Resistance to Apoptosis in Circulating α/ß and γ/δ T Lymphocytes from Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** T cell activation plays a pivotal role in the immunopathogenesis of systemic sclerosis (SSc). Lymphocyte processes are tightly controlled by molecules activating either proliferation or programmed cell death (apoptosis). We investigated whether an imbalance in apoptotic function, increasing the survival rate of autoreactive cells, may lead to persistent autoreactive phenomena.

Methods. We studied peripheral α/β and γ/δ T lymphocytes of 22 patients with SSc and 22 healthy controls for their spontaneous and stimulated (phytohemagglutinin, dexamethasone) apoptotic rate and surface phenotype including expression of Fas (CD95) and Bcl-2, determined by flow cytometry. sFas and sFas ligand in sera and supernatants were measured by ELISA. Caspase-3 activation in response to agonistic anti-Fas Mab treatment was assessed.

Results. Lymphocytes of SSc patients showed a significant decrease in the percentage of apoptotic cells over time, in both unstimulated and stimulated cultures, compared to controls. We observed no difference between patients and controls, in stimulated or unstimulated cells, in the phenotypic expression of apoptotic cells, including surface Fas. SSc T cells were less susceptible to undergoing apoptosis after anti-Fas stimulation. We observed a significant decrease of apoptotic cells from stimulated culture of isolated SSc γ/δ T cells. Serum levels of sFas in SSc patients were significantly higher compared to controls. Similar data were obtained in the supernatants of stimulated and unstimulated cultures. By contrast, sFas ligand was always reduced. Bcl-2 expression in SSc was significantly elevated. A significant decrease in caspase-3 activity was detected in SSc patients after treatment by agonistic anti-Fas antibody.

Conclusion. Resistance to apoptosis is present in α/β and γ/δ T cell lymphocyte subsets of patients with SSc, and several pathways seem to be connected in this setting. (J Rheumatol 2006;33:2003–14)

Key Indexing Terms:
SYSTEMIC SCLEROSIS γ/δ T CELLS APOPTOSIS

Systemic sclerosis (SSc) is an autoimmune disease that can be differentiated by the extent of skin involvement, presence of specific autoantibodies, and clinical course in 2 principal clinical subsets: limited and diffuse[1]. Immune T cell activation is supported by the presence of a CD3+ T cell infiltration in the affected skin[2], and by the evidence of high concentrations of several cytokines in the sera of patients with SSc and/or in the supernatants of their cultured peripheral blood mononuclear cells (PBMC)[3-5], showing a polarized Th2 responses[6,7]. Studies describe accumulation of activated T lymphocytes expressing a selective Vδ chain gene expansion of the γ/δ T cell receptor (TCR) in the lung[8] and in skin[9] of patients with SSc. In SSc, these cells display adhesion molecules[7,8], produce Th1-polarized cytokines[10], and kill endothelial cells (EC) significantly[11], suggesting their potential role in the development and maintenance of the disease. Despite intensive studies, the exact role of γ/δ T cells in the autoimmune response is poorly understood.

It is well known that several biological and pathological processes of lymphocytes are tightly controlled by molecules that activate either proliferation or programmed cell death (apoptosis), but the processes that allow T cell survival in contrast to cell death are incompletely understood. In addition, T cell activation can directly induce apoptosis. Activation-induced cell death (AICD) appears to be particularly important in controlling clonal size after repeated stimulation[12]. Thus, an imbalance in the apoptotic process, increasing the survival rate of autoreactive cells, may lead to persistent autoreactive phenomena. The Fas (CD95) antigen on the surface of lymphocytes has been identified as a key molecule that induces apoptosis. It belongs to the tumor necrosis factor (TNF) receptor superfamily and is expressed on a wide vari-

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Supported by PRIN 2004-06 from MIUR.

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Accepted for publication May 12, 2006.

ety of cell types[13]. Fas-encoding genes are responsible for the lymphoproliferative disorder and associated lupus-like syndrome in lpr and lpr^{cg} mice, and similar findings can be observed in humans[14]. In contrast, its specific ligand (FasL) displays a more restricted pattern of expression[15], and a mutation of FasL gene in gld mice resulted in a systemic autoimmune disease[16]. Normally, Fas expression alone is insufficient to account for an apoptotic cellular response. FasL and other factors are required to link the Fas-ligation event to distal death induction, including Fas-associating protein with death domain[17,18] and procaspases[19,20].

The caspase family has been categorized into 3 subfamilies as determined by their substrate specificity and function. The active forms of the caspases are multimers, the subunits of which are cleaved from the same proenzyme. It has been shown that caspase-3 plays a central executioner role in various pathways of apoptosis. Processing of procaspase-3 into an active enzyme results in cleavage of the proenzyme p32 into p20, p17, and p10 subunit proteins[21,22].

Further, various pathways of apoptosis are also differentially regulated by inhibitors of apoptosis, including Bcl-2 family members and cytokine response-modifier gene A[23-25]. Bcl-2 family members contain both anti- and pro-apoptotic molecules and play an important role in regulation of many different apoptotic pathways[26]. Bcl-2, an oncogene derived protein, confers negative control in the pathway of the cellular suicide process. The pro-apoptotic Bcl-2 family member Bax was isolated by virtue of its heterodimerization with Bcl-2[27]. Bax transgenic thymocytes show accelerated apoptosis in response to dexamethasone[28]. While Bax-Bax homodimers act as apoptosis inducers, Bcl2-Bax heterodimer formation evokes a survival signal for the cell[29].

