Cytokine production, serum levels and disease activity in systemic lupus erythematosus

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Abstract

Objective

T cell abnormalities, B cell hyperactivity and abnormal cytokine production have been implicated to be of pathogenic importance in systemic lupus erythematosus (SLE). The aim of this study was to investigate if ongoing production and serum levels of type 1 and 2 cytokines reflect disease activity and the presence of organ manifestations.

Methods

Fifty-two SLE patients and 29 healthy individuals were investigated. Blood samples were collected for assessment of anti-ds DNA antibodies, cytokine production and serum cytokine levels. Disease activity was simultaneously assessed using the Systemic Lupus Activity Measure (SLAM) index and SLE Disease Activity Index (SLEDAI). ELISPOT analysis of freshly isolated peripheral blood mononuclear cells (PBMC) was used to estimate the production of cytokines (γ-interferon (IFN-γ), interleukin-4 (IL-4), IL-6 and IL-10) using both unstimulated cells and cells stimulated with the T cell mitogen phytohaemagglutinin (PHA). Serum levels of IL-10 were determined using an ELISA method, serum levels of IL-6 were determined using a bioassay and anti-ds DNA antibodies were analysed by immunofluorescence.

Results

The SLE patient group had significantly increased numbers of cells spontaneously producing IL-10 and IL-6 as compared to healthy controls ($P = 0.01$ and 0.03, respectively). The number of cells producing IL-10 and IL-6 after PHA-stimulation was also increased in SLE patients ($P = 0.01$ and < 0.0004, respectively). Serum IL-10 and IL-6 levels were also significantly increased in SLE patients ($P < 0.0004$ and 0.0005, respectively). Serum IL-10 levels correlated with the titre of anti-ds DNA antibodies in the patients. No correlation was found between disease activity or clinical profiles and the production or serum levels of cytokines except for a weak correlation (not statistically significant) between levels of IL-10 in the sera and disease activity as measured by the SLEDAI but not by the SLAM index.

Conclusion

Our results confirm earlier reports that SLE patients have an increased production as well as increased serum levels of the type 2 cytokines IL-10 and IL-6. We found no significant correlation between IL-6 and IL-10 and disease activity or clinical profiles. Serum IL-10 levels correlated with the titre of anti-ds DNA antibodies in the SLE patients. In summary, our result indicate that the increased IL-10 production in SLE could be constitutive.

Key words

SLE, cytokine production, cytokine serum levels, disease activity, SLAM, SLEDAI, disease manifestations.

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Introduction

Systemic lupus erythematosus (SLE) is a multi-organ disease, clinically characterised by exacerbations and periods of remission. There is also a wide variation in the clinical profile from a mild disease to a severe life-threatening disorder. A characteristic laboratory feature is excessive antibody production, which is manifested both as increased overall serum levels of IgG antibodies and increased levels of specific autoantibodies. Both genetic and environmental factors have been proposed in the aetiology of SLE. Polymorphisms in MHC class II genes have been found to associate with susceptibility for SLE, suggesting that specific MHC-restricted immune responses are involved (1). Particularly interesting clues concerning cytokines have been provided from reports of the increased production of IL-10 in SLE patients as well as in their relatives (2, 3), suggesting a genetically determined tendency for high IL-10 production in at least some SLE patients. Such a tendency would be of special interest since IL-10 together with other type 2 cytokines such as IL-6 facilitates antibody production (4-7) and production of these cytokines seems to be increased in SLE. IL-10 has indeed been reported to stimulate SLE peripheral blood mononuclear cells (PBMC) to produce anti-DNA antibodies *in vitro* (8). In parallel, other reports have described a relationship between increased IL-6 and IL-10 serum levels and disease activity in SLE (5, 6, 9, 10). This suggests that certain features of the disease may be associated with a temporal increase in the production of these cytokines, although studies of this matter have been controversial. Still other papers have reported decreased production (11) as well as increased serum levels of type 1 cytokines such as IFN-γ (12), but these reports are more contradictory than the available reports on IL-6 and IL-10. While these observations raise a number of interesting questions regarding the appearance of and the role of various cytokines in SLE pathogenesis, they also point to the need for further studies addressing methodological issues concerning cytokine assessments, the correlations between several cytokines and finally the correlations between cytokine levels and various clinical manifestations of SLE. As to the methods for cytokine assessments, it is obvious that serum levels only relatively indirectly reflect the results of production and consumption of a locally produced cytokine. Cytokine production has in most cases been estimated from measurements of cytokine levels in the supernatants of PBMC cultures, in which we also face the problem of production versus consumption. As it is difficult in systemic diseases to gain access to cells from representative local inflammatory sites, we have to rely on studies of peripheral blood leukocytes. Use of single cell methods for production, such as the ELISPOT method, should theoretically overcome the consumption problem. So far, however, only one study has employed the single cell technique in the evaluation of cytokine production in SLE and the results were not compared with results using other methods such as serum analysis (11). Relatively few studies have previously correlated data from cytokine assessments to detailed clinical information on different organ manifestations (5, 10, 12-18).

