

HUMAN $T\gamma\delta$ lymphocytes constitute from 1 to 15% of all peripheral blood lymphocytes. Recent work has demonstrated that this population plays a major role in the pathogenesis of infectious and immune diseases. Increased numbers of $\gamma\delta$ T cells have been found in affected skin from systemic sclerosis and chronic cutaneous lupus erythematosus patients.

In our study, we have determined the numbers of $T\gamma\delta$ lymphocytes and their subpopulations in peripheral blood from 29 patients with systemic lupus erythematosus (SLE) and in 19 healthy volunteers using flow cytometry and specific monoclonal antibodies. The same cells in uninvolved skin from SLE patients and human controls using immunohistochemical analysis were estimated. T-Cell receptor (TCR) delta chain gene rearrangement was identified with primers for $V\delta 1$, $V\delta 2$ and $V\delta 3$ by the polymerase chain reaction. Statistical analysis showed a significantly decreased number of $\gamma\delta$ T cells in SLE patients ($26.4 \pm 16.9/\mu\text{l}$) compared with the control group ($55.3 \pm 20.6/\mu\text{l}$) ($p < 0.001$). The number of $V\delta 2$ TCR+ and $V\gamma 9$ TCR+ subpopulations was also lower in SLE patients than in healthy persons. No statistical correlation between disease activity and the number of $\gamma\delta$ T cells was demonstrated. The percentage of $T\gamma\delta$ lymphocytes in clinically normal skin from SLE patients was twice ($22.0 \pm 9.4\%$) that found in the skin from healthy persons ($11.1 \pm 5.5\%$) ($p < 0.002$). Higher percentages of the $V\delta 2$ TCR+ and $V\gamma 9$ TCR+ subpopulation of lymphocytes were found in the skin from SLE patients. We have also found positive correlation between the percentage of $T\gamma\delta$ lymphocytes in skin and the activity of SLE ($r = 0.594$, $p < 0.001$), and between subpopulation $V\delta 3$ TCR+ and disease activity ($r = 0.659$, $p < 0.001$). In conclusion, the results of our studies demonstrate that, in patients with SLE, accumulation of $T\gamma\delta$ lymphocytes can be seen in clinically normal skin, and the percentage of these cells correlates with the activity of the disease.

Key words: SLE, $\gamma\delta$ T cells, Peripheral blood, Skin, Disease activity, Prednisone, PCR, Immunohistochemistry

Lymphocytes $T\gamma\delta$ in clinically normal skin and peripheral blood of patients with systemic lupus erythematosus and their correlation with disease activity

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Introduction

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disorder of unknown origin with many different clinical manifestations. SLE can present with a wide range of immunological abnormalities, such as the production of a variety of autoantibodies by activated B cells, the presence of circulating immune complexes and a reduction in complement levels.^{1,2} T cells may have an important role in inducing B-cell hyperactivity.^{3,4} T cells in SLE show multiple abnormalities that include the presence of anti-T-lymphocyte antibodies, CD4 epitome deficiency, low levels of interleukin-2 (IL-2) receptor expression and increased levels of soluble forms of

the IL-2 receptor.⁵ The most common changes in peripheral blood mononuclear cell subsets are a reduction of CD4+ T cells and an increase of CD8+ cells with imbalance of the CD4/CD8 ratio.⁶

Most mature human T cells express T-cell receptors (TCRs) on the membrane in association with the signal transduction CD3 complex. These cells are the central lymphocytes in the immune system. They provide specific pathogen recognition and long-term memory, all within the context of distinguishing foreign antigens from self-antigens.^{7,8} Another CD3-associated TCR complex is composed of γ - and δ -polypeptides (TCR $\gamma\delta$), which has been identified in a small population of double-negative (CD4⁻/CD8⁻) or dimly CD8+ peripheral blood lymphocytes, tumor cell

lines, thymocytes and T-cell clones.⁹⁻¹¹ TCR $\gamma\delta$ molecules consist of disulfide-linked or non-disulfide-linked heterodimer depending on the use of C γ 2 or C γ 1 segment-derived TCR γ chains, respectively¹² TCR $\gamma\delta$ cells rearrange and express clonally diverse antigen receptors similarly to TCR $\alpha\beta$ cells.¹³ Human $\gamma\delta$ T cells range from 1 to 15% of peripheral blood lymphocytes and show a predilection for the red pulp of the spleen and gastrointestinal tract.^{14,15} Increased levels of $\gamma\delta$ T cells were detected in damaged skin from patients with infections and immune diseases, such as leprosy,¹⁶ cutaneous leishmaniasis,¹⁷ systemic sclerosis,¹⁸ and chronic cutaneous lupus erythematosus.¹⁹ Clonal expansion of TCR $\gamma\delta$ T cells has also been reported in the peripheral blood of patients with SLE.²⁰ In contrast, we and other workers have shown that the absolute number of $\gamma\delta$ T cells in peripheral blood was lower in SLE patients than in healthy controls.^{21,22}

The pathologic relevance of $\gamma\delta$ T cells in autoimmune diseases is suggested by their reactivity to highly conserved stress proteins and by the accumulation of these cells in affected organs.^{17,23,24} There are also suggestions that $\gamma\delta$ T cells participate in both the regulation and the propagation of systemic autoimmunity.²⁵

In the present study, we have measured the number of TCR $\gamma\delta$ cells and their subpopulations in peripheral blood and in clinically normal skin of SLE patients. We have also correlated the number of $\gamma\delta$ T cells with disease activity.