In this study, we report that peripheral T lymphocytes of patients with SSc are resistant to different apoptotic stimuli, mainly the γ/δ T cell subset, and this resistance might play a role in the immune phenomena associated with SSc.

MATERIALS AND METHODS

Twenty-two patients (20 women, 2 men) aged 39–68 years (mean 56.4 yrs) with SSc classified according to the American College of Rheumatology (formerly the American Rheumatism Association) criteria[30] were enrolled for the study, with their informed consent and institutional approval. Seventeen patients were classified as having the diffuse and 5 the limited form of SSc by accepted criteria[1]. Skin assessment was performed using a modified Rodnan total skin thickness score technique[31]. Patients were evaluated for involvement of the lungs (pulmonary function tests, chest high resolution computed tomography scan), esophagus (manometry, barium-swallow radiography), kidneys (blood urea, serum creatinine levels and urinalysis), and heart (electrocardiography, 2-mode echocardiography). Fifteen patients were considered as having active disease on the basis of recently published activity criteria[32]. Eighteen patients had never been treated with cytotoxic drugs or biologic response-modifying agents during the 12 months preceding the study; 4 patients who were receiving steroids (prednisone ≤ 10 mg/day) discontinued the treatment 30 days before immunological assays. Table 1 shows the characteristics of each patient. Twelve healthy volunteers matched with patients for sex, race, and age were enrolled as controls.

Cell cultures. Heparinized venous blood was obtained from all patients and controls, and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll-Hypaque gradients and counted. Cell viability assessed by trypan blue dye exclusion was routinely > 95%. PBMC at a density of 2×10 cells/ml (in RPMI-1640, with 10% heat-inactivated fetal calf serum supplemented with 1% L-glutamine, 1100 U/ml penicillin, 200 mg/ml streptomycin, 100 mg/ml gentamycin) were incubated in the presence of medium alone, medium plus phytohemagglutinin (PHA; Biochrom KG, Berlin, Germany) at a final concentration of 10 mg/ml, and dexamethasone (DEX) at a final concentration of 10^{-6} M, in 96-well culture plates and cultured at 37°C in a 5% CO_2 atmosphere. To avoid acute cytokine and growth factor deprivation, which in this experimental model could lead to an increased rate of apoptosis, we supplemented the media with 50% autologous plasma. Both unstimulated and stimulated cells and their supernatants were harvested after 24, 48, and 72 h of culture. Supernatants were stored at –80°C until use.

Measurement of apoptotic lymphocytes. Aliquots of 1.5×10^6 cells/ml were removed at 24, 48, and 72 h after initiation of cultures. Cells were washed twice with cold phosphate buffered saline (PBS), washed again with annexin binding buffer (apoptosis detection kit; R&D Systems, Minneapolis, MN, USA), resuspended in 100 μl binding buffer, and stained with annexin V-FITC for 15 min at room temperature in the dark. Annexin V binds to phosphatidylserine (PS), which is normally confined to the inner plasma membrane of non-apoptotic cells; the appearance of PS on the outer plasma membrane is an early event associated with apoptosis. Because PS translocation also occurs during necrosis, annexin V is used in conjunction with propidium iodide vital dye that binds to nucleic acids. The mixed cells were incubated in the dark at room temperature for 15 min and analyzed by flow cytometry (FACScan; Becton Dickinson). Cells that are annexin V-positive and vital dye-negative are in the early apoptosis stage as indicated by PS translocation, and still have an intact plasma membrane.

Flow cytometric staining to assess subsets of apoptotic lymphocytes. Annexin V-FITC, in combination with directly conjugated antibodies, can be used to detect apoptosis occurring in phenotypically defined subpopulations of cells within heterogeneous cell cultures[33]. Aliquots of the same stimulated cells removed at 24, 48, and 72 h of culture were stained with anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies conjugated with phycoerythrin (PE) in conjunction with annexin V-FITC. To assess the percentage of apoptotic γ/δ T cell subsets, cells were stained with anti-total TCR γ/δ (TCRδ1) and anti-δTCS1 (recognizing T lymphocytes bearing the Vδ1 chain of TCR) antibodies (T Cell Sciences, Cambridge, MA, USA). Phycoerythrin anti-mouse-specific antibody was used as second-step antibody.

Measurement of apoptosis of stimulated and expanded γ/δ T cells. To confirm data in the γ/δ T cell subset, different suspensions of PBMC at 2×10^6/ml concentration were cooled to 4°C and incubated with anti-total TCR γ/δ (TCRδ1; T Cell Sciences) for 45 min in ice. After several washings with PBS, cells were incubated with M450 dynabead particles precoated with sheep anti-mouse IgG (Dynal, Oslo, Norway). Rosette-forming cells were isolated by placing the tube on a flat magnet and successively washed 3 times using the same magnet technique. The cells obtained were > 95% pure for the intended surface marker, as determined by flow cytometry.

To carry out allogenic stimulation and expansion, γ/δ T cells (5×10^5/ml) from patients and controls were cocultured with irradiated (3000 rad) RPMI-8866 cells (1×10^5/ml) at 37°C in a humidified 5% CO_2 atmosphere for 10 days. On Day 10 the population was routinely composed of 90% TCRδ1-positive (total γ/δ T cells), CD16-positive, CD14-negative cells, as determined by flow cytometry. The cells were washed and resuspended in 100 μl annexin binding buffer (apoptosis detection kit; R&D Systems), and stained with annexin V-FITC for 15 min at room temperature in the dark. Apoptotic cells were evaluated by flow cytometry.

