Increased Fas and Bcl-2 Expression on Peripheral Mononuclear Cells from Patients with Active Juvenile-Onset Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To determine expressions of Fas and Bcl-2 on peripheral blood T and B lymphocytes from patients with juvenile-onset systemic lupus erythematosus (JSLE).

Methods. Thirty-eight patients with JSLE and 21 healthy controls were studied. Eleven JSLE patients with SLEDAI score ≥ 8 were categorized as active. Freshly isolated peripheral blood mononuclear cells were stained for lymphocyte markers CD3, CD4, CD8, and CD19 and for Fas and Bcl-2 molecules. Cell protein expression was measured by 3-color flow cytometry.

Results. Percentages of lymphocytes positively stained for Fas antigen and cytoplasmic expression of Bcl-2 measured by mean fluorescence intensity from patients were significantly increased compared to controls on CD3+, CD4+, and CD8+ T cells. Patients with active disease had higher percentages of CD19+ B cells positive for Fas antigen compared to patients with inactive lupus. A direct statistical correlation was observed between Fas and Bcl-2 expression on CD19+ B cells and SLE Disease Activity Index score.

Conclusion. Patients with juvenile-onset SLE show upregulation of apoptosis-related proteins. Patients with active and inactive disease have a different profile of Fas and Bcl-2 expression. (First Release May 15 2007; J Rheumatol 2007;34:1580–4)

Key Indexing Terms:
FAS BCL-2 APOPTOSIS JUVENILE-ONSET SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is the prototype of an autoimmune disease that presents a broad range of immunological abnormalities including dysfunction of T lymphocytes and polyclonal B cell activation causing a large production of autoantibodies[1]. The etiopathogenesis of SLE is not completely understood, and abnormalities of the apoptosis process at the stage of lymphocyte differentiation may be related to the presence of autoreactive T and B cells in peripheral blood. In addition, increased numbers of apoptotic cells or impaired clearance of apoptotic bodies may lead to the presence of large amounts of nuclear antigens at the extracellular tissue. These autoantigens can thus be presented to autoreactive lymphocytes and drive the development of SLE[1,2]. Indeed, lupus-specific autoantigens, including double-stranded-DNA, are exposed on the surface of apoptotic cells, and increased numbers of circulating apoptotic leukocytes and lymphocytes are observed in adult patients with SLE[1,3-5].

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Fas (APO1/CD95) is an apoptosis-promoting cell-surface receptor[2,5]. Whereas in the MRL/lpr mouse mutations of Fas gene led to the development of a lymphoproliferative syndrome with features that are similar to SLE[6,7], in patients with lupus increased Fas expression has been observed on peripheral T and B lymphocytes[8-10]. Through a different pathway, the cytoplasmic protein Bcl-2 inhibits apoptosis[11]. There is now considerable evidence that Bcl-2 expression is enhanced in peripheral T cells from patients with SLE, but some controversy remains concerning its expression in B cells[12-16]. Additionally, some studies have correlated Fas and Bcl-2 expressions with lupus disease activity in adults[10,14].

In patients with juvenile-onset SLE (JSLE), however, few studies into apoptosis and lymphocyte expression of Fas and Bcl-2 proteins have been performed[16]. Further, an altered expression of these proteins may be related to disease activity, as observed in adult patients with SLE[10,14]. We investigated the expression of Fas and Bcl-2 proteins on freshly isolated lymphocytes from patients with JSLE in order to determine their relationship with disease activity.

MATERIALS AND METHODS

Patients and controls. We studied 38 consecutive children and adolescents (32 girls; mean age 14.0 yrs, range 8.2–20.9 yrs; mean disease duration 3.2 yrs) fulfilling at least 4 of the American College of Rheumatology classification criteria for SLE[17,18] attending the Rheumatology Unit of the Children's Institute, University of São Paulo, from April 2004 through March 2005. At disease onset all patients were younger than 16 years. Disease activity was

assessed using the SLE Disease Activity Index (SLEDAI)[19] and 11 patients were categorized as having active disease, with a score ≥ 8. Twenty-one healthy controls matched for sex and age were included. Informed consent was obtained from all patients and volunteers after approval by the local ethics committee.

