**Cutting Edge: A Role for B Lymphocyte Stimulator in Systemic Lupus Erythematosus**


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Increased levels of B lymphocyte stimulator (BLyS) are associated with systemic autoimmunity in animal models of spontaneous autoimmune disease, and transgenic animals expressing BLyS develop typical autoimmune disease. Here, we demonstrate significant elevations of BLyS in the patients with systemic lupus erythematosus (SLE). The BLyS isolated from the sera of SLE patients had the same m.w. as the natural soluble form and was able to stimulate B cell activation in vitro. Increased BLyS in SLE patients was partially associated with higher levels of anti-dsDNA Ab of the IgG, IgM, and IgA classes, but not associated with the disease activity. Our results suggest that BLyS may be a useful marker for early activation of an autoimmune diathesis and likely plays a critical role in triggering activation of self-Ag-driven autoimmune B cells in human SLE. BLyS may provide an effective therapeutic target in systemic autoimmunity. *The Journal of Immunology, 2001, 166: 6–10.

Materials and Methods

Human subjects

Peripheral blood was obtained from 150 SLE patients meeting the American College of Rheumatology (ACR) criteria for the classification of disease (12). Serum from 40 patients was harvested and stored at −30°C to −70°C until use, and plasma from a second, independent cohort of 110 SLE patients was collected and also stored at −30°C to −70°C until use. Two additional subgroups of patients with positive antinuclear Abs (ANA) titers but who did not meet the ACR criteria were also selected. Disease activity was assessed by direct clinical assessment with the systemic lupus activity measure (13) usually on the same day as the blood specimen and always within 6 days. Cumulative disease damage was assessed with the Systemic Lupus International Cooperating Clinics (14) damage index at the same time. Thirty-eight normal control sera were obtained from the University of Alabama Blood Bank. Forty-four sera and 57 synovial fluids from patients fulfilling the ACR criteria for rheumatoid arthritis (RA) were also collected and stored as above. All studies were reviewed and approved by the Institutional Review Board, and written informed consent was obtained.

ELISA for measurement of BLyS

Monoclonal anti-human BLyS Ab was generated in BALB/c mice immunized with the recombinant soluble BLyS. 15C10 (IgG2a, κ) and 3D4 (IgG1, κ) recognize the soluble form of BLyS specifically and are able to neutralize the activity of BLyS. 9B6 (IgG1, κ) recognizes the membrane-bound form of BLyS and denatured BLyS in Western blot. Polyclonal anti-BLyS Ab was raised in rabbits immunized with the recombinant BLyS and affinity-purified by the BLyS-conjugated Sepharose column. Irrelevant murine IgG isotype controls were purchased from Southern Biotechnology.
Assays for B cell stimulatory activity
Flatt-bottom 96-well culture plates were coated with 10 ng/ml of anti-BLyS Ab or murine IgG1 isotype control at 4°C overnight. After washing and blocking with 3% BSA PBS, various concentrations of the trimerized recombinant BLYS Ab in 100 or 200 μl of serum were added to each of three wells and incubated for 1 h at 37°C. To ensure the maximum binding of BLYS to the plates, the incubation with fresh sera was repeated three times. The absorbed sera were incubated with 100 μl of the protein A-agarose beads at room temperature for 1 h. The absorbed sera were incubated with 100 μl anti-BLyS-conjugated beads at 4°C overnight. The beads were washed five times with PBS containing 0.1% Tween 20, and denatured in 50 μl of the SDS loading buffer. The samples were separated in 15% SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5% nonfat dry milk, the blots were probed with 1 μg/ml of a second monoclonal anti-BLyS Ab (clone: 9B6) at 4°C overnight. After washing, the blots were further incubated with HRP-conjugated goat anti-mouse IgG1 at room temperature. The reaction was developed by the tetramethylbenzidine substrate (Sigma, St. Louis, MO), and read in an E-Max plate reader (Molecular Device, Sunnyvale, CA). A standard curve using serial dilutions of the recombinant BLYS (5.57, 1.86, 0.62, 0.21, and 0 ng/ml) was incorporated into each assay. The absolute value of BLYS for each tested sample was calculated from the best fit of the standard curve, determined by nonlinear regression, and multiplied by the dilution factor.