Surface expression of Fas on lymphocyte subsets. The surface coexpression of Fas/APO1 (CD95) on mononuclear cells was determined in both uncultured and cultured cells by 2-color immunofluorescence staining, after incubation with a FITC or PE-conjugated anti-Fas/APO1 monoclonal antibody (PharMingen, La Jolla, CA/Becton Dickinson, Mountain View, CA, USA, respectively) in combination with anti-CD3, -CD4, -CD8, -CD25, and -WT31 mon-

Table 1. Clinical assessment of the 22 patients.

Patient	Sex	Age	Disease Subset	Skin Score	Auto-Antibodies	Disease Duration, yrs	Capillaroscopy	Raynaud	Fingertip Ulcers	DLCO, %	Cardiac	GI	Kidney	Active Disease
1.	F	39	Diffuse	25	ANA	2	Active	Yes	Yes	62	No	Yes	No	Yes
2.	F	40	Diffuse	15	ANA, Scl-70	13	Late	Yes	Yes	<60	Yes	Yes	No	Yes
3.	F	46	Diffuse	23	ANA, Scl-70	12	Late	Yes	Yes	60	No	Yes	No	Yes
4.	F	39	Diffuse	15	ANA, Scl-70	6	Active	Yes	No	91	No	Yes	No	Yes
5.	F	47	Diffuse	21	ANA, Scl-70	12	Late	Yes	Yes	86	Yes	Yes	No	Yes
6.	F	67	Diffuse	4	ANA, Scl-70	30	Late	Yes	Yes	87	Yes	Yes	No	No
7.	F	68	Diffuse	23	ANA, Scl-70	31	Late	Yes	Yes	99	No	Yes	No	Yes
8.	F	57	Diffuse	26	ANA, Scl-70	15	Late	Yes	Yes	64	Yes	Yes	No	Yes
9.	M	41	Diffuse	15	ANA, Scl-70	13	Late	Yes	No	37	Yes	Yes	No	Yes
10.	M	56	Diffuse	15	ANA, Scl-70	27	Active	Yes	Yes	80	No	Yes	No	Yes
11.	F	58	Diffuse	12	ANA, Scl-70	29	Late	Yes	Yes	>84	No	Yes	No	No
12.	F	39	Limited	3	ANA, ACA	5	Early	Yes	Yes	125	No	Yes	No	No
13.	F	68	Limited	5	ANA, ACA	4	Active	Yes	No	80	No	Yes	No	No
14.	F	66	Diffuse	13	ANA, Scl-70	16	Late	Yes	No	118	No	Yes	No	No
15.	F	39	Limited	4	ANA, ACA	4	Active	Yes	No	119	No	No	No	No
16.	F	39	Limited	6	ANA, ACA	3	Active	Yes	No	99	No	Yes	No	Yes
17.	F	68	Diffuse	13	ANA, Scl-70	13	Late	Yes	Yes	73	Yes	Yes	Yes	Yes
18.	F	39	Difusse	5	ANA, Scl-70	2	Late	Yes	No	112	No	Yes	No	Yes
19.	F	67	Diffuse	22	ANA, Scl-70	20	Late	Yes	Yes	61	Yes	Yes	No	No
20.	F	59	Limited	5	ACA	15	Late	Yes	Yes	63	No	Yes	No	Yes
21.	F	59	Diffuse	15	ANA, Scl-70	17	Late	Yes	Yes	58	No	Yes	No	Yes
22.	F	66	Diffuse	14	ANA, Scl-70	33	Late	Yes	Yes	52	No	Yes	No	Yes

ANA: antinuclear antibody; ACA: anticentromere antibody; GI: gastrointestinal

oclonal antibodies (Becton Dickinson) conjugated with FITC or PE and with anti-total TCR γ/δ (TCRδ1) or anti-δTCS1 (recognizing T lymphocytes bearing the Vδ1 chain of TCR) antibodies. Phenotypic analysis was by flow cytometry (FACScan; Becton Dickinson).

Assay for sFas and sFasL in sera and supernatants. Serum and supernatant sFas and sFasL concentrations were measured with an ELISA test kit (Cytelisa human sFas, Cytimmune, College Park, MD, USA; sFas ligand ELISA kit, MBL, Nagoya, Japan). The assays were based on the dual immunometric sandwich principle and were performed according to the manufacturer's instructions.

Fas engagement and apoptosis. To analyze AICD by the activity of surface Fas, T cells were isolated from PBMC after depletion of plastic-adherent cells by incubation for 90 min in plastic dishes at 37°C, in a humidified 5% CO_2 atmosphere, in RPMI-1640 with 10% fetal calf serum, and succesively using an immunomagnetic method with an anti-CD3 monoclonal antibody. After detachment of magnetic beads from isolated T cells, T cells were collected and plated on microplates precoated with 50 μg/ml anti-CD3 for 24 h. After stimulation, cells were recovered and incubated again with anti-Fas antibody (anti-CD95, CH11; Coulter/Immunotech, Westbrook, ME, USA) for 8 h at 37°C in an atmosphere with 5% CO_2. The cells were further stained with annexin V, as described, and finally analyzed by flow cytometry.