At admission, all patients underwent a complete physical examination; blood tests including white and red blood cells and platelet counts, erythrocyte sedimentation rate (ESR), serum complement factors C3 and C4; and urinalysis. Antinuclear antibodies (ANA) were detected by indirect immunofluorescence on HEp-2 cells and anti-dsDNA antibodies were determined by both indirect immunofluorescence on *Crithidia luciliae* and ELISA.

Considering current therapy, one patient was not using any drug, 30 were taking hydroxychloroquine, and 36 were taking oral prednisone (mean dose 19.4, median 10.0 mg/day); 16 patients with severe JSLE were under immunosuppressive therapy (cyclophosphamide, methotrexate, cyclosporine, or azathioprine) associated with prednisone, while one patient was taking only azathioprine.

Immunofluorescence staining. Immediately after venous blood was collected in EDTA tubes, peripheral blood mononuclear cells (PBMC) were separated by total blood lysis[20]. Expressions of Fas antigen and Bcl-2 protein on T and B lymphocytes were evaluated by 3- and 2-color immunofluorescence.

Surface and cytoplasmic staining were performed with the following monoclonal antibodies: fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against Fas [Becton-Dickinson-Pharmingen (BD) mouse IgG1 – 555673], FITC labeled anti-Bcl-2 (BD hamster IgG2a – 554234), phycoerythrin (PE) conjugated monoclonal antibodies against CD4 (BD mouse IgG1 – 555347), PE-labeled anti-CD8 (BD mouse IgG1 – 555635), PE-labeled anti-CD19 (BD mouse IgG1 – 555413), and anti-CD3-Cy5 (BD mouse IgG1 – 555334).

After one wash with phosphate buffered saline (PBS) supplemented with 0.1% sodium azide, 1×10^6/ml cells were resuspended in 50 μl PBS containing 2% fetal bovine serum and stained with CD4, CD8, CD19, CD3, and Fas monoclonal antibodies. After a 20 min period in the dark, cells were lysed with 15 min incubation in 10% FACS lysing solution and washed 2 times with PBS, then resuspended using 1% paraformaldehyde prior to flow cytometry analysis.

For Bcl-2 intracytoplasmic staining, cell membranes were permeabilized with 4% paraformaldehyde and 10% FACS lysing solution for 10 min and washed twice with 0.5% PBS-Tween, then FITC-labeled Bcl-2 was added. CD4, CD8, CD19, and CD3 stainings and all other procedures were performed in the same manner as for Fas staining. Isotype controls were included in all experiments.

Flow cytometry analysis. Immediately after staining, flow cytometry analysis was done on a Becton-Dickinson FACScan. Accurate physical and immunological gating was achieved using CD3-Cy5. Positive and negative controls were run for each sample in order to standardize FACS procedures. During the 11 month duration of this study there was no realignment of the cytometer. Instrument settings were constant, and alignment for forward scatter and green and red fluorescence was monitored before each experiment. Forward and side scatter profiles were identical for all patients and controls that were examined in parallel in each session. Ten thousand lymphocytes were analyzed per sample and double-positive cells identified by dot-plot histograms. Results were expressed as percentage of double-staining cells in relation to the cellular total count and as mean fluorescence intensity.

Statistical analysis. Data were analyzed using SPSS for Windows. To determine if there were significant differences between lymphocyte Fas and Bcl-2 expressions in JSLE patients compared to controls the nonparametric Kruskal-Wallis test was used[21]. Spearman's rank[21] was employed to study correlations between apoptosis measures and disease activity scores (SLEDAI), white blood cell counts, ESR, complement factors, dsDNA antibodies (ELISA), and steroid dose. P values < 0.05 were considered statistically significant.

