodies and then submitted to cytometric analyses. The results are expressed in percentages of positive cells.

Activation antigen expression on analyzed cells (CD25+, CD26+, CD69+, CD71+, HLADR+ was evaluated by a double staining procedure with monoclonal antibody (BD). Labeled cells were incubated in room temperature, washed in washing solution, and then fixed in CellFix (BD). The cells were analyzed using a BD FACS Calibur cytometer and CellQuest Software. The results were expressed as the percentage of gated double positive cells for antibodies used versus all lymphocytes tested.

ROI production by peripheral blood granulocytes was performed at the temperature of 37°C, within 45 minutes using MLX luminometer (Dymex USA) equipped in Revelation Software. We measured the luminol enhanced CL response of neutrophiles stimulated by N-fMLP-$2x10^{-6}$ M, OZ-0.3 mg/ml, PMA-200 ng/ml before and after priming with TNF-α 10 ng/ml.

The results were expressed in Relative Light Units (RLU) corrected by the whole blood neutrophils amounts and hemoglobin level according to the formula:

$$CL\ calculated = CL\ measured\ [RLU\ \max] \times \frac{Hb[\%]}{WBC\ [\text{thousand}/100\ \mu L] \times PMN\ [\%]}$$

WBC – white blood cell
CL – chemiluminescence
Hb – haemoglobin
PMN – polymorphonuclear leukocytes

The Priming Index was used to show the effect of TNF-α on neutrophils ROI production after their previous preincubation with TNF-α.

$$Priming\ Index = \left(\frac{CL_{TNF\alpha\ \text{and}\ fMLP} + CL_{unstimulated} - CL_{unstimulated\ with\ TNF\alpha}}{CL_{fMLP}} \times 100 \right) - 100$$

Using luminol-dependent chemiluminescence both extracellular and intracellular release of hydrogen peroxide and superoxide anion were detected in this system [8].

The Regional Ethics Committee approved this project.

Results

The diminished ROI production by lichen sclerosus peripheral blood cells (mostly neutrophils) and measurable, but low priming effect of TNF were noted (Table 1).

Table 1. Reactive oxygen intermediates (ROI) production by neutrophiles of lichen sclerosus patients measured in whole blood (Mean ± SD). The data are presented in RLU (Relative Light Units)

	Without TNF-α / Priming TNF-α	Resting neutrophils	fMLP	OZ	PMA
Control group	-	2.7 ± 1.66	4.8 ± 1.96	25.3 ± 9.66	20.5 ± 6.85
n=19	+	4.4 ± 1.99	7.9 ± 3.01[a]	24.2 ± 8.45	20.0 ± 5.61
Lichen sclerosus vulvae	-	1.3 ± 0.83*	3.3 ± 2.29*	15.7 ± 14.30*	7.9 ± 5.73*
n=22	+	2.6 ± 1.98*	4.5 ± 3.40*	19.0 ± 17.10	9.1 ± 6.82*

n - number of performed investigations
[a] - p<0.05 significant differences between primed and nonprimed ROI production
** - p<0.05 significant differences as compared to control group*

Table 2. The Th1/Th2 regulatory cytokines production by T-lymphocytes whole blood cells measured in the CBA system (Mean ± SD). The data are presented in pg/ml

	Without PHA / PHA	INF-γ	TNF-α	IL-10	IL-5	IL-4	IL-2
Control group	-	<10	<1	2.8 ± 2.80	<1	<1	1.2 ± 0.35
n=19	+	4467.7 ± 532.34	80.4 ± 23.16	178.4 ± 29.20	33.8 ± 5.46	22.5 ± 5.48	6.1 ± 1.34
Lichen sclerosus vulvae	-	<10	<1	30.1 ± 21.88*	<1	<1	1.5 ± 0.49
n=22	+	4179.7 ± 416.67	139.8 ± 25.11*	367.6 ± 95.56*	80.6 ± 18.52*	38.6 ± 9.25	23.7 ± 9.57*

n - number of performed investigations
** - p<0.05 significant differences as compared to control group*

Table 3. The cytokines production by T-lymphocytes isolated from the blood (Mean ± SD). The data are presented in pg/ml

	nonstimulated / PHA	IL-10	IL-12	TGF-β
Control group	-	9.7 ± 3.34	2.4 ± 1.05	1454.1 ± 250.26
n=19	+	254.9 ± 46.08	293.4 ± 65.57	2071.4 ± 254.17
Lichen sclerosus vulvae	-	10.0 ± 2.94	63.5 ± 27.96*	1694.6 ± 114.35
n=22	+	260.6 ± 67.58	254.8 ± 56.77	2161.5 ± 207.15

n - number of performed investigations
**- p<0.05 significant differences as compared to control group*

ROI production measured in whole blood after stimulation of responsive cells by receptor dependent (fMLP, OZ) and receptor independent stimuli (PMA) was significantly diminished in all analyzed systems of LS blood investigations. The celluar redox state regulates nuclear factor-kappa B (NF-κB) signaling system responsible for regulatory cytokine production. In table 2 and 3 enhanced cytokine production was observed after PHA stimulation of whole blood cells and isolated lymphocytes. The significant increase of TNF-α, IL-10, IL-5 and IL-2 was noted after stimulation of whole blood cells with PHA. Isolated lymphocytes baseline IL-12 production was increased (table 2 and 3).