BCL-2 staining of PBMC. Bcl-2 monoclonal antibody (Dako, Glostrup, Denmark) was used in intracytoplasmic staining of PBMC. Cells were fixed and permeabilized using Ortho-Permeafix (Ortho Diagnostics, Raritan, NJ, USA). Isotype controls were included in all the experiments. Results were expressed as mean florescence index (MFI, ratio between mean florescence intensity of samples and background) and as percentage of positive cells.

Evaluation of caspase-3 activity. To assess the specific role of caspase-3 in Fas-mediated lymphocyte cell death, we examined caspase-3 activation in response to agonistic anti-Fas monoclonal antibody treatment. Activation of caspase-3 was assessed using the PhiPhiLux G1D2 caspase 3 activity detection kit (AK304R1G, Oncoimmunin, College Park, MD, USA), according to the manufacturer's instructions. Briefly, after monoclonal antibody treatment 1×10^6 cells were washed twice with PBS, and 50 μl substrate solution [10 mM; GDEVDGID (single-letter amino acid code)] was added, followed by incubation for 60 min in a 5% CO_2, 95% air incubator at 37°C. This substrate emits fluorescence when it is cleaved within the GDEVDGID sequence, allowing determination of caspase-3-like activity by flow cytometry. Then 500 μl cold flow-cytometry solution was added to each sample, followed by analysis using a FACScan flow cytometer at 488 nm, FL1 channel.

Statistical analysis. The nonparametric Wilcoxon 2-tailed test (normal approximation) and the Rank correlation test were used when appropriate for statistical analysis of the data.

RESULTS

Measurement of apoptosis. Every time cells were removed for assessment of apoptosis, a total viable cell count was performed. Cell counts did not change significantly over 48 h of culture in any sample. As shown in Figure 1, lymphocytes of patients with SSc showed a gradual increase in the percentage of apoptotic cells over time, in both unstimulated and stimulated cultures. In contrast, control PBMC underwent apoptosis at a much more rapid rate, as follows:

3.6% (range 0.99–6.42%) vs 9.2% (5.9–11.6%), SSc vs control, respectively, in unstimulated cultures after 24 h ($p = 0.01$); 9.5% (range 6.5–14%) vs 23.4% (15–33%), respectively, in unstimulated cultures after 48 h ($p = 0.007$); and 15.6% (range 13–17.5%) vs 25.4% (16–33%), respectively, in unstimulated cultures after 72 h ($p = 0.003$).

For PHA stimulation: 3.9% (range 1.0–7.8%) vs 10.2% (6.1–14.5%) after 24 h PHA ($p = 0.01$); 11.5% (range 9.1–17.7%) vs 23.1% (15.5–31.4%) after 48 h PHA ($p = 0.01$); and 16.8% (range 10–21.2%) vs 28.18% (20–33.5%) after 72 h PHA ($p = 0.02$).

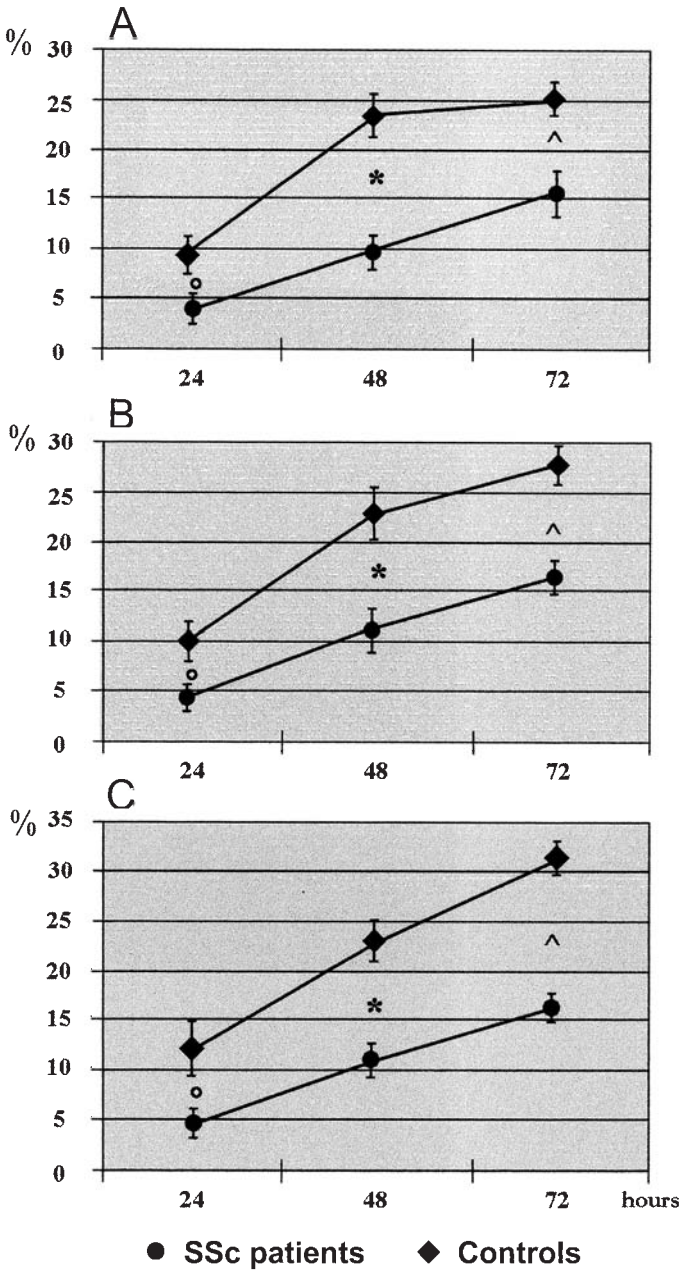

Figure 1. Percentage of apoptotic PBMC in (A) unstimulated, (B) PHA stimulated, and (C) DEX-stimulated cultures in SSc patients and controls. PBMC of SSc patients displayed a decreased apoptotic rate in every condition at each timepoint (°$p < 0.01$; $p < 0.007$; ^$p < 0.03$).

