Analysis of T-cell clones in systemic lupus erythematosus

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ABSTRACT

Background and Objectives. It is fairly well established that T-helper (TH) subpopulations play role in the pathogenesis of organ-specific autoimmune diseases, while their role and their relationship with TH1 cells is far from being defined in systemic lupus erythematosus (SLE). To address this issue, six female patients who fulfilled the American Rheumatism Association criteria for the diagnosis of SLE were studied.

Design and Methods. We analyzed the intracellular production of cytokines by T-cells from the peripheral blood (PB). Then, we established T-cell clones (TCC) from the peripheral blood (PB) of all cases as well as from the synovial fluid of one patient with an arthritic flare-up.

Results. The percentages of IL-4 positive and IFN-γ positive PB T-cells were not different between SLE patients and normal controls. When 93 TCC (67 CD4+, 23 CD8+) from the PB of 5 different SLE patients were compared to 118 TCC (94 CD4+, 23 CD8+) from 5 healthy controls no statistical difference was observed between SLE and controls in terms of TH1, TH2 or TH0 phenotype. However, SLE clones showed a reduced ability to secrete IL-10 (p = 0.002). In contrast, the analysis of the 30 clones obtained from synovial fluid revealed that 11/23 CD4+ clones were TH1, 12/23 were TH0, 2/7 CD8+ clones were TH1, and 5/7 were TH0. No TH2 clones were obtained from the synovial fluid.

Interpretation and Conclusions. The data suggest that the T-cell subsets operating in actively inflamed organs of SLE may belong to the TH1 and TH0 subsets.

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Key words: systemic lupus erythematosus, autoimmune diseases, T helper (TH) lymphocytes, cytokines, IL-10

Significant insight into normal T-cell function, it is now being evaluated whether pathologic conditions may be influenced by one of the two alternative polarized responses. It is fairly well established that TH1 cells play a role in the pathogenesis of organ-specific autoimmune diseases, while their role, as well as their relationship with TH2 cells, in systemic autoimmune diseases is far from defined. This is especially true in systemic lupus erythematosus (SLE), a multifactorial systemic autoimmune disease characterized by abnormal immune function with increased numbers of hyperactive B-cells together with impaired T-cell regulation and a dysfunction of antigen-presenting cells. A number of observations in murine SLE models suggest a role for TH2 cells. In NZB.H-2bm12 mice all TH cell clones able to provide help for anti-DNA production secrete IL-4, but not IFN-γ and the treatment of NZB/NZW mice with antibodies that block the interaction between B and TH2 cells significantly ameliorates the SLE-like disease. However, another set of observations indicate that TH1 cells may be significantly involved. Not only IL-4, but also IL-12 is important in the genesis of SLE in NZB/WF1 mice and IL-4 protects NZW x CS7BL/6. Yaa mice from the development of a lethal genetically linked lupus-like glomerulonephritis. Also, a study of the kinetics of cytokine production in experimental SLE has revealed that TH1 type or TH2 type cytokines predominate in different phases of the course of the disease.

To elucidate which T-cells play a role in human SLE and through which cytokines, we studied the T-cell intracellular production of cytokines and analyzed T-cell clones (TCC) generated from patients with SLE.

Design and Methods

Patients

Six female patients, aged 18-43 years, who fulfilled the American Rheumatism Association criteria for the diagnosis of SLE, were studied.

First, the intracellular cytokine profile was evaluated in peripheral blood lymphocytes (PBL) from patients (and from 7 controls matched for age and sex). Next, T-cell lines (TCL) and T-cell clones (TCC) were generated from the PB of 5 patients (and 5 healthy volunteers, matched for age and sex, used as controls). In one patient, who had a flare of disease activity characterized by non-erosive gonarthritis, TCC were also generated from the synovial fluid (SF). On the day of sampling, these patients' history and current treatment were recorded and disease activity...
assessed according to the European Consensus Study Group (ECLAM) scoring system. Two patients had not yet started any therapy, one was receiving low dose steroids, one low dose steroids plus antimalarials and one low dose steroids, antimalarials and azathioprine.