Immunoprecipitation and Western blot analysis of BLYS
Purified monoclonal anti-BLYS Ab (15C10) was conjugated to cyanogen bromide-activated Sepharose beads (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. The recombinant BLYS was serially diluted with 3% BSA PBS as control. One milliliter of each serum was pre-incubated with 100 μl of the protein A-agarose beads at room temperature for 1 h. The absorbed sera were incubated with 100 μl anti-BLYS-conjugated beads at 4°C overnight. The beads were washed five times with PBS containing 0.1% Tween 20, and denatured in 50 μl of the SDS loading buffer. The samples were separated in 15% SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5% nonfat dry milk, the blots were probed with 1 μg/ml of biotin-conjugated polyclonal anti-BLYS Ab at room temperature for 2 h. After addition washing, the plate was incubated with 1:30,000 diluted HRP-conjugated streptavidin (Southern Biotechnology) for an additional 1 h at room temperature. The reaction was developed by the tetramethylbenzidine substrate (Sigma, St. Louis, MO), and read in an E-Max plate reader (Molecular Device, Sunnyvale, CA). A standard curve using serial dilutions of the recombinant BLYS (5.57, 1.86, 0.62, 0.21, and 0 ng/ml) was incorporated into each assay. The absolute value of BLYS for each tested sample was calculated from the best fit of the standard curve, determined by nonlinear regression, and multiplied by the dilution factor.

Statistics
Statistical analysis was performed using the Student t test or ANOVA test for comparison of population samples. A value of p < 0.05 was used to reject the null hypothesis.

Results
Increased serum levels of BLYS in the patients with SLE
Using a sandwich ELISA, we measured the levels of BLYS in the sera of 150 patients with SLE, and 38 normal controls. Two independent sets of SLE sera (SLE1) and plasmas (SLE2) were collected and assayed. The serum levels of BLYS in both sets of samples from SLE patients were found to be significantly higher (p < 0.0001) than normal controls (Fig. 1). A very similar pattern of BLYS was found in both patient populations. The serum BLYS levels in majority of normal controls were below 5 ng/ml, and <10% were higher than 10 ng/ml. None of the normal controls was above 12 ng/ml. In contrast, the BLYS levels in most SLE patients were higher than 5 ng/ml, and >30% were above 10 ng/ml. Approximately 10% of SLE patients exhibited very high levels (>20 ng/ml) of BLYS. The BLYS levels in a few SLE patients were as high as nearly 40 ng/ml. Interestingly, the BLYS levels in the sera of 44 patients with RA and the synovial fluids of 57 RA patients were also significantly higher than that in normal sera.

BLYS can naturally exist in both membrane-bound and soluble forms (2). To determine whether the BLYS in sera corresponds to the predicted soluble form of BLYS, immunoprecipitation with an anti-BLYS mAb was performed. Using the recombinant soluble BLYS as a control, immunoprecipitation of BLYS revealed a 17 kDa protein (Fig. 2A). Dose-dependent immunoprecipitation of recombinant BLYS demonstrated a threshold for detection of about 15 ng/ml. The soluble form of BLYS was detected in all 16 sera of SLE patients tested, and the size of the immunoprecipitated BLYS exactly matched the 17 kDa of the recombinant BLYS (Fig. 2B). However, only one (no. 2) of eight normal controls was weakly detected (Fig. 2C). Taken together, these results indicate that serum levels of BLYS are elevated in the patients with SLE, and the increased BLYS in SLE1 exists in the soluble form, which is cleaved from cell surface.

BLYS in the SLE patients stimulates B cells
To determine whether the BLYS in the sera of SLE patients is functional, the B cell costimulation assay was performed using an anti-BLYS mAb to capture the BLYS in serum onto 96-well plates and then to costimulate B cells in the presence of anti-μ Abs. First, we compared the capacity of two monoclonal anti-BLYS Abs and a murine IgG1 isotype control to capture recombinant BLYS on
preabsorption of SLE1 with anti-BLyS Ab eliminated the activity of BLyS in the sera of SLE patients. The activity captured in SLE1 is specific for BLyS, because the preabsorption of SLE1 with anti-BLyS Ab eliminated the activity (data not shown), and excess mAb 15C10 or TACI-Fc added to the culture media inhibited the costimulation of BLyS (Fig. 3D). These results indicate that BLyS is not only increased in SLE patients but also can function as a B cell stimulator.