The assessment of whether an individual patient has more or less disease activity is crucial in the management of SLE patients. Many systems, both clinical and laboratory, for measuring SLE disease activity have been devised (19). In a study comparing some of the disease measurements used for SLE, the SLAM (20) and the SLEDAI (21) together with the British Isles Lupus Assessment Group index (BILAG) (22) were determined to be the most reliable (19). Both the SLAM and SLEDAI scores are based on SLE manifestations but they clinically differ in that constitutional symptoms can give a higher score in the SLAM index.

Against this background we considered it important to perform a further study in which the production of type 1 and 2 cytokines, as well as serum levels of the same cytokines, were assessed in SLE patients. We also determined the need for a detailed analysis of any potential correlation between the cytokines, anti-ds DNA antibodies, two different disease activity measurements and/or organ manifestations. No such previous comparative and comprehensive study has been published.

Patients and methods

Patients and controls

Fifty-two patients (46 women, 6 men) from the Department of Rheumatology at the Karolinska Hospital who fulfilled at least four ARA criteria (23) for SLE were investigated. The mean age was 45 years (range 20-79). ELISPOT data was available for 51 patients for IL-10 production, 46 patients for IL-6 production, 39 patients for unstimulated IFN-γ production and 47 patients for PHA-stimulated IFN-γ and IL-4 production. These differences were due to technical problems with the method (too few cells to perform the ELISPOT in some cases and unacceptable background after development in a few cases). For the analysis of sera, 50 patient sera were available for the IL-6 bioassay and 47 for the IL-10 ELISA.

Disease activity was assessed using the Systemic Lupus Activity Measure (SLAM) index (20) and the SLE-Disease Activity Index (SLEDAI) (21). As we wanted to compare clinical activity and immunological parameters such as cytokines and anti-ds DNA titres, the SLEDAI index was modified by the exclusion of immunologic laboratory items (complement and DNA binding) (24). The assessment was performed at the time of blood sampling by 3 of the authors. All patients gave their informed consent. Twenty-nine healthy controls (19 women, 10 men) were also included, their mean age being 40 years (range 23-52). For the IL-10 ELISA 24 control sera were available, for the IL-6 bioassay more controls were added.

Methods

Mononuclear cell preparation. Peripheral blood samples were collected in heparinized tubes at our department and transported to the laboratory without delay. Separation was conducted in no case later than 2 hours after sampling. Blood samples were diluted in equal amounts of phosphate buffered saline (PBS) at room temperature and separated on Ficoll-Hypaque (Pharmacia & Upjohn, Uppsala, Sweden). After 2 washes in PBS the cells were resuspended in RPMI-1640 (Flow Laboratories, Irvine, Scotland, UK) supplemented with glutamine, HEPES buffer, penicillin, streptomycin and 10% foetal calf serum (FCS) (Flow Laboratories, Irvine, Scotland, UK). The FCS batch used throughout the study was devoid of general mitogenic properties (25). Cell suspensions were adjusted to one million PBMC/ml.