Patients and methods

Clinical findings

The study involved 29 patients (27 females and two males), aged 18–66 years (mean, 42 years). The diagnosis of SLE was based on the revised criteria of the American Rheumatism Association (now known as the American College of Rheumatology).²⁶ The mean duration of the disease was 72 months (range, 1 month–11 years). Twenty-two patients had never been treated with steroids or any other immunosuppressive agents. Six patients were treated with prednisone at a dose of 5–20 mg/day during the study and one patient with prednisone and azathioprine.

Patients with both active and inactive disease were included in the study. In all patients, the activity of the disease was determined according to the systemic lupus activity measure (SLAM) scale, as described by Linker-Israeli *et al.*³ This scale lists 32 clinical and laboratory manifestations of SLE. The maximum score in this system is 84 points. In our group of patients, the number of points ranged from 9 to 25. In the present study, we considered the score of 0–15 points for inactive disease and the score of over 15 points for active disease. By this

Table 1. Clinical and laboratory characteristics of the patients with SLE

| Symptoms | Number of patients | % |
|---|--------------------|-------|
| Total | 29 | 100.0 |
| Active | 15 | 51.7 |
| Inactive | 14 | 48.3 |
| Fever | 6 | 20.7 |
| Arthritis | 24 | 82.7 |
| Skin symptoms | 14 | 48.3 |
| Cardiovascular symptoms | 6 | 20.7 |
| Neurologic symptoms | 24 | 82.8 |
| Antinuclear antibodies | 26 | 89.7 |
| Immunoglobulin deposits at the dermal-epidermal junction | 15 | 51.7 |
| Anemia (hemaglobin <12 g/dl) | 10 | 34.5 |
| Leukopenia (white blood cells <3.5 × 10 ⁹ /l) | 10 | 34.5 |
| Thrombocytopenia (<150 × 10 ⁹ /l) | 8 | 27.6 |
| Raised ESR* (>25 mm/h) | 24 | 82.8 |
| Treatment with steroids during the study | 6 | 200.7 |
| Treatment with steroids and cytotoxic agents during the study | 1 | 3.5 |

*ESR, erythrocyte sedimentation rate.

definition, active disease was found in 15 patients while 14 patients had inactive disease.

The clinical and laboratory features of SLE patients are presented in Table 1. The control group for peripheral blood investigation comprised 19 healthy volunteers (17 women and two men), aged from 35 to 58 years (median, 45 years). Each person underwent a thorough physical evaluation by one of the authors (E.R.). Samples of normal skin were excised from 10 individuals during an operation performed because of trauma. Skin samples from the patients and control group were collected from a clinically normal, sun-exposed area.

The patients with SLE and controls showed no clinical signs of an infectious or neoplastic disease and were not given antibiotics or any other antibacterial or antiviral medication for at least 4 weeks before skin or blood donation. Informed consent was obtained from all patients and normal individuals participating in the study, which was approved by the local ethics committee.

Blood samples and immunophenotype analysis

Venous blood samples obtained from all patients and control individuals were collected at the time of clinical assessment in pyrogen-free tubes containing anticoagulant (ethylenediamine tetraacetic acid at a final concentration of 25 nM). Lymphocyte immunophenotyping and TCR diversity analysis was performed by EPICS flow cytometer S-XL (Coulter, Hialeah, FL, USA) and standard two-color immunofluorescence measurement. The details of the procedure have been described in our previous studies.^{21,27,28} Briefly, a combination of phycoerythrin-conjugated and fluorescein isothiocyanate-conjugated monoclonal antibodies (MoAbs) was

used. In polystyrene tubes, 100 μ l samples of whole blood were directly stained with 10 μ l of appropriate MoAbs in the dark, at room temperature. Immunoglobulin (Ig) G₁ isotype control antibody conjugates were included to establish the background fluorescence. After incubation for 30 min, the samples were placed to Q-prep (Coulter) for lysis of erythrocytes and fixation of nuclear cells. At least 10,000 cells were then analyzed. Gate check was used to gate lymphocyte population defined by FS/SS and anti-CD14 and CD45 RO MoAbs. Analysis was performed using XLv2 software.

Immunohistochemistry of skin biopsies

In SLE patients, biopsies from clinically normal skin were taken from the wrist during routine diagnostic procedure of Ig deposit examination. Control skin samples were also collected from sun-exposed areas. The samples were kept at -80°C . Frozen section was carried out with a microtome (cryocut) with 3 μ m layers each for histochemical study in the detection system LSAB PLUS PAP (DAKO), according to the method described by Hsu *et al.*²⁸ The examination was carried out using monoclonal murine antibodies DAKO against CD4, CD3, CD8 and Immunotech against panTCR $\gamma\delta$, V δ 2, V δ 3 and V γ 9. The presence or absence of expression of a particular antigen in lymphoid T cells was evaluated in several randomly chosen visual fields of a given preparation. Using Hogg's grid, all cells in the infiltrate were calculated, determining the percentage of cells with positive reactions in relation to all cells of the infiltrate in the examined visual fields.