RESULTS

Expression of Fas antigen. Percentages of freshly isolated PBMC from JSLE patients positively stained for Fas antigen were significantly higher compared to controls on CD3+, CD4+, and CD8+ T lymphocytes and on CD19+ B cells. The density of Fas antigen expressed on cell surfaces from patients measured by mean fluorescence intensity (MFI) was increased only on CD3+, CD4+, and CD8+ T lymphocytes (Table 1).

Expression of Bcl-2 protein. There was no difference in the percentages of T and B lymphocytes positively stained for Bcl-2 protein in patients and controls. In contrast, MFI of Bcl-2-positive cells from patients were significantly increased compared to controls in CD3+, CD4+, and CD8+ T lymphocytes, but not in B cells (25.5 ± 9.6 vs 21.5 ± 3.6; p = 0.06; Table 2).

Correlation between Fas expression and disease activity. JSLE patients with active disease showed significantly higher percentages of CD19+ B cells positive for Fas antigen than patients with inactive disease and controls (Table 3). A statistically direct correlation between CD19+ B cells positive for Fas antigen and SLEDAI score was observed (p = 0.02, r = 0.38; Figure 1). In addition, there were significant inverse correlations between percentages of B lymphocytes positive for Fas antigen and serum complement factors C3 (p = 0.02, r = –0.38) and C4 (p = 0.01, r = –0.38). No correlation between Fas expression and white blood cell count, ESR, anti-dsDNA, or steroid dose was observed. Compared to controls, patients with active disease showed higher percentages of CD4+ T lymphocytes positive for Fas, while patients with inactive lupus had significantly increased MFI of Fas antigen on CD3+, CD4+, and CD8+ T cells (Table 3).

Correlation between Bcl-2 expression and disease activity. MFI of Bcl-2-positive cells from patients with both active and inactive disease were significantly increased compared to controls in CD3+, CD4+, and CD8+ T lymphocytes (Table 4). Patients with inactive disease had lower percentages of CD19+ B cells positively stained for Bcl-2 compared to controls (8.8 ± 6.6 vs 10.8 ± 3.3; p = 0.03). Additionally, there was a significant direct correlation between percentage of CD19+ B cells positive for Bcl-2 and the SLEDAI score (p = 0.004, r = 0.47; Figure 2). Otherwise, there was no correlation between Bcl-2 positively stained PBMC and white blood cell count, complement factors C3 and C4, anti-dsDNA, ESR, or steroid dose.

DISCUSSION

We observed that the surface expression of Fas antigen and the cytoplasmic detection of Bcl-2 were increased on T and B lymphocytes from patients with juvenile-onset SLE. More importantly, a significantly higher percentage of B cells positively stained for Fas antigen occurred in JSLE patients with active disease, but not in those with inactive lupus.

Although it is well established that Fas and Bcl-2 proteins play significant roles in lymphocyte proliferation, survival, and apoptosis, it is not clear whether the latter relates to the

Table 1. Percentage of peripheral blood mononuclear cells (PBMC) positively stained and mean fluorescence intensity (MFI) of Fas antigen in patients with JSLE and healthy controls. Data are mean ± SD.

Cells	Percentage of PBMC		MFI	
	JSLE, n = 38	Controls, n = 21	JSLE, n = 38	Controls, n = 21
CD3+ T	43.7 ± 10.3**	28.9 ± 9.4	36.4 ± 7.6*	32.3 ± 3.7
CD4+ T	20.3 ± 6.7*	16.2 ± 6.2	45.3 ± 10.7*	39.4 ± 4.9
CD8+ T	21.5 ± 9.6**	12.3 ± 5.8	26.1 ± 5.5**	21.9 ± 2.4
CD19+ B	2.1 ± 1.4*	1.4 ± 0.7	30.6 ± 10.2	29.7 ± 5.5

* $p < 0.05$ patients vs controls, Kruskal-Wallis test. ** $p < 0.01$ patients vs controls, Kruskal-Wallis test.