And for DEX stimulation: 4.52% (range 1.4–8.5%) vs 11.64% (8.9–19.7%) after 24 h DEX ($p = 0.03$); 11.1% (range 9.2–17.7%) vs 22.8% (15.2–31.4%) after 48 h DEX ($p = 0.01$); and 16.7% (range 12.4–20.2%) vs 31.3% (28.3–34.7%) after 72 h DEX ($p = 0.002$). Results are summarized in Table 2.

Flow cytometric staining to assess subsets of apoptotic lymphocytes. After 24 h and 48 h of incubation with PHA and DEX, unstimulated and stimulated cells were recovered and assayed to determine the apoptotic subsets and analyzed for the coexpression of surface markers CD4 and CD8, total γ/δ, Vδ1, and annexin V. Our results are summarized in Table 3. Cultured cells maintained the same phenotype represented in the original cell samples. We did not observe any difference in the phenotypic expression of apoptotic cells between patients and controls, in each phase, for both stimulated and unstimulated cells, and observed a trend to a decreased apoptotic rate in the patients' γ/δ T cell subsets, although these findings did not achieve statistical significance.

In contrast, in isolated γ/δ T cell cultures, we observed a significant decrease of apoptotic cells in the stimulated cultures of SSc patients' γ/δ T cells [27.8% (range 5–55%) vs 12.3% (4–18%); $p < 0.0005$], suggesting a decreased apoptotic rate of this subset during SSc.

Surface expression of Fas. Fas surface expression on freshly isolated CD3+, CD4+, CD8+, and total γ/δ T cells, as shown in Table 4, did not reveal significant differences between patients and controls. Similarly, no difference was observed in cultured cells. In our experiments the PHA stimulation induced an increased expression of CD95 on the surface of PBMC, with no differences between patients and controls.

Fas engagement and apoptosis. T cells of SSc patients were analyzed for their ability to undergo AICD, after stimulation with anti-CD95 monoclonal antibody CH11, to verify if this increased survival is a more general feature of these cells. The results (Figure 2) demonstrated that T cells of SSc patients were less susceptible to undergoing AICD. Patient and control cells displayed a similar cell-surface expression of CD95.

sFas and sFasL in sera and supernatants. Serum levels of sFas in SSc patients were significantly higher than those in controls: 2.44 ng/ml (range 1.37–3.89) vs 1.15 ng/ml (range 0.50–2.01) ($p = 0.002$; Figure 3).

Raised sFas levels were detected in cell supernatants at 24, 48, and 72 h of stimulated cultures from SSc patients compared with control cultures [at 24 h: 2.11 ng/ml (range 0.15–3.25) vs 1.05 ng/ml (range 0.10–1.70); 48 h: 2.61 ng/ml (range 0.65–3.80) vs 1.25 ng/ml (range 0.10–2.01); and 72 h: 3.95 ng/ml (range 0.40–5.25) vs 1.05 ng/ml (range 0.90–1.70); $p = 0.002$ for all assays] (Figure 4).

By contrast, serum levels of sFasL in our patients were significantly lower than in controls [0.15 ng/ml (range 0–0.4) vs 0.25 ng/ml (range 0–0.7), respectively; $p < 0.0005$] (Figure 3).

Similarly, in the supernatants sFasL levels were significantly decreased in SSc patients vs controls [at 24 h: 0.11 ng/ml (range 0–0.25) vs 0.35 ng/ml (range 0.10–0.70); 48 h: 0.17 ng/ml (range 0.05–0.60) vs 0.35 ng/ml (range 0.10–0.70); and 72 h: 0.15 ng/ml (range 0–0.25) vs 0.30 ng/ml (range 0–0.70), respectively; $p = 0.002$ for all assays] (Figure 5).

Expression of Bcl-2. The MFI of Bcl-2 in our patients was 490 (range 320–650), and this value was significantly elevated compared to controls [278 (range 92-480); $p < 0.001$]. This value was independent of subset of disease, disease duration, and clinical findings.

Table 2. Percentage of apoptotic cells (annexin V+) in SSc patients and controls from unstimulated (US) and stimulated (PHA, DEX) cultures. SSc lymphocytes showed a significant decrease in percentage of apoptotic cells over time, in both unstimulated and stimulated cultures.

	24 Hours			48 Hours			72 Hours		
	US	PHA	DEX	US	PHA	DEX	US	PHA	DEX
	% (range)			% (range)			% (range)		
Control	3.6 (0.99–6.42)	3.9 (1.0–7.8)	4.52 (1.4–8.5)	9.5 (6.5–1.4)	11.5 (9.1–17.7)	11.1 (9.2–17.7)	15.6 (13–17.5)	16.8 (10–21.2)	16.7 (12.4–20.2)
SSc	9.2 (5.9–11.6)	10.2 (6.1–14.5)	11.64 (8.9–19.7)	23.4 (15–33)	23.1 (15.5–31.4)	22.8 (15.2–31.4)	25.4 (16–33)	28.18 (20–33.5)	31.3 (28.3–34.7)
p	0.01	0.01	0.03	0.007	0.01	0.01	0.003	0.02	0.002

PHA: phytohemagglutinin, DEX: dexamethasone, US: unstimulated.