Human cytokine ELISPOT method. The method has recently been described (25). Briefly, primary monoclonal antibodies [IFN-γ (Mabtech), IL-4 (Mabtech), IL-6 (Pharmingen) and IL-10 (Pharmingen)] were diluted in PBS and adsorbed to plastic ELISA plates (Nunc Maxisorb, Roskilde, Denmark), 50 µl/well at 4°C in a moist chamber overnight. The plates were then washed 4 times in PBS and directly afterwards 100 µl of cell suspension was added in duplicate. Cytokine production of MNC was studied either unstimulated or stimulated with 1 µg/ml phytohaemagglutinin (PHA; Murex Diagnostics, Dartford, UK).

Plates were kept in a 37°C incubator with 5% CO_2 for 18-20 hours. Next the plates were washed in PBS and 50 ml of diluted secondary antibody [IFN-γ (Mabtech), IL-4 (Mabtech), IL-6 (Pharmingen) and IL-10 (Pharmingen)] were added to each well and they were thereafter left overnight at 4°C. After 3 new washes, 50 µl of avidin-alkaline phosphatase (Dakopatts AS, Glostrup, Denmark) was added at 1.2 µg/ml in PBS and left for 2 hours at room temperature. The plates were then washed and 50 µl of BCIP phosphatase substrate solution (Sigma, St. Louis, MO, USA) was added and the plates were developed at room temperature for 3.5 hours. Plates were finally washed in deionized water and left to dry. Spot-forming cells (SFC) were counted using an inverted microscope with a 2x objective; the readings were taken blindly by 2 of the investigators.

Serum sampling. Blood samples for serum analysis were centrifuged after being allowed to clot at room temperature for one hour. The serum recovered was then stored at -70°C.

IL-6 bioassay. Serum levels of IL-6 were determined by using the IL-6-dependent B9 cell line. Cells were diluted to 5 x 10^4 cells/ml and 100 µl of cell suspension in culture medium added to flat bottomed 96-well plates. 100 µl of serum adequately diluted was then added to each well in triplicate in parallel with recombinant cytokine standards. After 72 hours of incubation, 10 µl ^{3}H-thymidine was added to each well for 4 hours, after which the cells were harvested onto nitrocellulose filters and incorporated thymidine was determined in a β-counter.

Serum IL-10 analysis. Serum levels of IL-10 were determined using a high sensitivity ELISA kit for human IL-10 from R&D Systems. The ELISA was performed according to the instructions provided by the manufacturer.

Analysis of anti-ds-DNA antibodies. Titres of anti-ds DNA antibodies were determined using immunofluorescence with Crithidia Luciliae as antigen source (Department of Clinical Immunology, Karolinska Hospital, Stockholm).

Statistical analysis. For unpaired comparisons between different groups of patients or between patients and controls, Mann-Whitney's U test was used. Spearman's rank correlation test was applied for calculations of the correlation between parallel variables in single patients. Bonferroni correction was used for the cytokines studied. A P-value < 0.05 was considered significant.

Results

Number of cytokine producing unstimulated mononuclear cells. The ELISPOT method was used to determine the number of unstimulated mononuclear cells producing IFN-γ, IL-4, IL-6 and IL-10. The SLE patients as a group had significantly higher numbers of cells producing IL-10 and IL-6 (P = 0.01 and 0.03, respectively) compared to healthy controls (Figs. 1 and 2). No differences were observed between the patients and controls concerning the spontaneous production of IFN-γ or IL-4 (Table I). There was no correlation between the unstimulated cytokine production and anti-ds DNA titres.

Number of cytokine producing PHA-stimulated mononuclear cells. The same cytokines were studied with the ELISPOT method after stimulation with the T cell mitogen PHA. Here the patients

Table I. Cytokine production using the ELISPOT method.

