Blys Levels in Sera of Patients with Systemic Lupus Erythematosus: Clinical and Serological Correlation

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ABSTRACT: Background: Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by disturbance of the innate and adaptive immune systems with the production of auto-antibodies by stimulated B lymphocytes. The BlyS protein (B lymphocyte stimulator) is secreted mainly by monocytes and activated T cells and is responsible for the proliferation, maturation and survival of B cells.

Objectives: To study sera BlyS level and its clinical significance in Israeli lupus patients over time.

Methods: The study population included 41 lupus patients (8 males, 33 females; mean age 35.56 ± 15.35 years) and 50 healthy controls. The patients were followed for 5.02 ± 1.95 years. We tested 221 lupus sera (mean 5.4 samples/patient) and 50 normal sera for BlyS levels by a capture ELISA. Disease activity was determined by the SLEDAI score.

Results: Sera BlyS levels were significantly higher in SLE patients than in controls (3.37 ± 3.73 vs. 0.32 ± 0.96 ng/ml, P < 0.05). BlyS levels were high in at least one sera sample in 80.5% of the patients but were normal in all sera in the control group. There was no correlation between sera BlyS and anti-ds-DNA autoantibody levels. BlyS levels fluctuated over time in sera of lupus patients with no significant correlation to disease activity.

Conclusions: Most of our lupus patients had high sera BlyS levels, suggesting a role for BlyS in the pathogenesis and course of SLE. Our results support the current novel approach of targeting BlyS (neutralization by antibodies or soluble receptors) in the treatment of active lupus patients.

KEY WORDS: BlyS, BAFF, systemic lupus erythematosus (SLE), lupus pathogenesis, anti-ds-DNA autoantibodies

Systemic lupus erythematosus is an autoimmune disease affecting almost any organ in the body including the nervous system, kidneys, lungs, cardiovascular system, skin, eyes, digestive system and the hematological system. The disease appears at any age in both genders but is more common in females of reproductive age [1]. Although the precise etiology of SLE has not yet been determined, it has been shown that genetic, environmental and hormonal factors play a role in its pathogenesis. SLE is characterized by disturbances of both the innate and the adaptive immune systems with dys-regulation of T and B cells, cytokines and the complement system, as well as in the production of autoantibodies [1].

The basis for the development of autoimmune diseases, like SLE, is the loss of self tolerance by the immune system. The latter involves dysregulation of CD4 and CD8 cells with upregulation of soluble and intracellular pro-inflammatory cytokines (e.g., interleukins-6 and 18 and interferon-gamma), and downregulation of suppressive cytokines (e.g., transforming growth factor-beta) [2,3]. Recently, reports from several groups demonstrated a low prevalence of CD4+ CD25+ FoxP3+ regulatory T cells in mice and humans with SLE [3]. In addition to the dysregulation of T cells, SLE is characterized by the production of autoantibodies by “autoreactive” B cells [2]. Thus, antinuclear antibodies can be detected in the sera of almost all SLE patients though these autoantibodies are not highly specific for lupus [4]. More specific autoantibodies such as anti-ds-DNA and anti-Sm autoantibodies were demonstrated in the sera of 80–85% and 20–30% of SLE patients, respectively [1].

B lymphocyte stimulator (BlyS), also designated B cell-activating factor (BAFF), is a 250 amino acid protein that belongs to the tumor necrosis factor ligand superfamily [5]. It is produced and secreted mainly by monocytes and activated T cells. The three BlyS receptors – BlyS receptor 3 (BR3, also known as BAFF-R), transmembrane activator-1 and calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) – are expressed by B cells [6]. BlyS plays a major role in the stimulation, proliferation and maturation of B cells to plasma cells which produce immunoglobulins [5,6]. Mice with overexpression of BlyS demonstrated high immunoglobulin levels, including high levels of autoantibodies and SLE-like disease with immune

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SLE = systemic lupus erythematosus
BlyS = B lymphocyte stimulator
complex glomerulonephritis [7]. Furthermore, NZB/NZW F1 mice (murine model for spontaneous SLE) revealed high BLyS levels in their sera, while neutralization of BLyS in those mice by soluble TACI receptor led to amelioration of the disease and a reduced mortality rate [8].

Similar to the mice studies, high BLyS levels were reported in sera obtained from 40–50% of SLE patients [9,10]. The correlation between BLyS levels in the sera of lupus patients and disease activity has not yet been defined. It was suggested that high mRNA BLyS levels in monocytes of SLE patients correlate with disease activity better than BLyS sera levels do [11].

The present study was aimed at determining BLyS levels in the sera of a cohort of SLE patients as compared to healthy matched controls over 5 years of clinical and serological follow-up and to define the correlation of sera BLyS levels, disease activity and levels of anti-ds-DNA autoantibodies.