Table 3. Percentage of apoptotic cells (annexin V+) in different T cell subsets from PBMC of SSc patients and controls, from both unstimulated and stimulated (PHA, DEX) cultures. As shown, no differences were observed.

T Cell Subset	Unstimulated		PHA		DEX	
	SSc	Control	SSc	Control	SSc	Control
	% (range)		% (range)		% (range)	
CD4	31.5 (14–80)	27 (13–60)	30 (21–70)	28 (19–52)	32 (20–64)	26 (21–48)
CD8	21 (12–25)	25 (15–29)	23 (20–28)	20 (7–29)	22 (19–27)	16 (7–26)
γδ	2 (1.5–7)	2.7 (2–3.6)	2.4 (1.8–3.9)	2.5 (0.8–4.6)	2.7 (1.5–4.2)	2.1 (0.8–4.3)
δ1	0.5 (0.3–2)	0.6 (0.2–2.3)	0.2 (0.1–1.3)	0.7 (0.6–1.3)	0.3 (0.2–1.5)	0.9 (0.5–1.1)

PHA: phytohemagglutinin, DEX: dexamethasone.

Table 4. Surface expression of Fas in unstimulated and stimulated (PHA, DEX) T cell subsets from SSc patients and controls. Data are mean percentage ± SD. As shown, no differences on Fas surface expression were observed.

	Fas Expression							
	Unstimulated		PHA		DEX			
T Cell Subset	SSc	Control	SSc	Control	SSc	Control	SSc	Control
CD3	81 ± 3	84 ± 6	34 ± 4	37 ± 2	79 ± 3	85 ± 5	75 ± 3	81 ± 4
CD4	45 ± 4	47 ± 4	20 ± 3	29 ± 3	42 ± 4	35 ± 2	40 ± 3	31 ± 3
CD8	39 ± 4	33 ± 3	22 ± 4	28 ± 3	30 ± 4	40 ± 2	34 ± 4	42 ± 5
γ/δ	4.6 ± 3	3.8 ± 2	16 ± 2	20 ± 1	25 ± 2	33 ± 1	22 ± 1	31 ± 3
δ1	3.3 ± 2	1.17 ± 1	20 ± 2	23 ± 2	27 ± 3	32 ± 2	25 ± 3	31 ± 2

PHA: phytohemagglutinin, DEX: dexamethasone.

Double-staining experiments showed that both α/ß and γ/δ subsets of SSc patients displayed significantly higher levels of Bcl-2 compared to controls, as shown in Figure 6 [SSc α/ß 460 (range 320–600) vs control α/ß 290 (156–480), $p < 0.01$; SSc γ/δ 527 (range 395–650) vs control γ/δ 251 (92–462), $p < 0.001$].

Expression of caspase-3 activities. To determine activation of caspase-3 in our lymphocytes, after anti-Fas treatment the PhiPhiLux-G2D2 cell-permeable fluorescent substrate for caspases was used. As shown in Figure 7, which is representative of the experiments, a significant decrease in the mean of fluorescence index displaying caspase-3 activity, after treatment by agonistic anti-Fas antibody, was detected in SSc patients compared to controls [13% (range 9–30%) vs 51% (27–66%), respectively; $p < 0.01$].

There was no correlation with skin involvement and other clinical indicators. Further, no significant difference was found between the diffuse and limited forms of the SSc.

DISCUSSION

Dysregulation of apoptosis may lead to longevity of autoreactive T cell clones, perpetuating the autoimmune process. Oligoclonal expansion of T cell subsets has been described in patients with SSc[9,34]. To analyze whether the ability for clonal growth was correlated with resistance to apoptosis-inducing mechanisms, total T cells and γ/δ T cell subsets of patients with SSc were tested for apoptotic stimuli. We observed a significant decrease in the apoptotic rate of total T cells from SSc patients compared to controls, the latter showing a more rapid rate of apoptosis at every study point. Further, these data were confirmed after incubation with PHA and DEX. No difference was observed between patients and controls in the surface