Reagents
Phytohemagglutinin (PHA) was purchased from Gibco BRL (Grand Island, NY, USA), phorbol myristic acid (PMA), ionomycin and brefeldin from Sigma Chemical Co. (St. Louis, MO, USA). Human recombinant IL-2 was a kind gift from Eurocetus (Milan, Italy). OKT3 (anti-CD3) monoclonal antibody (MAb) was purchased from Ortho Pharmaceuticals (Raritan, NJ, USA).

The monoclonal antibodies (MAbs) used for flow cytometric analysis were CD3-FITC (Leu 4, Becton Dickinson, cat. n. 92-0001), HLA-DR-PE (Becton Dickinson, cat. n. 7367), CD4-Tri-color (Caltag, San Francisco, CA, cat. n. MHCDO406), CD8-Tri-color (Caltag, cat. n. MHCDO806), IL-4-PE (Becton Dickinson, cat. n. 340451), γIFN-FITC (Becton Dickinson, cat. n. 340449).

Cell separation
PBL were obtained by separation on Ficoll-Hypaque (PH: Pharmacia-LKB, Uppsala, Sweden) gradient and washed twice in phosphate buffered saline (PBS). Cells from synovial fluid were collected by centrifugation after PBS dilution.

Intracellular cytokine analysis
Cells were cultured in 24-well plates (Celbio, Milan, Italy) at a concentration of 2 × 10⁶/mL for 10 hours at 37°C in an humidified atmosphere containing 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were then fixed and permeabilized with FACS permeabilizing solution (Becton Dickinson, cat. n. 340457) according to the manufacturer's instructions. Fixed cells were washed with PBS containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and 0.1% sodium azide and then reacted with MAb to IL-4 and IFN-γ for 30 min at room temperature.

Flow cytometric analysis
An aliquot of cells was resuspended in PBS containing 1% BSA and 0.02% sodium azide (staining medium) at a concentration of 10 × 10⁶/mL. An appropriate amount of fluorochrome-labeled antibody, at optimal concentration, was added to 100 µL of cell suspension. Negative controls were incubated with isotype irrelevant Abs. After 30 min incubation at 4°C, cells were washed twice with 2 mL of staining medium and resuspended for flow cytometric analysis. All samples were analyzed using a FACScan Research cytometer (Becton-Dickinson) equipped with a 488 nm argon ion laser (BDIS). Data acquisition was performed using the FACScan Research software (BDIS). Forward light scattering, orthogonal light scattering, and three fluorescence signals (FITC, PE, Tricolor) were determined for each cell and stored in listmode data files. Each measurement acquired 20,000 cells.

Generation of T-cell lines and clones
Mononuclear cells obtained from the PB of 5 patients and 5 healthy volunteers and from the synovial fluid of 1 patient were resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 2 × 10⁻⁵ M 2ME and 10% heat-inactivated FCS (complete medium) at the concentration of 10⁶/2 mL. To generate TCL, mononuclear cells were stimulated in 24-well plates for 10 days in the presence of human IL-2 (50 U/mL) and PHA 1% in an atmosphere with 5% CO₂ in air.

To generate TCC, T-cell blasts obtained from TCL were seeded under limiting dilution conditions (1 cell per well) in 10 round-bottomed microwell plates containing 10⁵ irradiated PBMC (6,000 rad) as feeder cells and PHA (0.5% vol/vol) in a final volume of 0.2 mL of complete medium supplemented with IL-2 (70 U/mL) and 10% FCS (Hyclone Laboratories Inc.).

Growing microcultures were then supplemented, at weekly intervals, with IL-2 (50 U/mL) and 10⁵ irradiated feeder cells.