Table 2. Percentage of PBMC positively stained and mean fluorescence intensity (MFI) of Bcl-2 protein in patients with JSLE and healthy controls. Data are mean ± SD.

Cells	Percentage of PBMC		MFI	
	JSLE, n = 38	Controls, n = 21	JSLE, n = 38	Controls, n = 21
CD3+ T	55.9 ± 15.4	52.9 ± 8.6	28.8 ± 8.4**	22.9 ± 4.2
CD4+ T	28.4 ± 11.2	28.7 ± 6.3	28.6 ± 8.2**	22.9 ± 4.4
CD8+ T	25.8 ± 8.8	22.2 ± 6.8	29.4 ± 9.3**	22.8 ± 3.6
CD19+ B	9.7 ± 6.5	10.8 ± 3.3	25.5 ± 9.6	21.5 ± 3.6

** $p < 0.01$ patients vs controls, Kruskal-Wallis test.

Table 3. Percentage of PBMC positively stained and mean fluorescence intensity (MFI) of Fas antigen from JSLE patients with active (SLEDAI ≥ 8) and inactive disease and healthy controls. Data are mean ± SD.

Cells	Percentage of PBMC			MFI		
	Active JSLE, n = 11	Inactive JSLE, n = 27	Controls, n = 21	Active JSLE, n = 11	Inactive JSLE, n = 27	Controls, n = 21
CD3+ T	47.1 ± 10.1†	42.4 ± 10.2†	28.9 ± 9.4	33.9 ± 7.4	37.5 ± 7.5†	32.3 ± 3.7
CD4+ T	23.9 ± 7.8†	18.8 ± 5.7	16.2 ± 6.2	41.9 ± 9.2	46.7 ± 11.1†	39.4 ± 4.9
CD8+ T	23.4 ± 10.7†	20.7 ± 9.3†	12.3 ± 5.8	24.2 ± 3.8	26.8 ± 5.9†	21.9 ± 2.4
CD19+ B	3.2 ± 1.7*†	1.7 ± 1.0	1.4 ± 0.7	27.3 ± 5.2	31.9 ± 11.4	29.7 ± 5.5

* $p < 0.05$ active JSLE vs inactive JSLE, Kruskal-Wallis test. † $p < 0.05$ patients vs controls, Kruskal-Wallis test.

pathogenesis of autoimmune diseases[1,2]. In this regard, accelerated apoptosis of circulating lymphocytes might lead to the release of increased amounts of intact nuclear antigens that could drive an autoimmune response, combining with autoantibodies to form immune complexes[1,5].

It is likely that greater expression of Fas antigen can increase the exposure of hidden antigens, and overexpression of Bcl-2 protein inhibits the removal of autoreactive cells, leading to an autoimmune disease[1]. In tissue cultures from patients with SLE, increased rates of apoptotic lymphocytes in comparison to healthy controls have been observed[4,22]. Higher levels of apoptosis-related molecules have also been described in adult patients with SLE[8-10,14,15,23-27], although this remains controversial[22,28] and may be related to patient selection and to the method used to quantify these proteins. Regarding juvenile SLE the literature is scarce[16] and data are not conclusive: in the few studies available the assessment also included adult patients[8,14]. Further, the work of Falcini, *et al*[16], with rigorous selection of JSLE patients, analyzed only the Bcl-2 protein.

Our study evaluating solely juvenile-onset SLE showed increased expression of both Fas antigen and Bcl-2 protein, and greater expression of the latter particularly in T lymphocytes is in accord with Falcini's report[16]. Concerning Fas expression, our findings are similar to reports from adult populations that described increased expression of Fas antigen on CD3+ T cells[23], on CD4+ and CD8+ T subtypes[9], and on B lymphocytes[10].