Cytokine production	SLE patients		Controls		SLE vs. controls
	Range	Median	Range	Median	P value*
IFN-γ (unstimulated)	0 - 33.5	4	0 - 39	4	NS
IL-4 (unstimulated)	0		0		
IFN-γ (PHA stimulated)	50 - 8000	815	120 - 2470	1090	NS
IL-4 (PHA stimulated)	0 - 321.5	36.5	3 - 268.5	80.5	NS

*Mann Whitney U test; NS = not significant

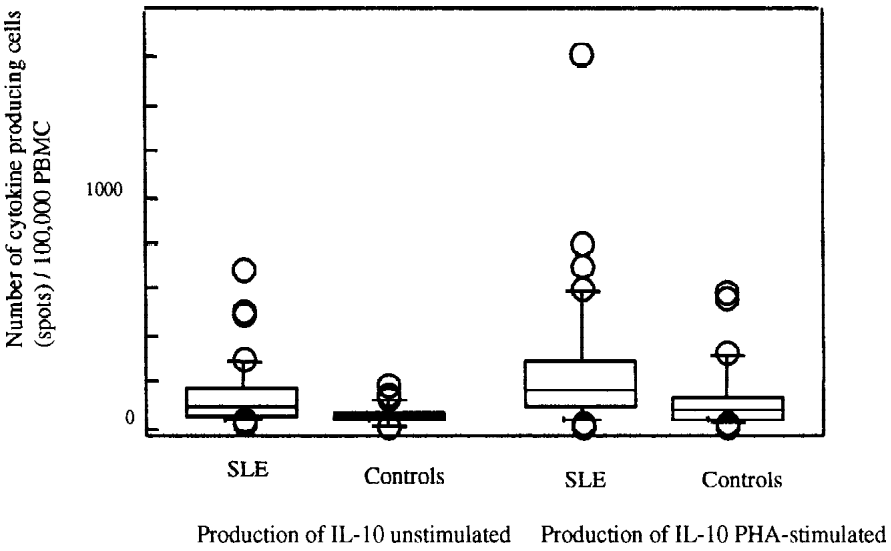

Fig. 1. Number of IL-10 producing cells per 100,000 PBMC, both unstimulated and PHA-stimulated, in SLE patients (n = 51) and controls (n = 27). There was a significant difference between the groups both for the unstimulated and stimulated production of IL-10 (P = 0.01 and 0.01, respectively).

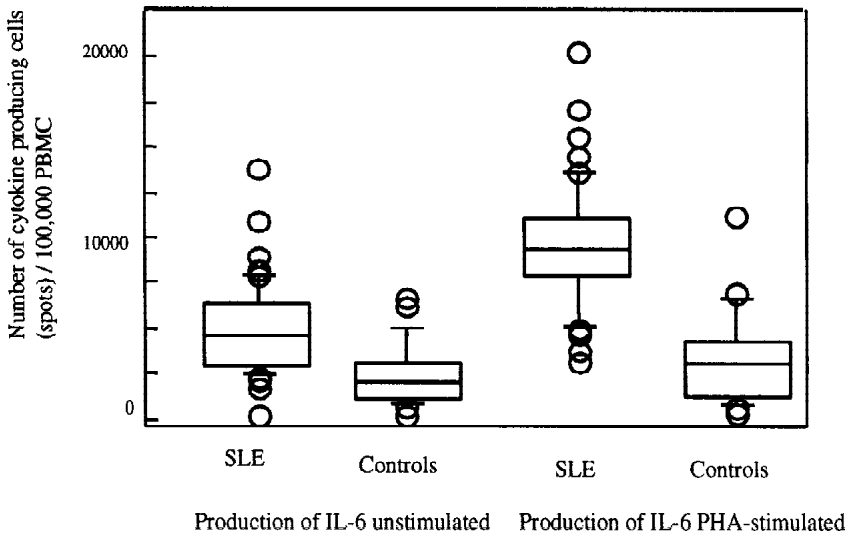

Fig. 2. Number of IL-6 producing cells per 100,000 PBMC both unstimulated and PHA-stimulated in SLE patients (n = 46) and controls (n = 24). There was a significant difference between the groups for both the unstimulated and stimulated production of IL-6 (P = 0.03 and < 0.0004, respectively).

also had higher numbers of cells producing IL-10 and IL-6 (P = 0.01 and < 0.0004, respectively) compared to controls (Figs. 1 and 2). In contrast, no difference was apparent for IFN-γ or IL-4 (Table I). No correlation was observed between stimulated cytokine production and anti-ds DNA titres.