Characterization of BLyS in the sera of SLE patients. BLyS was first immunoprecipitated with anti-BLyS mAb-conjugated Sepharose beads, and separated in 15% SDS-PAGE. The presence of BLyS was determined by Western blot analysis using a second monoclonal anti-BLyS Ab. Immunoprecipitation of the recombinant BLyS. The serially diluted recombinant soluble form of BLyS was immunoprecipitated and detected by Western blot analysis. B. The BLyS was immunoprecipitated from 16 sera of SLE patients. C. Eight normal sera were processed by the same procedure. The upper bands were the IgG light chain from the Ab from the affinity beads. The lower bands were the soluble form of BLyS as indicated by the apparent molecular mass of 17 kDa.

96-well plates. Two anti-BLyS Abs (clone: 15C10 and 3D4) exhibited the ability to capture of recombinant BLyS while one the isotype control showed no significant capture activity. With 15C10 as a capture Ab, the captured BLyS was able to bind a BLyS receptor fusion protein (TACI-Fc). This binding correlated well with the binding of polyclonal anti-BLyS (Fig. 3A). In coculture of anti-μ F(ab’)2-stimulated B cells with the captured and immobilized recombinant BLyS, dose-dependent B cell proliferative responses were observed with mAbs 15C10 and 3D4 as capture Ab (Fig. 3B). A significantly increased B cell proliferation response was seen in the presence of ≥10 ng/ml of the Ab-captured recombinant BLyS. Taken together, these results indicate that some anti-BLyS Abs, when immobilized, are able to capture functional BLyS. Because both 15C10 and 3D4 in solution are able to block the functional epitope of BLyS (data not shown), we infer that the recombinant BLyS includes polymeric forms and that all functional epitopes of polymeric BLyS are not blocked by the immobilized 15C10 and 3D4 capture Abs. Indeed, this is supported directly by the availability of TACI-binding epitopes in our ELISA (Fig. 3A). Furthermore, because BLyS has to be trimerized to function, this capture method may detect and mimic polymeric forms of BLyS in the biological samples.

Using mAb 15C10 to capture BLyS activity in SLE1, the B cell costimulatory activity in the sera of SLE patients was significantly higher (p < 0.001) than that in normal control sera (Fig. 3C). Although normal sera showed no significant costimulatory activity, most sera from SLE patients exhibited increased costimulatory activity in anti-μ induced B cell proliferation. The B cell costimulatory activity captured in SLE1 is specific for BLyS, because the preabsorption of SLE1 with anti-BLyS Ab eliminated the activity (data not shown), and excess mAb 15C10 or TACI-Fc added to the culture media inhibited the costimulation of BLyS (Fig. 3D). These results indicate that BLyS is not only increased in SLE patients but also can function as a B cell stimulator.

Correlation of BLyS with increased levels of anti-dsDNA Ab

To determine whether increased levels of BLyS play a role in the production of autoantibodies, the sera of SLE patients were divided into two groups according to their BLyS levels: SLEhigh (BLyS > 15 ng/ml) and SELow (BLyS < 5 ng/ml) (Table I). The SLE patients with high levels of BLyS exhibited significantly higher levels of anti-dsDNA Ab in each of the IgG, IgM, and IgA classes compared with the SLE patients with low levels of BLyS and normal controls (p < 0.0001). The percentage of positive anti-dsDNA Ab was also significantly higher in the patient group with high BLyS. The SLEhigh patients were 80%, 80%, and 60% IgG, IgM, and IgA anti-sdDNA Ab positive, compared with 30%, 20%, and 10% in SELow group, respectively. Total IgA and IgG levels were slightly but significantly higher in the SLEhigh group compared with the SELow and control groups (p < 0.005 and p < 0.05, respectively), while total IgM levels showed no differences. Two major anti-nuclear protein autoantibodies, anti-Sm and anti-Sm ribonucleoprotein, were also measured in two SLE and control groups. Both autoantibody levels were significantly higher in both SLE groups compared with normal controls but there was no consistent difference between the BLyShigh and BLysLow groups (data not shown). These results indicate that increased levels of BLyS in SLE patients are associated with increased production of anti-dsDNA Abs, which may participate in disease pathogenesis, but not with other anti-nuclear protein Abs.