**PATIENTS AND METHODS**

The participants in this study were 41 SLE patients and 50 healthy volunteers matched for age, gender and ethnic background. All 41 lupus patients were diagnosed with SLE according to at least four diagnostic criteria of the American College of Rheumatology [12]. Disease activity score was determined according to the Systemic Lupus Erythematosus Disease Activity Index [13]. The mean follow-up was 5.02 ± 1.95 years (range 2–10 years). All patients (and healthy volunteers) signed an informed consent form before entering the study. The study was approved by the Kaplan Medical Center ethics committee and was performed according to all GCP guidelines.

**BLOOD SAMPLES**

Sera obtained from SLE patients or healthy controls were frozen and stored at -70°C prior to BLyS measurement. At every time point that we obtained a blood sample, once every 3–6 months, disease activity was evaluated by SLEDAI, and ANA and ds-DNA autoantibodies were also measured. For every patient we obtained at least two different samples at two different time points during the follow-up. Overall we obtained 221 sera samples from our 41 SLE patients during the study: the mean number of samples per patient was 5.4 ± 2.18 (range 2–10).

**BLyS DETERMINATION**

The sera levels of BLyS were determined by a capture enzyme-linked immunosorbent assay [14]. Briefly, plates (96 wells, Nunc, Waltham, MA, USA) were covered overnight (at least 6 hours) with 50 µl of mice monoclonal antibodies at a concentration of 10 µg/ml directed against human BLyS. After washing (phosphate-buffered saline Tween 0.001%), blocking with fetal calf serum (10%) (200 µl per well) at room temperature for 3 hours, plates were washed again and sera samples were added in triplicate at 1:10 to 1:1000 dilutions for one hour at room temperature. Following another wash (x 3), polyclonal anti-human BLyS rabbit antibodies were added (1 hour, room temperature) [14,15]. Known concentrations of recombinant BLyS were used as positive controls (standard curve). The lower limit for BLyS detection of our capture ELISA was 0.32 ng/ml. Normal upper level (3.2 ng/ml) was defined as mean BLyS levels in the sera of 50 healthy volunteers plus 3 standard deviations (mean + 3SD). BLyS levels higher than 10 ng/ml, about three times the normal level, were (arbitrarily) considered “very high.”

**STATISTICAL ANALYSIS**

Data are presented as means ± SD. Mann-Whitney, unpaired Student’s *t*-test and chi-square tests were used for statistical analysis. Correlation between variables was assessed by the Pearson’s coefficient correlation test. A value of *P* ≤ 0.05 was considered statistically significant.

**RESULTS**

The study group comprised 41 SLE patients, 33 (80.5%) females and 8 (19.5%) males. Their mean age at study entry was 35.56 ± 15.35 years (range 9–79). Fifty healthy volunteers matched for age, gender and ethnic background served as a control group. The mean time of follow-up was 5.02 ± 1.95 years (range 2–10 years).

**CLINICAL AND SEROLOGICAL CHARACTERISTICS**

The 41 lupus patients in our study demonstrated typical SLE-related serological and clinical disease manifestations [Table 1]. Arthritis was present in 78% of the patients, photosensitivity was observed in 29%, lupus-related skin involvement (either malar rash or discoid lupus erythematosus) in 20%, mucosal ulcers in 22%, serositis (pleuritis and/or pericarditis) in 24%, and kidney disease (proteinuria > 0.5 g/24 hr or nephritis) in 48% of the patients. Hematological disease (hemolytic anemia and/or leukopenia and/or lymphopenia and/or thrombocytopenia) was observed in 42% of our SLE patients and central nervous system involvement (psychosis and/or epilepsy) in 12%. In all 41 lupus patients, significant (≥ 1:320) titers of ANA were detected whereas anti-ds-DNA autoantibodies were observed in the sera of 33 (85%) (at least at one time point during the follow-up period) [Table 1]. SLE disease activity (SLEDAI) was determined at every study visit with a mean score of 6.95 ± 5.94 (range 0–28).
The patients in our cohort were treated with one or more of the following modalities at the time of the study and since SLE diagnosis:

- corticosteroids (68% of the patients at the time of the study and 85% since SLE diagnosis)
- non-steroidal anti-inflammatory drugs (17% and 46%, respectively)
- antimalarials (e.g., plaquenil) (66% and 88%, respectively)
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- cytotoxic agents (e.g., cyclophosphamide or methotexate) (39% and 54%, respectively).