The results were expressed as percentages. Throughout the whole period of study, pathologists were not informed about the source of tissue samples.

Analysis of TCR gene rearrangements by polymerase chain reaction

Frozen slices of skin specimens from studied patients were used for DNA isolation by routine proteinase K digestion and the phenol-chloroform extraction procedure.²⁹

The multiplex polymerase chain reaction (PCR) procedure for the identification of TCR δ chain gene rearrangement described by Sambrook *et al.*³⁰ was used to identify the repertoire of TCR $\gamma\delta$ expressed on T lymphocytes in skin specimens. Three pairs of primers were used together:

Forward primers:

V δ 1: 5'-ACTCAAGCCCAGTCATCAGTATCC-3'

V δ 2: 5'-ACCAAACAGTGCCTGTGTCAATAGG-3'

V δ 3: 5'-TGAGGTGGTACTGCTCTGCACTTACG-3'

Reverse primer:

J δ 1: 5'-ACCTCTTCCCAGGAGTCCTCC-3'

Briefly, the genomic DNA was amplified in 20 μ l of 1 \times PCR buffer containing 200 μ mol of each dNTP, 3 pmol of each primer and 1 U of Taq polymerase. The amounts of rearranged DNA obtained from the analyzed samples (especially from normal skin infiltrating lymphocytes) were low. In that case, the PCR reaction was performed with α -³²P-dCTP (370 MBq/ml) to improve the sensitivity of amplification (0.25 μ l per tube). Thirty cycles were run on a thermocycler (Perkin Elmer 2400) under the following conditions: denaturation at 94 $^{\circ}\text{C}$ for 1 min (10 min in the first cycle), annealing at 64 $^{\circ}\text{C}$ for 1 min, extension at 72 $^{\circ}\text{C}$ for 1.5 min (7 min in the last cycle). The radioisotope-labeled PCR sample products were resolved using horizontal 3% agarose gel electrophoresis. Then gel was vacuum-dried and the reaction products were visualized by autoradiography. The intensity of bands (with sizes for different rearrangements between 200 and 400 base pairs) was analyzed with Molecular Analyst software using the Gel Doc 1000 Bio-Rad system. The relative proportions of different V δ lymphocyte subsets were calculated.

Statistical analysis

To statistically calculate the obtained data the range of measured variables has been given (min-max), and mean arithmetic values (\bar{x}) and standard deviation (SD) were calculated. The type of distribution of the studied features was evaluated with the Shapiro-Will test. The comparison of values of type variables in two groups were performed depending on the distribution of features with the Mann-Whitney test or a test for two means from small samples (in this case, the condition of homoscedasticity was checked by the Fischer test). If the condition of homoscedasticity was not met, comparison between mean values was carried out with the Cochran-Cox test. The differences in mean values in three groups were evaluated with the Kruskal-Wallis test or by the analysis of variance. Then, the Mann-Whitney test or Tukey test performed indirect comparisons. The correlation between features was evaluated by straight-line correlation coefficient (r) or by Spearman rank coefficient.

Results

In the present study, we have evaluated the number of T $\gamma\delta$ cells and their subpopulations in the peripheral blood and in normal skin of 29 SLE patients. The characteristics of the studied patients are presented in Table 1. The control group comprised of 19 healthy sex- and age-matched volunteers, and 10 patients undergoing cosmetic surgery procedures.

The expression of TCR on peripheral blood lymphocytes and also the number of CD3+, CD4+ and CD8+ lymphocytes were calculated by standard, double color immunofluorescence measurement. The results

are presented in Table 2. Our results show a significantly lower number of $\gamma\delta$ T cells in SLE patients ($26.4 \pm 16.9/\mu\text{l}$) than in healthy persons ($55.3 \pm 20.6/\mu\text{l}$) ($p < 0.001$). However, no statistically significant correlation between the concentration of these cells and clinical activity of the disease was found ($p > 0.05$) (Fig. 1). Among three $\gamma\delta$ T subpopulations, only the concentration of V δ 3 TCR+ was over twice higher in SLE (4.8 ± 6.8) patients, compared with the control group (2.2 ± 3.1), both in the active (5.0 ± 8.4) and inactive stage of the disease (4.5 ± 4.8), but the difference was not significant statistically ($p > 0.05$). The concentration of other $\gamma\delta$ T subpopulations and CD3+, CD4+ and CD8+ cells was statistically significantly lower in the patients with SLE than in the control group (Table 2). T $\gamma\delta$ lymphocytes in clinically normal skin of SLE patients and control patients were evaluated by the immunohistochemical method. TCR gene rearrangement analysis was carried out by the