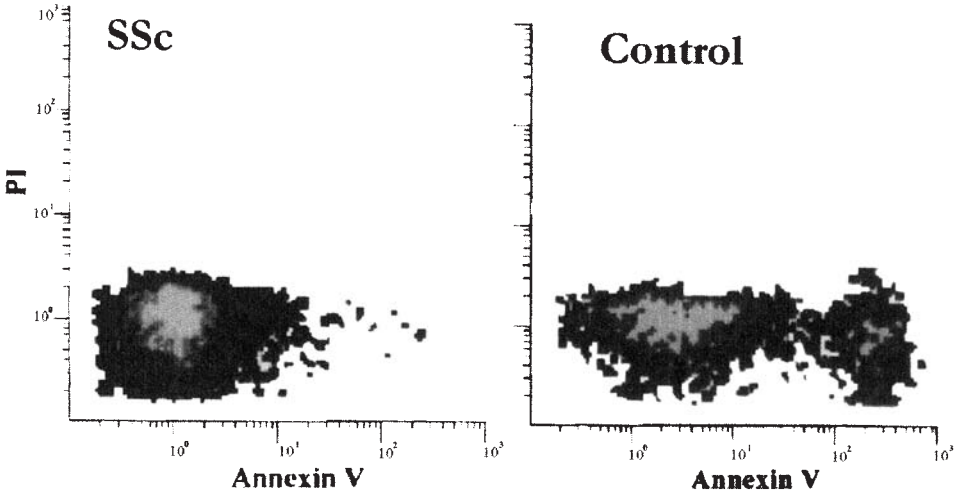

Figure 2. T cells of SSc patients were analyzed for their ability to undergo AICD, after stimulation with anti-CD95 Mab CH11. AICD values from 22 SSc patients and 12 controls: SSc median 8 (range 4–9), controls median 35 (range 22–69) ($p < 0.005$, statistically significant). Cytofluorimetric assay result, representative of different experiments, illustrates that cells of SSc patients were less susceptible to undergoing AICD, as shown by the lower number of cells stained with annexin V in the SSc plot compared to control. SSc and control cells displayed a similar cell-surface expression of CD95. PI: phosphorescence intensity.

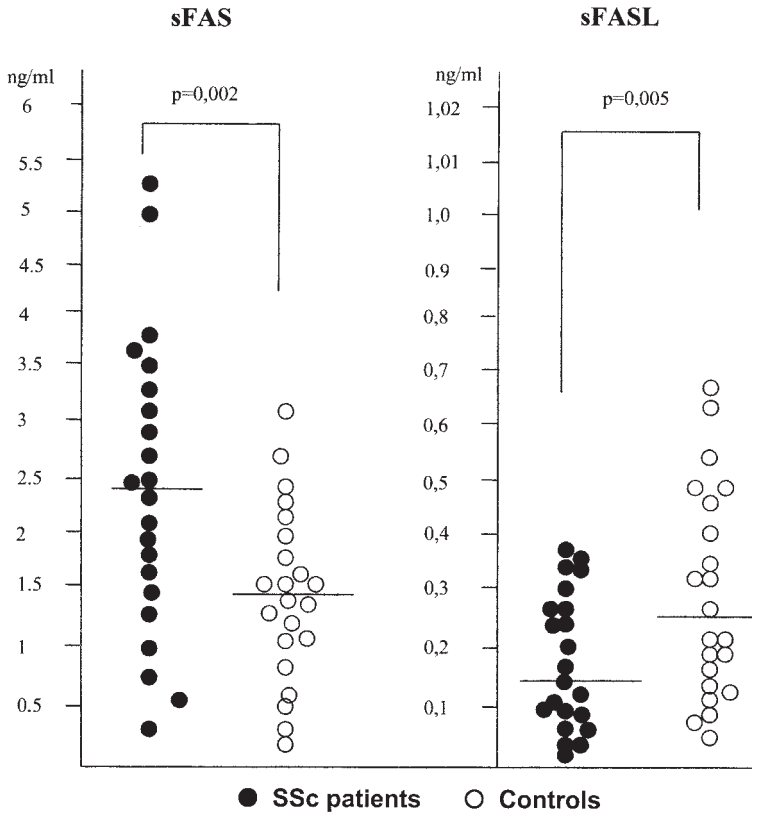

Figure 3. Serum levels of soluble FAS (sFAS) and soluble FAS ligand (sFASL) in SSc patients and controls. Differences between patients and controls were statistically significant.

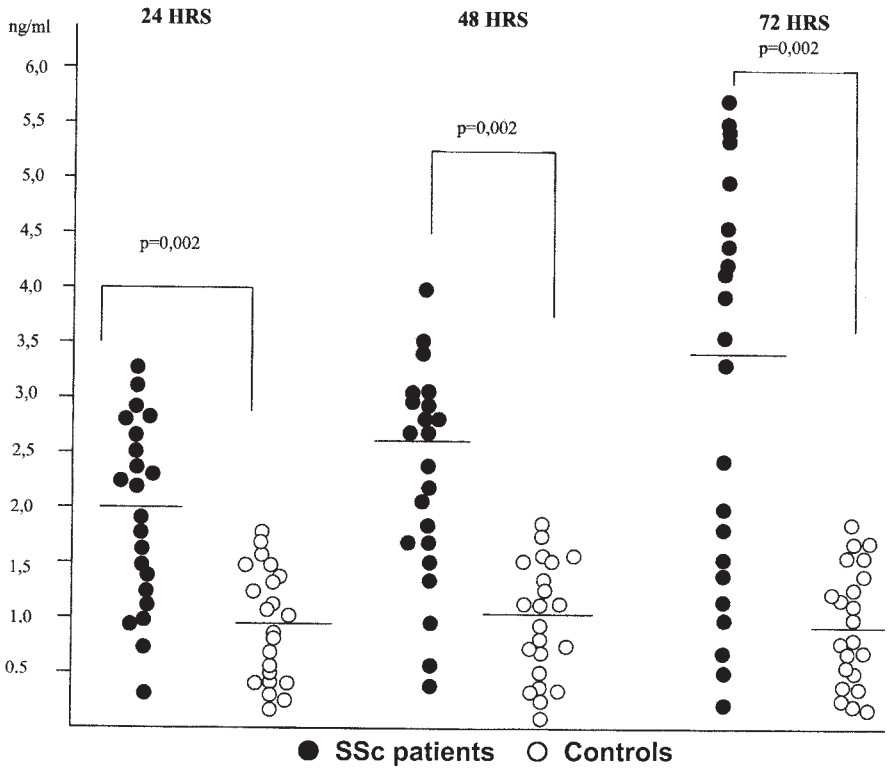

Figure 4. Soluble FAS levels in supernatants from stimulated cultures of SSc patients and controls at 24, 48, 72 hours. Differences between patients and controls were statistically significant for every assay.