**BLYS LEVELS IN THE SERA OF SLE PATIENTS**

The mean BLYS level in the sera of the 50 healthy volunteers was 0.32 ± 0.96 ng/ml. Thus, we defined 3.2 ng/ml (mean of normals + 3SD) as the upper normal limit for BLYS sera levels. The mean BLYS level in all 221 lupus sera samples (3.37 ± 3.73, range 0.3–22.6 ng/ml) was significantly higher (P < 0.05) than levels in the normal control group (0.32 ± 0.96 ng/ml). BLYS levels were high (above the upper limit of normal, 3.2 ng/ml) in about a third (71/221) of the sera from the SLE patients, and within the normal range in the healthy control group. At the time of study entry (first blood sample), 20 of the 41 SLE patients (49%) had high (> 3.2 ng/ml) BLYS sera levels. During the follow-up period an additional 13 patients demonstrated high levels (at least once in one sera sample). Thus, 33 (80.5%) of our SLE patients revealed high BLYS levels at least once during a mean follow-up period of 5.02 years. In the sera of the other eight patients BLYS levels were within the normal range (similar to levels observed in the healthy controls) during the entire follow-up period. There was no difference in the number of sera samples (tested for BLYS levels) between the "positive" and "negative" SLE patients.

The determination of BLYS levels during the follow-up period revealed fluctuations in sera BLYS levels with different patterns (e.g., high-normal-high, high-high-normal, normal-high-high, etc.). In none of our SLE patients were high BLYS levels consistently demonstrated during the follow-up period.

**CORRELATION BETWEEN BLYS SERA LEVELS AND SLE-RELATED CLINICAL MANIFESTATIONS**

All lupus males in our study had high BLYS sera levels as compared to 76% of the female patients. However, this difference was not statistically significant (P = 0.14). The 33 patients who had elevated sera BLYS levels during the course of the study (as compared to the 8 SLE patients who had never had elevated BLYS levels) had a higher rate of arthritis (P = 0.04). In addition, an insignificant higher rate of renal (P = 0.40) and hematological (P = 0.29) involvement was observed in patients with high BLYS levels. On the other hand, the rate of CNS involvement was non-significantly higher (25% vs. 9%, P = 0.26) in the 8 patients with normal sera BLYS levels. There was no significant difference regarding treatment modality (e.g., corticosteroids) between patients with elevated BLYS levels and those with normal sera BLYS levels.

Nine of our lupus patients (27% of patients with elevated BLYS levels) demonstrated very high (≥ 10 ng/ml) BLYS levels in their sera at least at one time point during the study. There were no significant differences in the demographic, clinical or serological manifestations between the 9 patients with very high sera BLYS levels and the 24 patients with moderately high (3.2-10 ng/ml) levels or the 8 patients with normal levels.

**CORRELATION BETWEEN ANTI-DS-DNA AUTOANTIBODIES AND BLYS LEVELS**

We also looked for a possible correlation between BLYS and anti-ds-DNA autoantibody levels in the same sera samples of our lupus patients. The anti-ds-DNA autoantibody levels were determined by the *Crithia luciliae* assay. The ds-DNA reactivity was set as 0 (negative reaction) to +1, +2, +3, +4 (semiquantitative estimation of fluorescence intensity). There was no correlation (R = 0.06) between BLYS and anti-ds-DNA autoantibody levels. Thus, some sera samples demonstrated high BLYS levels and low ds-DNA reactivity and vice versa. The correlation between BLYS and other ANAs (e.g., anti-SM) was not assessed due to the small number of patients in our cohort with such autoantibodies.
In another SLE male patient whose major SLE-related manifestation was renal disease, we observed a similar significant ($R = 0.70$) correlation between sera BLyS levels and disease activity (SLEDAI) during 9.5 years of follow-up [Figure 3A & B].

**CORRELATION BETWEEN SERA BLyS LEVELS AND SLEDAI**

For the evaluation of SLE activity we used the SLEDAI score [13] and erythrocyte sedimentation rate. Overall, there was no significant correlation between sera BLyS levels and ESR ($R = 0.127$) or SLEDAI scores ($R = 0.26$) (at the same visit) [Figure 1A & B]. Nevertheless, in some patients (4 of 33), close follow-up revealed a significant correlation between sera BLyS levels and disease activity (SLEDAI). Thus, a female SLE patient aged 20 whose major clinical problem was vasculitis demonstrated a close correlation ($R = 0.65$) between sera BLyS levels and lupus-related disease exacerbations (presented as high SLEDAI scores) during 10 years of follow-up [Figure 2A & B].

In another SLE male patient whose major SLE-related manifestation was renal disease, we observed a similar significant ($R = 0.70$) correlation between sera BLyS levels and disease activity (SLEDAI) during 9.5 years of follow-up [Figure 3A & B].