Assessment of cytokine secretion profile of TCC
The cytokine secretion profile of T-cell clones was evaluated by stimulating 10⁵ T-cell blasts from each clone in 1 mL of complete medium with PMA (10 ng/mL) plus anti-CD3 MAb (100 ng/mL) to achieve maximal stimulation. After 36 h stimulation, the culture supernatants were collected and stored at −80°C until used.

Supernatants were assayed for IFN-γ, IL-4, IL-5 and IL-10 content. The quantitative determinations of IFN-γ, IL-4, IL-5 and IL-10 were performed by commercial ELISA (Benfer-Scheller, Keystone Laboratories, CA, USA) according to the manufacturer’s instructions. Values of cytokine content 5 standard deviations (SD) greater than those detected in control supernatants (obtained by stimulation of irradiated feeder cells alone) were regarded as positive. The following values were considered positive: IFN-γ > 0.8 ng/mL, IL-4 > 150 pg/mL, IL-5 > 400 pg/mL, IL-10 > 200 pg/mL.

Clones producing IFN-γ but not IL-4 and/or IL-5 were classified as TH₁-like, clones producing both IFN-γ and IL-4 and/or IL-5 were classified as TH₁₂-like, and clones producing IL-4 and/or IL-5 but not IFN-γ were classified as TH₂-like.

Statistical analysis
Student’s t-tests were used: p values < 0.05 were considered to be statistically significant.

Results
Cytokine expression in PB CD4+ T-cell subsets
As expected from literature data, the percentage of IL-4 positive cells was low and that of IFN-γ positive cells was higher among PB CD4+ T-lymphocytes. By intracellular staining and subsequent FACS analysis the proportion of IL4-positive and of IFN-γ positive elements was similar in CD4+ T-cells from the PB of SLE and of normal controls (Table 1).
Phenotype of T cell clones from peripheral blood

Ninety-three TCC were established from PBMC of 5 different SLE patients and 118 TCC from 5 healthy control subjects (Table 2). Cloning efficiency ranged between 15 and 20% and no differences were observed between SLE and controls. Sixty-seven out of the 93 TCC from SLE were CD4+ (72.04%), 23 were CD8+ (24.73%), 1 was CD4, CD8 double positive (1.07%) and 2 were CD4, CD8 double negative (2.15%). As for normal donors, 94 TCC were CD4+ (79.66%), 23 were CD8+ (19.49%) and 1 was CD4, CD8 double positive (0.84%). Therefore, there was no statistical difference between the phenotype of PB-generated clones from SLE or normal donor PBMC.

The clones were assessed for their ability to produce IL-4 (and/or IL-5) and IFN-γ by measuring, with ELISA, the cytokine levels in the clone supernatants. Of the SLE CD4+ TCC, 17 clones were TH1 (26%), 7 were TH2 (11%) and 42 were TH0 (64%) (Table 2). No difference was observed with normal controls among which 28 CD4+ TCC were TH1 (30%), 11 were TH2 (12%) and 53 were TH0 (58%) (Table 2). As for SLE CD8+ TCC, 7 clones were TH1 (30.4%), 3 were TH2 (13%) and 13 were TH0 (56.5%) (Table 2). Again no difference was observed with the functional phenotype of CD8+ TCC from normal controls in whom 8 clones were TH1 (34.7%), 3 were TH2 (13%) and 12 were TH0 (52.1%) (Table 2).

Cytokine production by T-cell clones from peripheral blood

The amounts of cytokines secreted by the individual clones from SLE and normal control TCC were compared. No individual differences were observed in the production of cytokines, so the data were pooled together. IL-4 production by SLE CD4+ clones was 417.28±116.08 pg/mL (65 TCC examined) as compared to 394.68±133.15 pg/mL observed in the 58 normal donor TCC examined. IL-5 production was 692.08±103.32 pg/mL (33 SLE TCC) as compared to 692.08±11.75 pg/mL (35 normal donor TCC). IFN-γ production by SLE CD4+ clones was 5.9±1.1 ng/mL as compared to 5.8±0.7 ng/mL observed in the normal donors. No statistical difference was observed.