PCR method (Fig. 2). The accumulation of particular T-lymphocyte subpopulations in the skin of SLE patients and in healthy persons is presented in Table 3. In SLE patients, the percentage of T $\gamma\delta$ lymphocytes in the skin was twice higher ($22.0 \pm 9.5\%$) than in the skin of healthy persons ($11.1 \pm 5.5\%$) ($p < 0.002$). A higher percentage of these cells was also noted in patients with active disease ($27.0 \pm 9.4\%$) than in non-active SLE ($16.6 \pm 5.6\%$) ($p < 0.002$). Similar differences were noted in the percentage of V δ 2 TCR+ and V γ 9 TCR+ subpopulations in SLE patients and in the skin of healthy persons (Table 3). However, the differences in the percentage of V δ 3 TCR+ subpopulations were not statistically significant. Similarly, a higher percentage of this subpopulation was observed in patients with active SLE ($10.5 \pm 4.8\%$) than in patients with non-active SLE ($6.8 \pm 3.5\%$; $p < 0.03$) and in healthy persons ($3.6 \pm 3.1\%$, $p < 0.02$). It should also be pointed out that the percentage of

Table 2. Analysis of $\gamma\delta$ TCR expression on peripheral blood T cells in patients with SLE and healthy donors (cell donors/ μl)

| Cell subpopulation | SLE (n = 29) | | | Control (n = 19) | Comparison | p value |
|-------------------------|--------------------|---------------------|-----------------------|--------------------|-------------|---------|
| | Total (n = 29) (a) | Active (n = 15) (b) | Inactive (n = 14) (c) | | | |
| Pan $\gamma\delta$ TCR+ | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 26.4 \pm 16.9 | 25.7 \pm 14.6 | 27.3 \pm 19.7 | 55.3 \pm 20.6 | (b)-(c)-(d) | <0.001* |
| Median | 27.0 | 27.09 | 23.765 | 50.0 | (b)-(c) | >0.05 |
| Range | (2.0-68.8) | (8.0-68.8) | (2.0-59.4) | (20.0-90.0) | (b)-(d) | <0.001* |
| | | | | | (c)-(d) | <0.001* |
| V δ 2 TCR+ | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 14.2 \pm 12.6 | 16.5 \pm 12.7 | 11.7 \pm 12.4 | 38.1 \pm 23.6 | (b)-(c)-(d) | <0.001* |
| Median | 12.5 | 12.88 | 9.225 | 30.0 | (b)-(c) | >0.05 |
| Range | (0.0-53.7) | (0.0-53.7) | (1.1-49.3) | (4.0-70.0) | (b)-(d) | <0.002* |
| | | | | | (c)-(d) | <0.001* |
| V δ 3 TCR+ | | | | | (a)-(d) | >0.05 |
| Mean \pm SD | 4.8 \pm 6.8 | 5.0 \pm 8.4 | 4.5 \pm 4.8 | 2.2 \pm 3.1 | (b)-(c)-(d) | >0.05 |
| Median | 2.24 | 1.40 | 2.905 | 0.20 | (b)-(c) | >0.05 |
| Range | (0.0-25.0) | (0.0-25.0) | (0.0-17.4) | (0.08-9.0) | (b)-(d) | >0.05 |
| | | | | | (c)-(d) | >0.05 |
| V γ 9 TCR+ | | | | | (a)-(d) | <0.002* |
| Mean \pm SD | 16.5 \pm 13.5 | 16.7 \pm 12.0 | 16.2 \pm 15.4 | 41.3 \pm 31.1 | (b)-(c)-(d) | <0.005* |
| Median | 13.86 | 13.86 | 13.65 | 30.0 | (b)-(c) | >0.05 |
| Range | (0.0-57.1) | (0.0-49.8) | (1.1-57.1) | (4.0-100.0) | (b)-(d) | <0.01* |
| | | | | | (c)-(d) | <0.004* |
| CD3+ | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 737.9 \pm 485.1 | 897.85 \pm 419.0 | 566.6 \pm 506.6 | 1174.9 \pm 336.7 | (b)-(c)-(d) | <0.001* |
| Median | 775.0 | 872.16 | 486.445 | 1201.0 | (b)-(c) | >0.05 |
| Range | (12.0-1782.5) | (376.1-1782.5) | (12.0-1741.7) | (510.0-1770.0) | (b)-(d) | <0.02* |
| | | | | | (c)-(d) | <0.001* |
| CD4+ | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 203.4 \pm 188.3 | 267.5 \pm 215.7 | 134.6 \pm 128.2 | 471.2 \pm 185.2 | (b)-(c)-(d) | <0.001* |
| Median | 148.12 | 161.0 | 111.86 | 518.0 | (b)-(c) | >0.05 |
| Range | (6.0-723.0) | (44.0-723.0) | (6.0-449.1) | (197.0-891.0) | (b)-(d) | <0.008* |
| | | | | | (c)-(d) | <0.001* |
| CD8+ | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 281.8 \pm 214.0 | 340.5 \pm 215.0 | 218.9 \pm 201.6 | 451.7 \pm 118.9 | (b)-(c)-(d) | <0.001* |
| Median | 232.0 | 256.68 | 199.43 | 430.0 | (b)-(c) | >0.05 |
| Range | (4.0-815.0) | (114.5-815.0) | (4.0-619.1) | (271.0-632.0) | (b)-(d) | <0.005* |
| | | | | | (c)-(d) | <0.001* |

* Statistically significant difference.