**DISCUSSION**

The main finding of this study was the high prevalence of elevated serum BLyS levels in the SLE patients (80.5%). BLyS levels fluctuated over time in the patient sera without significant correlation to lupus disease activity. The SLE cohort in our study is typical in terms of age, organ involvement, moderate disease activity (SLEDAI 0–28, mean 6.95 ± 5.94) and serological markers (ANA, anti-ds-DNA) [Table 1]. Similarly,

ESR = erythrocyte sedimentation rate
the treatment modalities – which included NSAIDs, steroids, plaquenil and cytotoxic agents (especially cyclophosphamide) – used during both the study period and the entire course of the disease were not different from those in other SLE cohorts [14,16]. Our cohort, however, included a relatively high proportion of males (19.5%) as compared to others (about 10%) [1].

The upper normal limit of BLyS sera levels in the present study (3.2 ng/ml) was relatively high (3SD above the mean in 50 healthy matched controls). If a lower normal limit for BLyS was determined (e.g., mean +2SD) the proportion of SLE patients with sera BLyS levels above the normal range would be even higher. It is important to note that in all samples from the healthy matched control volunteers, BLyS levels were within the normal range. Thus, the mean sera BLyS levels in our SLE cohort (3.37 ± 3.73 ng/ml) were significantly higher (P < 0.05) than those in the control group (0.32 ± 0.96 ng/ml). At the time of study entry, high BLyS sera levels were noted in only 49% of our SLE patients, but this rose to 80.5% by the end of the follow-up period (mean 5.02 years). Only 8 patients (19.5%) demonstrated normal BLyS sera levels during the entire follow-up period. Since fluctuations (negative-positive levels) occurred in all our lupus patients with high BLyS levels during the follow-up period, it is essential to test BLyS levels periodically in order to define the prevalence of lupus patients with high sera BLyS levels (currently without any clinical significance).

The prevalence of SLE patients with elevated BLyS levels was higher in our study than in previous studies (80.5% vs. 40–50% respectively) [9,17,18]. The relatively long follow-up period (5 years) as compared to previous reports [14-16] and the repeated measurements performed in our study may explain this difference. In addition, the high prevalence of males in our study, all with elevated BLyS levels, may also have contributed to the high rate of patients with high BLyS levels. It is also possible that SLE patients in Israel are genetically different and/or have different environmental exposure (e.g., to the sun) which would result in higher BLyS levels. More studies with a larger number of SLE patients (including males) with longer follow-up periods are needed in Israel and in other parts of the world to define the exact prevalence of lupus patients with elevated BLyS levels.

Similar to previous studies [15,16,18], we did not find a significant correlation between the high sera BLyS levels and disease manifestations or disease activity. Recently, Vincent et al. [9] reported the presence of high BLyS levels in 44% of their lupus cohort. Similar to our results, there was no significant correlation between lupus-related disease activity and BLyS levels. Nevertheless, we observed several patients (4 of 33) [Figures 2 & 3] whose high sera BLyS levels correlated significantly with disease activity (flares and remissions). It should be noted that the reduction in BLyS levels, which was correlated with clinical improvement, may be due to the treatment (corticosteroids or cytotoxic agents) given to the patients at the time of lupus flares. Some studies suggested a better clinical correlation for BLyS mRNA levels (rather than the sera protein levels) [19], but this was not confirmed by other studies, mainly in pediatric lupus [20]. Interestingly, anti-BLyS treatments were shown to be effective in lupus patients with moderate disease activity regardless of sera BLyS levels. Thus, there is no clinical need to measure BLyS levels in the sera of lupus patients prior to the initiation of anti-BLyS treatment [21].

Previous studies [14] reported a significant correlation between BLyS levels and anti-ds-DNA autoantibodies in the

**Figure 3.** Correlation between sera BLyS levels and SLE disease activity (in an 11 year old male lupus patient) determined by SLEDAI during a follow-up period of 9.5 years [A]. The correlation is statistically significant (R = 0.70) [B]

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NSAIDs = non-steroidal anti-inflammatory drugs
sera of lupus patients. Although the prevalence of anti-dsDNA autoantibodies in patient sera in our study (85%) was similar to other reports [4,22], we did not observe any correlation between these autoantibodies and BLyS levels. Different methods for detection of anti-ds-DNA autoantibodies (ELISA vs. *Crithidia luciliae*) may explain the different observations.

To conclude, we were able to demonstrate that a high proportion of Israeli lupus patients have elevated BLyS levels in their sera. BLyS levels fluctuated during the follow-up period without significant correlation to disease activity. Our results support the rationale for the current novel therapeutic approach aimed at neutralizing BLyS in lupus patients (either by monoclonal antibodies or by specific soluble receptor) [21,23-25].

### References