The decreased expression of CD3+/CD25+ and CD3+/CD26+ cells was noted in peripheral blood of LS patients. PHA stimulation enhanced these cells number (Table 4). The T lymphocyte redistribution in the skin demonstrate the decrease of CD3/CD26 and increased number CD3/HLA-DR bearing cells in LS patients (Table 5).

Discussion

The lichenoid infiltrate of lichen sclerosus affected tissue consists predominantly of T-cells as it was proved in biopses analyses [9] and our own data [11].

Table 4. Resting and PHA stimulated T-lymphocytes distribution in peripheral blood (Mean ± SD)

	Resting / With PHA	CD3/CD25	CD3/CD26	CD3/CD69	CD3/D71	CD3/HLA-DR
Control group	-	18.1 ± 3.90	50.4 ± 7.87	7.0 ± 2.16	3.1 ± 1.66	10.4 ± 4.60
n=19	+	66.2 ± 14.54	72.2 ± 14.08	65.8 ± 12.94	20.0 ± 5.61	61.1 ± 17.70
Lichen sclerosus vulvae	-	13.0 ± 5.97*	42.2 ± 10.57*	7.5 ± 4.26	2.5 ± 2.20	12.2 ± 6.76
n=22	+	57.1 ± 21.70	62.6 ± 17.96	48.3 ± 16.27*	40.9 ± 24.62*	60.3 ± 18.30

n - number of performed investigations
**- p<0.05 significant differences as compared to control group*

Table 5. The distribution of T-lymphocytes in skin samples of healthy people compared to lichen sclerosus suffering patients (Mean ± SEM)

		CD3/CD25	CD3/CD26	CD3/CD69	CD3/D71	CD3/HLA-DR
Control group	Tissue	7.9 ± 3.31	21.1 ± 8.06	11.7 ± 4.56	6.6 ± 2.51	4.9 ± 1.10
n=19	whole blood	18.1 ± 1.23	50.4 ± 2.49	7.0 ± 0.68	3.1 ± 0.63	10.4 ± 1.45
Lichen sclerosus vulvae	Tissue	6.0 ± 1.99	12.9 ± 5.20*	7.7 ± 8.49	7.9 ± 3.04	10.5 ± 5.61*
n=22	whole blood	13.0 ± 5.97*	42.2 ± 10.57*	7.5 ± 0.89	1.9 ± 0.31	12.2 ± 1.64

n - number of performed investigations
**- p<0.05 significant differences as compared to control group*

T cells play a major role in inflammatory skin disorders as psoriasis vulgaris or atopic dermatitis [6]. ROI generated derivatives (hydrogen peroxide and chloramines) degrade of NF-κB inhibitor and activate cytokine production in the nucleus of the cells [12]. Human neutrophils contain a significant amount of NF-κB inhibitor, stimulation of these cells resulted in degradation of inhibitor [13]. The interrelationship between ROI production and inflammatory cytokine generation by peripheral blood leukocytes was observed in vitro in children with food allergy [14]. In the presented paper the contrary dependence was observed, diminished ROI production and increased cytokine production by PHA stimulated lymphocytes. Recently it has been demonstrated that regulatory suppressor T cells are present in the peripheral blood of healthy human, and exert function via cell-to-cell contact of regulatory CD3+/CD25+ T cells and suppress T cell proliferation and cytokine production [15, 16]. Consequently the decrease of CD25+ suppressors T cells in peripheral blood of lichen sclerosus patients may lead to regulatory dysbalance in favour of pro-inflammatory mediators production. To verify this assumption the regulatory cytokine overproduction was analyzed both in whole blood (Table 2 – CBA system) and in isolated lymphocytes. The collective conclusion supporte the interrelationship between enhanced production of almost all cytokine tested while the percentages of CD25+ T cells were diminished (Table 4 and 5).

The increased baseline secretion of IL-10 by nonstimulated lymphocytes in patients with lichen sclerosus may also argue for suppressive action of these cell not only by cell to cell contact [17]. Moreover the overproduction of IL-10 is a strong inhibitor of ROI production [18, 19] and may be responsible for diminished ROI production as it was noted in our patients.

Regulatory T-cells (CD25+) downregulate of T-cell response and selectively inhibits the host immune response and therefore their decrease could contribute to the progression of lichen sclerosus. The suppressive effect of anergic T-cells was explained by their ability to induce the inhibition of the IL-2 production [20].

CD26 is a lymphocyte associated dipeptidyl peptidase, which is able to inactivate chemokines and can be regarded as an anti-inflammatory molecule. The decreased expression of CD3+/CD26+ as observed in presented paper may lead to increased proinflammatory mediators production [6, 7]. The diminished number of cells heaving CD26 molecules support the assumption that failed chemokine inactivation by CD3+/CD26+ may be involved in LS pathogenesis. The increase of IL-12 baseline production by cultured lymphocytes, diminished the ROI production, taken together

with depressed cytokine production and decrease number of suppressive cells have prove that immune reactions are involved in the pathogenesis of LS.

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