FIG. 1. Correlation between the number of $\gamma\delta$ T cells and their subpopulations in peripheral blood with SLE activity score. The disease activity was determined according to the SLAM scale as described by Linker-Israeli *et al.*³ Lymphocyte immunophenotyping analysis was performed by the direct staining of cells with commercially prepared MoAbs and standard two-color immunofluorescence measurement using EPICS flow cytometer S-XL. The correlation between features was evaluated by Spearman rank coefficient. No statistically significant correlation between the concentration of investigated cells and clinical activity of SLE was found ($p > 0.05$).

CD3+ lymphocytes in the skin of SLE patients and healthy persons was similar (81.4 ± 7.9 and $83.0 \pm 13.4\%$, respectively; $p > 0.05$). We have also found a positive correlation between the percentage of T $\gamma\delta$ lymphocytes in the skin and the activity of the disease ($r = 0.594$, $p < 0.001$) and between the V δ 3 TCR+

subpopulation and the activity of SLE ($r = 0.659$, $p < 0.001$) (Fig. 3). The correlation between infiltration with T $\gamma\delta$ lymphocytes and their subpopulations in the skin of SLE patients and the number of these cells in peripheral blood was also evaluated. A negative correlation between these parameters was noted, but

FIG. 2. Resolution of multiplex PCR products for the identification of TCR δ gene rearrangements. Electrophoresis in 3% agarose gel and autoradiography were performed. Lanes 1–8, control skin specimens; lanes 9–14, unevolved skin specimens of SLE patients; lane B, peripheral blood sample from healthy donor.

Table 3. Accumulation of $\gamma\delta$ T cells in normal skin from SLE patients and normal controls (percentage of cells with positive reactions relative to all cells of the infiltrate in the examined visual fields)

| Cell subpopulation | SLE (n = 29) | | | Control (n = 10) (d) | Comparison | p value |
|-------------------------------------|--------------------|---------------------|-----------------------|----------------------|-------------|---------|
| | Total (n = 29) (a) | Active (n = 15) (b) | Inactive (n = 14) (c) | | | |
| Pan $\gamma\delta$ TCR ⁺ | | | | | (a)–(d) | <0.002* |
| Mean \pm SD | 22.0 \pm 9.4 | 27.0 \pm 9.4 | 16.6 \pm 5.6 | 11.1 \pm 5.5 | (b)–(c)–(d) | <0.02* |
| Median | 20.0 | 26.0 | 16.5 | 11.5 | (b)–(c) | <0.002* |
| Range | (7.0–50.0) | (11.0–50.0) | (7.0–29.0) | (4.0–20.0) | (b)–(d) | <0.001* |
| | | | | | (c)–(d) | <0.03* |
| V δ 2 TCR ⁺ | | | | | (a)–(d) | <0.002* |
| Mean \pm SD | 8.9 \pm 4.5 | 10.5 \pm 4.8 | 6.8 \pm 3.5 | 3.6 \pm 3.1 | (b)–(c)–(d) | <0.002* |
| Median | 8.0 | 10.0 | 6.5 | 2.5 | (b)–(c) | <0.03* |
| Range | (2.0–18.0) | (2.0–18.0) | (2.0–15.0) | (1.0–10.0) | (b)–(d) | <0.002* |
| | | | | | (c)–(d) | <0.02* |
| V δ 3 TCR ⁺ | | | | | (a)–(d) | >0.05 |
| Mean \pm SD | 8.0 \pm 4.5 | 10.1 \pm 4.8 | 5.7 \pm 3.0 | 5.1 \pm 2.5 | (b)–(c)–(d) | <0.003* |
| Median | 7.0 | 11.0 | 6.0 | 4.5 | (b)–(c) | <0.008* |
| Range | (1.0–20.0) | (3.0–20.0) | (1.0–11.0) | (2.0–10.0) | (b)–(d) | <0.007* |
| | | | | | (c)–(d) | >0.05 |
| V γ 9 TCR ⁺ | | | | | (a)–(d) | <0.003* |
| Mean \pm SD | 7.3 \pm 4.6 | 7.9 \pm 5.0 | 6.7 \pm 4.4 | 2.6 \pm 2.3 | (b)–(c)–(d) | <0.008* |
| Median | 6.0 | 7.0 | 6.0 | 1.5 | (b)–(c) | >0.05 |
| Range | (1.0–19.0) | (1.0–19.0) | (1.0–16.0) | (1.0–7.0) | (b)–(d) | <0.004* |
| | | | | | (c)–(d) | <0.02* |
| CD3 ⁺ | | | | | (a)–(d) | >0.05 |
| Mean \pm SD | 81.4 \pm 7.9 | 82.3 \pm 7.3 | 80.4 \pm 8.7 | 83.0 \pm 13.4 | (b)–(c)–(d) | >0.05 |
| Median | 85.0 | 85.0 | 82.5 | 90.0 | (b)–(c) | >0.05 |
| Range | (70.0–95.0) | (70.0–95.0) | (70.0–95.0) | (50.0–95.0) | (b)–(d) | >0.05 |
| | | | | | (c)–(d) | >0.05 |
| CD4 ⁺ | | | | | (a)–(d) | <0.001* |
| Mean \pm SD | 45.0 \pm 8.3 | 46.3 \pm 9.3 | 43.6 \pm 7.2 | 57.0 \pm 5.4 | (b)–(c)–(d) | <0.002* |
| Median | 45.0 | 45.0 | 40.0 | 57.5 | (b)–(c) | >0.05 |
| Range | (30.0–60.0) | (35.0–60.0) | (30.0–55.0) | (45.0–65.0) | (b)–(d) | <0.02* |
| | | | | | (c)–(d) | <0.001* |
| CD8 ⁺ | | | | | (a)–(d) | <0.001* |
| Mean \pm SD | 55.0 \pm 8.3 | 53.7 \pm 9.2 | 56.4 \pm 7.2 | 43.0 \pm 5.4 | (b)–(c)–(d) | <0.002* |
| Median | 55.0 | 55.0 | 60.0 | 42.5 | (b)–(c) | >0.05 |
| Range | (40.0–70.0) | (40.0–65.0) | (45.0–70.0) | (35.0–55.0) | (b)–(d) | <0.02* |
| | | | | | (c)–(d) | <0.001* |