The great difference of our study is the simultaneous analysis of apoptosis-related proteins in both T and B cells, in contrast to studies in which data were obtained for either Fas or Bcl-2 in a particular lymphocyte subpopulation. Since disease activity and therapy are known to interfere with the expression of these proteins[8,10,29], concomitant interpretation of different studies may be hampered by the analysis of heterogenous patient populations.

Expression of apoptosis-inhibitory protein Bcl-2 in freshly isolated lymphocytes has been a matter of controversy. In adult SLE, some investigators[8,14] have shown increased Bcl-2 expression in T cells, but not in B cells, as we observed.

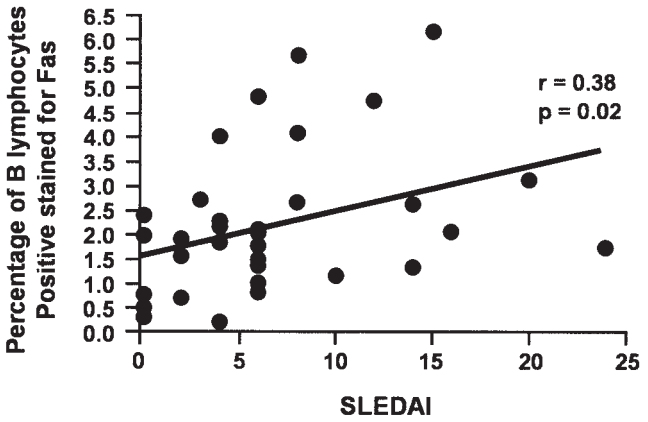

Figure 1. Correlation between percentages of B lymphocytes (CD19+) from patients with JSLE positively stained for Fas and SLEDAI score (Spearman rank correlation).

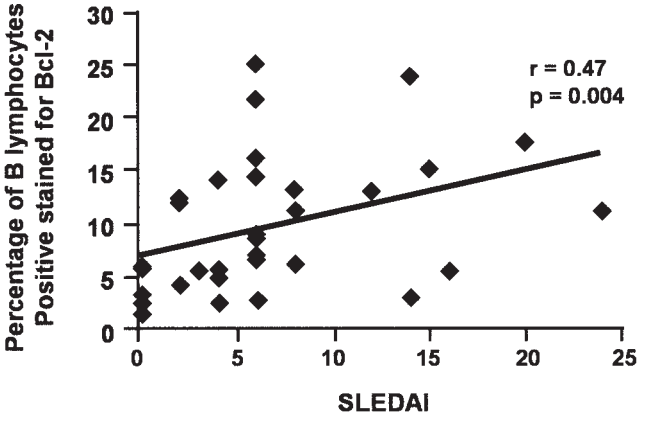

Figure 2. Correlation between percentages of B lymphocytes (CD19+) from patients with JSLE positively stained for Bcl-2 and SLEDAI score (Spearman rank correlation).

Similar quantities of Bcl-2 in unfractionated lymphocytes[28], increased expression in both T and B lymphocytes[24], and decreased Bcl-2 expression have also been observed[22]. In our study, cytoplasmic Bcl-2 was significantly increased in CD3+, CD4+, and CD8+ T cells compared to healthy controls, although a similar Bcl-2 expression was observed in B cells ($p = 0.06$). Our results are in agreement with those of Falcini, *et al*[16] showing greater expression of Bcl-2 protein in T lymphocytes but not in B cells from patients with JSLE.

Remarkably, when JSLE patients were categorized according to disease activity we observed that patients with active and inactive disease presented different profiles of Fas and Bcl-2 expressions compared to healthy controls. Fas expression on CD4+ T lymphocytes and CD19+ B cells was increased only in patients with active disease, but not in controls, as also observed in studies of adult SLE[8,10]. Moreover, the percentage of B lymphocytes positive for Fas was significantly higher in patients with active disease compared to those with inactive lupus, whereas Bcl-2 expression was similar in these 2 groups. Of interest, an inverse correlation was observed between Fas expression on B cells and C3 and C4 levels, but not specifically with white blood cell count or anti-dsDNA, a well established marker for lupus nephritis. The indistinct target-organ association is reinforced by the positive correlation with SLEDAI score but not with its individual components.