IL-10 production could be measured in 33 SLE and 58 normal control CD4+ TCC. SLE clones belonged to patients with both active and inactive disease. Overall, the production of IL-10 by SLE CD4+ clones was 630.09±237.0 pg/mL as compared to 995.77±643.8 pg/mL observed in the normal donors. No statistical difference was observed.

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TCC from SLE synovial fluid

The SLE synovial fluid yielded 30 clones (Table 3): 23 TCC were CD4+ (76.6%) and 7 were CD8+ (23.3%). Of the CD4+ clones 11 were TH1 (47.8%) and 12 were TH0 (52.2%). Of the CD8+ clones 2 were TH1 (28.5%) and 5 were TH0 (71.4%). No TH2 clones were obtained from the synovial fluid.

The cytokine production of synovial fluid TCC was

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<th>Pt. N.</th>
<th>CD4+/IL4+ %</th>
<th>CD4+/IFN-γ+ %</th>
<th>CD4+/IL4+/IFN-γ+ %</th>
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<tr>
<td>1</td>
<td>1</td>
<td>9.3</td>
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<tr>
<td>2</td>
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<td>14.2</td>
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<td>3</td>
<td>0.2</td>
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<tr>
<td>4</td>
<td>0.5</td>
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<tr>
<td>5</td>
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<td>3.5</td>
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<tr>
<td>6</td>
<td>0.7</td>
<td>4.7</td>
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<td>Normal controls (7 cases)</td>
<td>0.4±0.29</td>
<td>8.16±8.3</td>
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<th>CD4+ clones</th>
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<th>CD8+ clones</th>
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Clone supernatants were assayed for IFN-γ, IL-4, IL-5 and IL-10 content by ELISA. Clones producing IFN-γ but not IL-4 and/or IL-5 were classified as TH1, clones producing both IFN-γ and IL-4 and/or IL-5 were classified as TH2, and clones producing IL-4 and/or IL-5 but not IFN-γ were classified as TH0; NC = normal controls.
T-cell clones in SLE

This study was aimed at investigating which T-cell subsets are preferentially involved in SLE and through which cytokines they operate. These in vitro studies and their interpretation are complicated by the difficulty in patients’ sampling because of the clinical heterogeneity of the disease and the modulation of the immune response exerted by the drugs used in the treatment of SLE. Further, T-cells in SLE have multiple abnormalities that include the presence of anti-T-lymphocyte antibodies, CD4 epitope deficiency, low levels of IL-2 receptor expression and increased levels of soluble forms of the IL-2 receptor. Finally, the proportion of T-cells that recognize presumptive auto-antigen is variable and imperfectly defined. In an attempt to overcome these difficulties we used two strategies. First, we analyzed the intracellular production of cytokines by T-cells from the PB of SLE patients. Second, we established non-antigen specific T cell clones from the PB of several cases as well as from the synovial fluid of a patient with an articular flare-up, stimulating T-cells with the polyclonal mitogen PHA and IL-2 before limiting dilution. Admittedly, this approach reflects the full T-cell repertoire rather than the T-cells activated by self-antigen, hence the in vitro data may not be fully representative of the in vivo situation. However, taking into account all the mentioned caveats it may be reasonably concluded that, while PB T-cells do not reveal any difference between SLE and normal controls (Table 1), likewise, T-cell clones from PB of SLE do not differ from those obtained from normal controls in terms of TH1, TH2 or TH0 phenotype (Table 2). In contrast, the non-antigen specific T-cell clones obtained from the synovial fluid of a patient presenting with an articular flare-up were of the TH1 and TH0 type and no TH2 T-cell clones were generated (Table 3), thereby suggesting that the T-cell subsets operating in actively inflamed organs may belong to the TH1 and TH0 subsets. These observations are in the same vein as the results of antigen-specific T-cell clones obtained from the PB of SLE patients and those obtained with non-antigen-specific TCC from patients with rheumatoid arthritis (RA) and Sjögren’s syndrome. Taken together, these data suggest that there is a tendency to a TH0-TH1 polarization in inflamed tissues of patients with systemic autoimmune diseases. As for SLE, two caveats still have to be considered before a similar statement can be definitely made. First, additional patients and with other tissue localizations of the disease must be studied. Next, antigen-specific T-cell clones need to be obtained and carefully analyzed in order to expand those T-cells that are likely to be pathogenic.