We also analyzed the clinical data of the second group of SLE patients. In general, there was no correlation of increased BLyS levels with clinical SLE activity as determined by the systemic lupus activity measure index and by ESR. Although BLyS levels were associated with anti-dsDNA Ab, they were not associated with global organ damage as determined by the Systemic Lupus International Cooperating Clinics index or with renal damage as...
Table I. Correlation of high BLyS levels with anti-dsDNA Ab

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>BLyS\textsuperscript{S\textsubscript{high}}</th>
<th>BLyS\textsuperscript{S\textsubscript{low}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>BLyS</td>
<td>4.87 ± 0.99</td>
<td>20.77 ± 2.37\textsuperscript{***}</td>
<td>4.61 ± 0.51</td>
</tr>
<tr>
<td>Anti-dsDNA-IgG</td>
<td>0.108 ± 0.017</td>
<td>1.615 ± 0.427\textsuperscript{***}</td>
<td>0.242 ± 0.080</td>
</tr>
<tr>
<td>Anti-dsDNA-IgM</td>
<td>0.058 ± 0.008</td>
<td>0.712 ± 0.292\textsuperscript{***}</td>
<td>0.104 ± 0.016</td>
</tr>
<tr>
<td>Anti-dsDNA-IgA</td>
<td>0.055 ± 0.005</td>
<td>0.170 ± 0.038\textsuperscript{***}</td>
<td>0.060 ± 0.009</td>
</tr>
<tr>
<td>Total IgG (µg/ml)</td>
<td>1898 ± 188</td>
<td>2560 ± 221\textsuperscript{*}</td>
<td>1729 ± 183</td>
</tr>
<tr>
<td>Total IgM (µg/ml)</td>
<td>182 ± 21</td>
<td>193 ± 30</td>
<td>266 ± 20</td>
</tr>
<tr>
<td>Total IgA (µg/ml)</td>
<td>437 ± 85</td>
<td>693 ± 138\textsuperscript{**}</td>
<td>610 ± 47</td>
</tr>
</tbody>
</table>

\textsuperscript{a} SLE samples were divided into BLyS\textsuperscript{S\textsubscript{high}} and BLyS\textsuperscript{S\textsubscript{low}} groups and compared to normal controls. Anti-dsDNA Ab and total IgG, IgM, and IgA were determined by ELISA. The results are presented as mean ± SEM. The positive value was determined by the mean + 3 SD of normal control. The \( p \) value was determined by \( t \) test and ANOVA test between the BLyS\textsuperscript{S\textsubscript{high}} and BLyS\textsuperscript{S\textsubscript{low}} groups.

\( ***, p < 0.0001; **, p < 0.005, *, p < 0.05. \)

determined by serum creatinine (data not shown). These results indicate that single point increases in the levels of BLyS are unlikely markers for the activity and severity of SLE. However, we also analyzed two subgroups of the patients with positive ANA but who did not yet meet the formal ACR criteria for classification as SLE. The first group with only a positive ANA exhibited slightly higher BLyS levels (8.59 ± 0.82; \( n = 8 \)) compared with normal controls. In contrast, the second group with a positive ANA and the clinical impression of lupus had significantly increased BLyS levels (14.94 ± 2.99; \( n = 5; p < 0.01 \) between two groups). These results suggest that an elevated level of BLyS precedes the formal fulfillment of the criteria and may be a useful measure of immune activation. Such an interpretation is consistent with findings of normally elevated BLyS in sera from patients with RA (6.68 ± 0.43 ng/ml; \( n = 44; p < 0.0003 \) compared with normal) and in RA synovial fluid (13.51 ± 0.79; \( n = 57; p < 0.0001 \) compared with both normal and RA sera).

**Discussion**

SLE is a systemic autoimmune disease characterized by autoantibody production against self Ags. Autoreactive B cells are driven by self Ag, but the factors that promote the loss of B cell tolerance and drive autoantibody production are still unknown (9). Endogenous B cell stimulatory factors are attractive candidates in this process, and BLyS has been recently identified as a potent B cell stimulator comparable to recombinant human IL-6 (1). Because deficiencies in apoptosis can lead to the development of lupus-like disease (10), BLyS might well play an anti-apoptotic role in B cell tolerance loss. Indeed, blockade of BLyS function with a soluble form of BLyS receptors can decrease disease severity and prolong the survival in animal lupus models (8). Thus, our results suggest that anti-BLyS might be a potential therapy for human SLE and other autoimmune disease.

**Acknowledgments**

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References