These findings suggest that the increased expression of Fas antigen observed on B lymphocytes from patients with active disease might reflect *in vivo* B cell activation, probably due to interactions with activated CD4+ T lymphocytes. Our findings are also in accord with the suggestion that increased Fas expression and enhancement of apoptotic material may be a hallmark of SLE[4,8-10,22,23]. In addition, higher level of expression of Bcl-2 protein in T lymphocytes may lead to longevity and persistence of autoreactive cell clones, and thus perpetuate autoimmunity, considering that lymphocytes with increased Bcl-2 expression are destined to remain viable and can be selected for further differentiation[1,2,11,30]. Similarly, as obtained from SLE patients, peripheral B lymphocytes that are producing autoantibodies may easily avoid apoptosis even though they are prepared for it[2].

The role of steroids in inducing apoptosis[29] could not be ruled out in our study, since the great majority of patients were taking this drug. Nevertheless, the expected correlation with steroid dose described in an *in vitro* study[31] was not observed in our study, reinforcing the possible influence of disease activity.

In addition, it remains to be elucidated whether the Fas and Bcl-2 overexpression observed in patients with JSLE can be considered a primary pathogenic event, or alternatively, a secondary epiphenomenon. The former hypothesis is supported

Table 4. Percentage of PBMC positively stained and mean fluorescence intensity (MFI) of Bcl-2 protein from JSLE patients with active (SLEDAI ≥ 8) and inactive disase and healthy controls. Data are mean ± SD.

Cells	Percentage of PBMC			MFI		
	Active JSLE, n = 11	Inactive JSLE, n = 27	Controls, n = 21	Active JSLE, n = 11	Inactive JSLE, n = 27	Controls, n = 21
CD3+ T	61.0 ± 10.5	53.9 ± 16.7	52.9 ± 8.6	31.5 ± 7.1†	27.8 ± 8.7†	22.9 ± 4.2
CD4+ T	33.8 ± 11.0	26.2 ± 10.7	28.7 ± 6.3	31.3 ± 6.5†	27.4 ± 8.6†	22.9 ± 4.4
CD8+ T	23.9 ± 5.1	26.6 ± 9.8	22.2 ± 6.7	32.6 ± 8.2†	28.1 ± 9.5†	22.8 ± 3.6
CD19+ B	11.9 ± 6.1	8.8 ± 6.6†	10.8 ± 3.3	25.9 ± 7.1	25.4 ± 10.5	21.5 ± 3.6

† $p < 0.05$ patients vs controls, Kruskal-Wallis test.

by an ongoing study by our group showing that Fas and Bcl-2 expressions on T and B lymphocytes from patients with juvenile idiopathic arthritis and juvenile dermatomyositis are similar to healthy controls (data not shown).

Moreover, increased expression of Fas and Bcl-2 proteins on freshly isolated T and B lymphocytes could reflect an imbalance of apoptosis-inducing and apoptosis-inhibitory proteins, leading to a disturbed apoptosis process and possibly to different clinical symptoms such as those observed in JSLE patients with active disease. Although we did not perform a simultaneous analysis with a functional assay using annexin V, precluding a definitive conclusion in children, increased apoptotic lymphocytes have been described in adult patients with SLE[4,23].

Our results showed an upregulation of apoptosis-related proteins in patients with juvenile-onset SLE. We also observed that patients with active and inactive disease presented different profiles of Fas and Bcl-2 expression. Further studies are necessary to determine the relevance of these findings in the etiopathogenesis of juvenile SLE.

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