Serum levels of IL-6, IL-10 and anti-ds-DNA antibodies. SLE patients had higher levels of IL-10 and IL-6 as compared to healthy controls (P < 0.0004 and 0.0005, respectively) (Table II). Anti-ds DNA antibodies were detected in 24 of the 50 patients tested (Table III). A significant correlation was found between the IL-10 levels in sera and the titre of anti-ds DNA antibodies (Rho 0.44, P = 0.01). There was a tendency for a correlation between the IL-6 in sera and the titre of anti-ds DNA antibodies, but this was not statistically significant.

Cytokine production and serum levels. No correlation was evident between the number of cytokine-producing cells and the serum cytokine levels.

Disease activity and cytokine production/serum levels/antibodies. Disease activity was assessed using the SLAM and SLEDAI indices, the mean value for SLAM being 8.1 (range 2 - 18) and 6.2 for SLEDAI (range 0 - 22). Information about disease activity and treatment was not available for 4 patients. There was a correlation between the SLAM and the SLEDAI indices (Rho 0.55, P = 0.0002). No correlation was detected between the number of cytokine-producing cells or IL-6 serum levels and disease activity or manifestations of SLE. Only a weak correlation was observed between serum IL-10 levels and the SLEDAI (Rho 0.32), but this did not reach statistical significance after the Bonferroni correction. No correlation was found for IL-10 in the serum and the SLAM index or specific manifestations of SLE. The anti-ds DNA titres also correlated better with the SLEDAI score as compared to the SLAM score, although this was not significant. The clinical characteristics of the patients are depicted in Table III.

Twenty-eight of 48 patients were receiving medical therapy at the time of the study. Of these patients, 21 were on prednisolone, but only 4 of them had a daily dose over 10 mg; 14 patients were being

Table II. Cytokine levels in sera using a bioassay and ELISA.

Cytokine in sera	SLE patients		Controls		SLE vs. controls
	Range	Median	Range	Median	P value*
IL-6 (bioassay, pg/ml)	< 13.5 - 156	23.7	< 13.5 - 29	< 13.5	< 0.0005
IL-10 (ELISA, pg/ml)	1.0 - 23.9	5.6	0 - 3.3	0.6	< 0.0004

*Mann Whitney U test

Table III. Clinical profile of the SLE patients investigated.

Fulfilled criteria	Ever (%)	At the time of study (%)
1. Malar rash	19 (38.8)	8 (16.3)
2. Discoid rash	4 (8.2)	0
3. Photosensitivity	27 (55.1)	ND
4. Oral ulcers	12 (24.5)	0
5. Arthritis	37 (75.5)	18 (36.7)
6. Serositis	19 (38.8)	3 (6.2)
7. Renal disorder	21 (42.8)	11 (22.4)
8. Neurological disorder	11 (22.4)	11 (22.4)
9. Hematological disorder	29 (59.2)	25 (51.0)
10. ANA	48 (98.0)	ND
11. Anti-ds DNA	29 (55.8)	24 (48.0)

ND: not determined

treated with antimalarial medication, 7 with azathioprine and 1 with cyclophosphamide. The medication did not always clearly represent the activity of the disease as patients with active disease were often investigated at admission before any treatment was started.

For the production of cytokines, serum levels, and anti-ds DNA antibodies, there was no difference between treated and untreated patients. Furthermore, there was no difference between patients treated with corticosteroids or not.

Discussion

The main finding in this study was the increased production of IL-10 as well as IL-6 among SLE patients as detected using the ELISPOT method. In SLE patients an increase in serum levels of IL-10 and IL-6 was also evident and a significant correlation was noted between serum levels of IL-10 and anti-ds DNA antibodies in the SLE patients. Neither the levels of production of cytokines studied nor the serum IL-6 levels correlated with clinical activity measured by the SLAM or SLEDAI indices. No relationship was observed between cytokine production and/or serum cytokine levels and clinical features. Furthermore, there was no correlation between serum levels and the levels of production of a given cytokine.