* Statistically significant difference.

FIG. 3. Correlation between the percentage of pan $\gamma\delta$ T cells and their subpopulations determined by the immunohistochemical method in clinically normal skin and SLE activity score. The examination was performed using murine MoAbs against TCR $\gamma\delta$, V δ 2, V δ 3, and V γ 9. Estimation of the percentage of the cells with positive reactions in relation to all the cells in the infiltrate was evaluated. Hogg's grid in the examined visual fields was used. The correlation was evaluated by straight-line correlation coefficient (r) or by Spearman rank coefficient. Positive correlation between the percentage of the T $\gamma\delta$ lymphocytes as well as the V δ 3 TCR+ subpopulation and SLE activity was found.

the differences were not statistically significant ($p > 0.05$) (Fig. 4).

In the analyzed group of 29 SLE patients, six were treated with prednisone during the study. All of them were with inactive disease. The influence of treatment with prednisone on the number $\gamma\delta$ T cells in periph-

eral blood and their percentage in clinically normal skin is presented in Table 4.

The percentage of T $\gamma\delta$ cells in clinically normal skin was lower in the patients with SLE treated with prednisone (mean, 15.3 ± 5.2) than in the patients who were not treated with this agent (mean, $23.7 \pm$

9.5) ($p < 0.04$). Similarly, the percentage of V δ T CR+ subpopulation was lower in patients who were not treated with prednisone (mean, $5.5 \pm 2.7\%$) than in the treated patients (mean, $9.5 \pm 4.6\%$).

The results of the identification of TCR δ gene rearrangements by the PCR method and evaluation of amplified product density on gel are presented in Fig. 2 and in Table 4. It is difficult to compare, on the basis

of molecular assays, the number of T $\gamma\delta$ cells infiltrating skin specimens. Our semiquantitative estimates suggest that the total number of T $\gamma\delta$ cells vary in the range of error between samples from SLE patients and healthy individuals (data not shown). However, we observed the appearance of the V δ 3+ T-cell subset in T-lymphocyte population infiltrating the skin specimens from SLE patients ($22.4 \pm 9.4\%$ for the total

FIG. 4. Correlation between the number of $\gamma\delta$ T cells and their subpopulations in peripheral blood and the percentage of these cells in clinically normal skin of SLE patients. The correlation between features was evaluated by Spearman rank coefficient. Negative correlation between investigated parameters was noted, but the differences were not statistically significant.

Table 4. $\gamma\delta$ T cells in peripheral blood and accumulation of $\gamma\delta$ T cells in clinically normal skin from SLE patients treated and not treated with prednisone during the study