One problem is how to reconcile these findings with the observation that high levels of soluble (s)CD30, a marker of TH2 activation, are detected in the serum of SLE patients and may point to the existence of active TH2-type cells in SLE. Of interest, high levels of sCD30 are present in SLE patients with specific organ damage and have also been detected in patients with rheumatoid arthritis and Sjögren’s syndrome. Conceivably, these data may indicate that in some tissues TH2 cells are activated without necessarily implying that they are pathogenic. Rather, their activation might be an attempt to divert T-cells from TH1 activation and production of inflammatory cytokines or reflect a human counterpart of the kinetics of cytokine production observed in experimental SLE, in which TH type or TH2 type cytokines predominate in different phases of the course of the disease. In keeping with this possibility is the observation that SLE patients have an increased concentration of nitric oxide in the air they exhale, which is inversely related to functional lung involvement. Nitric oxide has an immunoregulatory role primarily targeted at limiting the activity of TH1-type T-cells. Finally, it has to be taken into account that CD30 is also distributed on activated B-cells, so that high levels of sCD30 may merely imply the presence of high proportions of activated B-lymphocytes.

We have shown that T-cells from SLE patients have a reduced ability to secrete IL-10 upon activation. This reduced ability appears to be an intrinsic feature of SLE T-cells, as emerges from the analysis of individual T-cell clones that, having been kept in culture for several weeks, cannot obey feedback mechanisms nor are influenced by other cell types. Such a property appears to be SLE-specific since T-cell clones obtained from a salivary gland biopsy from a patient with Sjögren’s syndrome, as well as from synovial fluid from patients with olioartricular juvenile arthritis and rheumatoid arthritis have been shown to...
produce high levels of IL-10. Our data are in contrast with those of Voll et al.; however, the different experimental conditions (antigen-specific T-cell clones vs antigen-non-specific TCC) and the different number of clones tested may account for this discrepancy. The reduced production of IL-10 by SLE CD4+ T-cells might reflect intrinsic T-cell abnormal regulation of cytokine production, as already shown for IL-2, IL-6, TNF-α, and TGF-β. It has been demonstrated that B-cells and macrophages from SLE PB produce high levels of IL-10. Thus, it is plausible to consider that IL-10 is present at high levels in SLE, as confirmed by the high levels that are detected in the serum of patients with active disease. Still, the fact remains that, in SLE inflamed tissues, most infiltrating immune cells belong to the T-cell lineage, while B-cells are very rare. This cellular distribution has been proved by immunocytochemistry both in the kidneys and the skin and indicates a role for T-T interactions in inflamed areas. To what extent the reduced production of IL-10 by SLE CD4+ T-cells might influence T-T interactions in inflamed tissues remains to be investigated.

Contributions and Acknowledgements

FCC and M TB conceived and designed the study and wrote the manuscript. M C and AV designed the experimental strategy, performed the cell culture (MC) and cytofluorography (AV) studies and interpreted the results together with the other authors. The authors are indebted to Prof. S. Romagnani, Prof. E. Maggi and Dr. P. Parronchi, Clinica Medica, Florence for invaluable help and assistance. The secretarial assistance of Ms. G. Tessa, Fondazione R. Favretto, is gratefully acknowledged.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- Our findings imply that an extended investigation of T cells infiltrating SLE inflamed tissues and an analysis of which cytokines they produce is warranted to understand some central issues in the pathophysiology of SLE.

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