The increased production of IL-10 and IL-6 could not be related to disease activity as measured by the SLAM or SLEDAI indices. These data thus support some earlier reports that IL-10 production should be independent of disease activity (4, 13, 26), indicating that it may be genetically determined or dependent on environmental factors acting over long periods of time. One previous study using another variant of the ELISPOT method to estimate cytokine production reported a correlation between the increased ratio of IL-10/IFN-γ and disease severity as measured by the VAS scale (11). We could not confirm this in our study as we found no correlation between this ratio and the SLEDAI or SLAM indices (data not shown). These divergent results could be related to both methodological differences in the cytokine assessments and to patient selection and classification. In our study patients were characterized according to their disease activity but not disease severity. Furthermore, it is less likely that the absence of correlation between IL-10 production and disease activity was due to the selection of low active lupus patients, since our patient group represented a wide range of disease activity and quite a few blood samples were taken early in a flare, before the introduction of immunosuppressives, thus explaining the relatively low number of patients on corticosteroids or immunosuppressive drugs.

The finding of increased serum levels of IL-10 in SLE confirms a previous report in which a correlation between IL-10 and anti-ds DNA antibodies as well as a correlation (albeit weak) between the IL-10 in sera and the SLEDAI was observed (9). The correlation between IL-10 in sera and anti-ds-DNA antibodies is not surprising since it has been reported that IL-10 has the ability to stimulate SLE PBMC to produce anti-DNA antibodies in *in vitro* experiments (8).

For the other cytokines investigated we only recorded an aberration as compared to healthy controls for IL-6 but not for IL-4 or IFN-γ. In fact the interaction between IL-6 and IL-10 seems to be abnormal in SLE, as in normal controls IL-10 suppresses the IL-6 production of monocytes and B-cells, but apparently fails to do so in SLE (27). This supports the hypothesis that IL-10 overproduction represents an early pathogenic event in SLE rather than being secondary to an aberrant production of other cytokines. In our study, exactly which PBMC were the producers of the respective cytokines was not investigated, but previous studies have identified monocytes as the main source of IL-10, IL-6 and the T-cells of IFN-γ and IL-4 in SLE patients (11).

In the present study two different, widely used disease activity measurements were compared. The SLEDAI score (modified version) had a stronger correlation to the immunological aberrations observed (increased IL-10 and anti-ds-DNA antibodies in sera) as compared to SLAM. The main clinical difference between these two measurements is that some constitutional symptoms such as fatigue are included in the SLAM score. A recent study reported that fatigue in patients with SLE was not correlated to disease activity (28). This could be the reason for the difference between SLEDAI and SLAM when correlating these indices to immunological parameters. There is a possibility that the modified SLEDAI score

more closely reflects immunological changes, which could be related to disease activity such as anti-ds DNA antibody and IL-10 serum levels, than the SLAM index.

The lack of correlation between serum levels and the production of various cytokines should not be surprising; serum levels result from the compound production and consumption in the body after eventual binding of the remaining cytokine not only to specific cytokines receptors but also to various serum proteins. In our study IL-10 serum levels but not production correlated to anti-ds DNA antibodies and weakly to the SLEDAI score. We would therefore prefer to regard the increased serum levels of IL-10 as a serum marker in SLE, which could eventually come to be clinically useful. Previous studies have demonstrated increased levels of immunoglobulins and autoantibodies in SLE patients as well as in some of their relatives (29), indicating a tendency towards a general activation of the humoral immune response. It is feasible that this activation of the humoral immune system is in part due to an underlying increase in IL-10 and IL-6 production. An attractive possibility is that environmental factors such as sun exposure or infections could induce IL-10 production and thereby further trigger the induction of SLE in these genetically predisposed individuals.

In summary, we believe that the present study has added some needed information regarding the relationship between the production and presence of cytokines in the blood and the manifestations and disease activity of SLE, thus further strengthening the concept of an important role of an increased IL-10 production, probably constitutive, in this disease. Additional clinical studies in different populations will certainly be essential to provide a more complete picture of the relationship between the cytokines studied here on the one hand and disease risk and the profile of disease manifestations in SLE on the other hand.

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