| Cell subpopulation | Cells in peripheral blood/ μ l | | | Percentage of cells with positive reaction relative to all cells | | |
|-------------------------------------|------------------------------------|----------------------------|---------|--|----------------------------|---------|
| | Prednisone (+) (n = 6) | Prednisone (-) (n = 23) | p value | Prednisone (+) (n = 6) | Prednisone (-) (n = 23) | p value |
| Pan $\gamma\delta$ TCR ⁺ | | | | | | |
| Mean \pm SD | 20.8 \pm 13.5 | 27.9 \pm 17.7 | | 15.3 \pm 5.2 | 23.7 \pm 9.5 | |
| Median | 23.765 | 27.09 | >0.05 | 15.2 | 24.0 | <0.04* |
| Range | (3.4–39.0) | (2.0–68.8) | | (9.0–24.0) | (7.0–50.0) | |
| V δ 2 TCR ⁺ | | | | | | |
| Mean \pm SD | 6.5 \pm 5.7 | 16.3 \pm 13.2 | | 5.5 \pm 2.7 | 9.5 \pm 4.6 | |
| Median | 4.225 | 12.88 | >0.05 | 6.0 | 9.0 | <0.05* |
| Range | (1.1–14.0) | (0.0–53.7) | | (2.0–9.0) | (2.0–18.0) | |
| V δ 3 TCR ⁺ | | | | | | |
| Mean \pm SD | 2.5 \pm 2.4 | 5.4 \pm 7.5 | | 6.2 \pm 3.4 | 8.4 \pm 4.7 | |
| Median | 1.665 | 2.31 | >0.05 | 5.5 | 7.0 | >0.05 |
| Range | (0.0–6.8) | (0.0–25.0) | | (2.0–11.0) | (1.0–20.0) | |
| V γ 9 TCR ⁺ | | | | | | |
| Mean \pm SD | 8.9 \pm 7.2 | 18.5 \pm 14.1 | | 5.5 \pm 3.9 | 7.8 \pm 4.8 | |
| Median | 7.645 | 17.94 | >0.05 | 5.0 | 6.0 | >0.05 |
| Range | (1.1–18.0) | (0.0–57.1) | | (1.0–11.0) | (1.0–19.0) | |
| CD3 ⁺ | | | | | | |
| Mean \pm SD | 563.2 \pm 432.6 | 783.5 \pm 496.4 | | 79.2 \pm 10.2 | 82.0 \pm 7.3 | |
| Median | 617.75 | 775.0 | >0.05 | 77.5 | 85.0 | >0.05 |
| Range | (20.1–1044.1) | (12.0–1782.5) | | (70.0–90.0) | (70.0–95.0) | |
| CD4 ⁺ | | | | | | |
| Mean \pm SD | 124.5 \pm 86.7 | 223.9 \pm 203.2 | | 42.5 \pm 8.2 | 45.7 \pm 8.4 | |
| Median | 122.35 | 148.12 | >0.05 | 42.5 | 45.0 | >0.05 |
| Range | (10.1–235.4) | (6.0–723.0) | | (30.0–55.0) | (35.0–60.0) | |
| CD8 ⁺ | | | | | | |
| Mean \pm SD | 218.4 \pm 218.4 | 298.4 \pm 214.7 | | 57.5 \pm 8.2 | 54.3 \pm 8.4 | |
| Median | 189.3 | 232.0 | >0.05 | 57.5 | 55.0 | >0.05 |
| Range | (6.7–605.7) | (4.0–815.0) | | (45.0–70.0) | (40.0–65.0) | |

* Statistically significant difference.

group). Practically, this subpopulation was not found in skin samples from healthy control patients (1.4 \pm 1.9%). We noted a significant relative decrease in the V δ 2⁺ T-cell subset in skin specimens of SLE patients. The relative proportion of the V δ 1⁺ T-cell subpopulation was not significantly changed. The V δ 2⁺ T-cell subpopulation dominates in the blood from healthy donors (more than 80% of pan T lymphocytes), confirming the results of earlier studies.²¹ The diffuse electrophoretic bands of PCR-amplified δ TCR genes revealed their extensive junctional diversity. This may reflect the polyclonal T-cell response to a diverse array of recognizable antigens.

Discussion

The aim of the present study was to quantitatively evaluate the number of T $\gamma\delta$ lymphocytes in peripheral blood and clinically healthy skin of SLE patients and to compare these results with their respective values in healthy individuals. The number of T $\gamma\delta$ lymphocytes in peripheral blood of SLE patients was found to be much smaller than in healthy persons, irrespective of SLE disease activity. Similar differences were observed for

subpopulations V δ 2 TCR⁺, V γ 9 TCR⁺, CD3⁺, CD4⁺, and CD8⁺. However, mean values of V δ 3 TDR⁺ lymphocytes were twice as high in SLE patients compared with the control group. These differences did not correlate with the activity of disease. The number of T $\gamma\delta$ lymphocytes in peripheral blood of SLE patients was also lower than in healthy persons, even though the majority of patients in the present study had not been treated with corticosteroids or other immunosuppressive drugs. In the previous study group, 22 patients among 32 (68.7%) received corticosteroids. We cannot therefore exclude the possibility that the administration of corticosteroids does not affect significantly the level of T $\gamma\delta$ lymphocytes and V δ 2 TCR⁺ subpopulation in peripheral blood.²¹ A similar study was conducted by Lunardi *et al.*³¹ in a group of 35 SLE patients and 36 healthy persons. They found almost a 50% reduction in the percentage of T $\gamma\delta$ lymphocytes in the blood of SLE patients compared with the control group. However, similar to our study, no correlation was found between the number of these cells and the activity of SLE.

Determination of the percentage of $\gamma\delta$ T lymphocytes and their subpopulations in clinically healthy

skin in SLE patients revealed very interesting results. Immunohistochemical studies revealed that the percentage of T $\gamma\delta$ lymphocytes and their subpopulations V δ 2TCR+ and V γ 9TCR+ is significantly higher in SLE patients than in healthy persons, and is higher in patients with active SLE than with inactive disease. Similar results were seen for all three subpopulations. We have also found a positive correlation between the activity of SLE and the percentage of T $\gamma\delta$ lymphocytes ($r = 0.594$, $p < 0.001$) and V δ 3 TCR+ lymphocytes ($r = 0.659$, $p < 0.001$). Our results were also confirmed by molecular analysis. The presence of lymphocyte populations with gene rearrangements characteristic for V δ 3+ cells in the skin of SLE patients has been confirmed beyond doubt (Fig. 2 and Table 5). If these data are compared with the results of the immunohistochemical test, it should be kept in mind that the percentage of cells with V δ 3+ rearrangement was determined in relation to the sum of amplified PCR product and may not be applied for the particular number of skin cells. Moreover, mature and selected lymphocytes with a particular rearrangement type may not have such a TCR δ gene on the membrane surface. It has been demonstrated that effective rearrangement of the TCR δ gene is not solely limited to T $\delta\gamma$ lymphocytes, but may also be present in the genome of cellular clones presenting receptor TCR $\alpha\beta$.³² In addition, the phenomenon of 'allelic exclusion' in the case of T $\gamma\delta$ lymphocytes is not always present,³³ and the process of negative selection of these cells most probably occurs in peripheral target tissues.³⁴ Our results also suggest that these cells may play a role in the pathogenesis of SLE and that the determination of T $\gamma\delta$ lymphocytes in clinically healthy skin may have prognostic value.

T $\gamma\delta$ lymphocytes in the skin have been previously determined in other autoimmune diseases. Volc-Platzer *et al.*¹⁹ found the accumulation of these cells in pathologically changed skin of patients with chronic cutaneous lupus erythematosus. They claim that T $\gamma\delta$ lymphocytes, due to their cytotoxic properties, may be responsible for epithelial damage. T $\gamma\delta$ lymphocytes have also been evaluated in patients

with systemic sclerosis, similar to our study, where the percentage of these cells in systemic sclerosis patients was found to be higher than in the control group.¹⁸ Similar observations were made in some infectious skin diseases such as leprosy¹⁶ and cutaneous leishmaniasis.¹⁷ However, like the published studies of systemic sclerosis and cutaneous lupus erythematosus patients, accumulation of T $\gamma\delta$ lymphocytes was studied in pathologically changed skin but not in clinically healthy skin, like in our study. We believe that our study is the first to demonstrate that, in an autoimmune disease like SLE, these cells accumulate in clinically healthy skin as well as in diseased skin. The differences in the number of T cells between the blood and tissues may suggest their migration from blood to skin in SLE patients, mainly during exacerbation of disease.

In the present study, we have shown lower percentage of lymphocytes T $\gamma\delta$ and their V δ 2 TCR+ subpopulation in the skin of patients treated with prednisone than in the patients who were not treated with this agent during the study. It has been shown earlier that long-continued immunosuppressive therapy determined the disappearance of expanded $\gamma\delta$ T cells in target tissues of patients with polymyositis or other autoimmune diseases.^{35,36} The *in vitro* study indicated that all T lymphocytes bearing the $\gamma\delta$ TCR isolated from SLE patients and healthy individuals were susceptible to dexamethasone, and steroid-induced apoptosis was the basic mechanism responsible for cell death.³⁷ The same study has shown that 6-month glucocorticoid treatment normalized SLE $\gamma\delta$ T-cell subfraction in blood simultaneously with clinical remission.

The mechanism that leads to the accumulation of T $\gamma\delta$ lymphocytes in SLE patients has not been fully elucidated. Lymphocyte trafficking is thought to be due to surface expression of three different families of cellular adhesion molecules (immunoglobulin superfamily receptors, integrins and selectins).³⁸ This mechanism may be supported by the fact that in SLE patients there are deposits of immunoglobulins IgG or IgM, and less commonly IgA in the dermo-epidermal junction.

Table 5. The V δ 3 gene rearrangement in T cells infiltrating skin specimens of SLE patients and healthy controls (percentage of V δ 3 amplified material in relation to total PCR product visible on the gel)

| Characteristics | SLE (n = 29) | | | Control (n = 8) | Comparison | p value |
|----------------------------|--------------------|---------------------|-----------------------|-----------------|-------------|---------|
| | Total (n = 29) (a) | Active (n = 15) (b) | Inactive (n = 14) (c) | | | |
| V δ 3 subpopulation | | | | | (a)-(d) | <0.001* |
| Mean \pm SD | 22.4 \pm 9.4 | 25.7 \pm 7.9 | 18.9 \pm 10.8 | 1.4 \pm 1.9 | (b)-(c)-(d) | <0.001* |
| Median | 25.5 | 28.0 | 22.5 | 0.5 | (b)-(c) | <0.05* |
| Range | (2.0-36.0) | (8.0-33.0) | (2.0-36.0) | (0.0-4.5) | (b)-(d) | <0.001* |
| | | | | | (c)-(d) | <0.001* |

* Statistically significant difference.

In conclusion, it has been confirmed that T $\gamma\delta$ lymphocytes accumulate in clinically healthy skin in SLE patients, and that the percentage of these cells correlates with the activity of the disease.

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