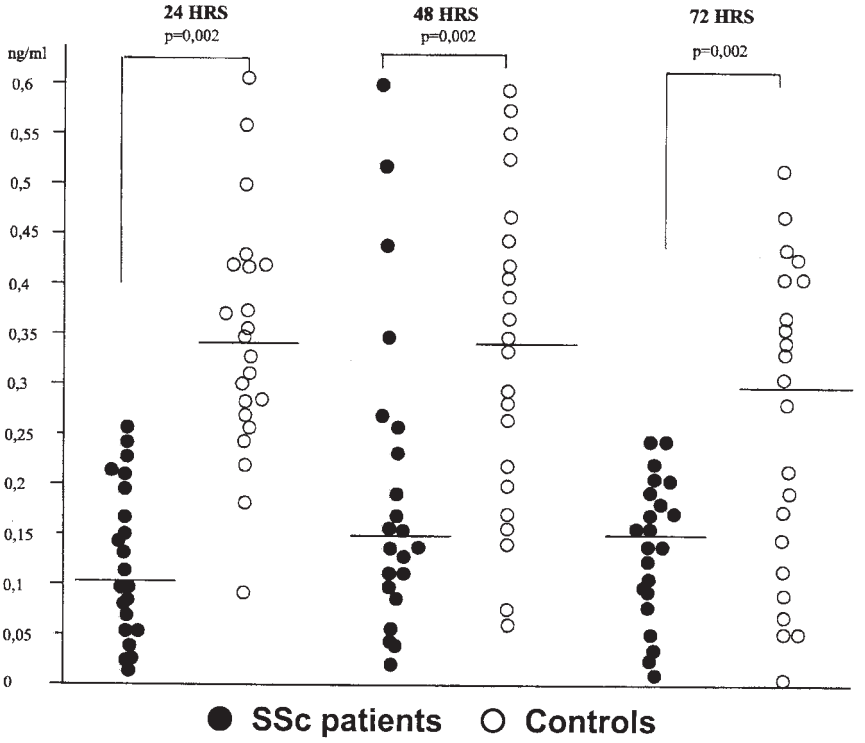

Figure 5. Soluble FAS ligand levels in supernatants of stimulated cultures from SSc patients and controls. Differences between patients and controls were statistically significant for every assay.

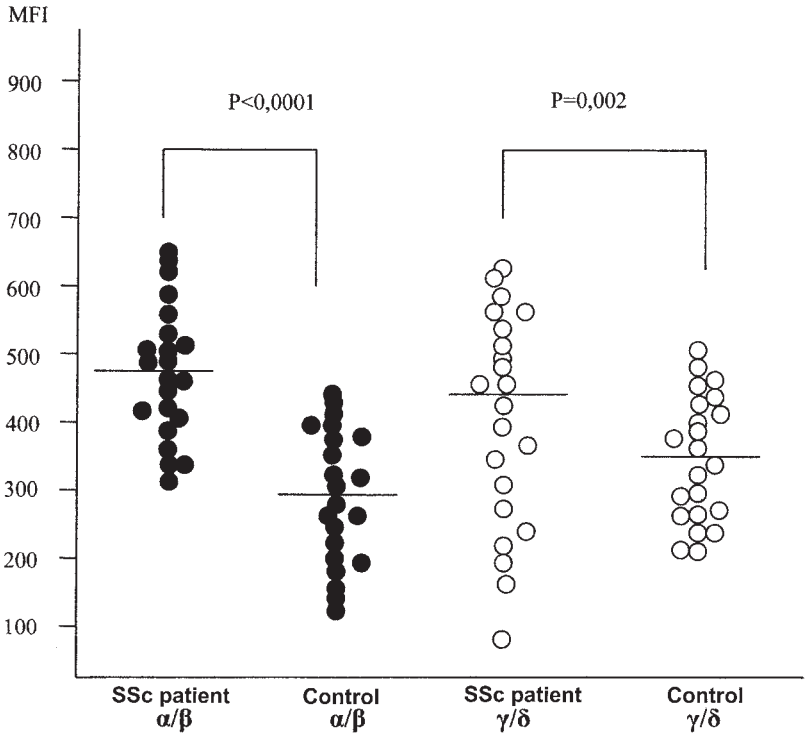

Figure 6. Levels of Bcl-2 expressed as median fluorescence index (MFI) in α/ß and γ/δ subsets of T lymphocytes from SSc patients and controls.

expression of Fas in the T cell subsets, also after PHA stimulation. After PHA exposure, T cells display an activated phenotype and activation-induced cell death, a homeostatic mechanism involving engagement of death receptors on lymphocytes by death ligands on the same lymphocyte (suicide) or on adjacent lymphocytes (fratricide)[35,36]. It is well known that AICD is dependent on prior upregulation in expression of Fas/FasL on T cells[35,37]. In the immune system, AICD acts as a feedback mechanism for terminating an ongoing immune response[38] and has the function of maintaining peripheral tolerance and regulating immune responses in disease[39]. In patients with SSc, after PHA stimulation, AICD displays a lower activity compared to healthy controls. To confirm that T lymphocytes of SSc patients were less susceptible to undergoing AICD, these cells were further stimulated with an anti-CD95 monoclonal antibody, and we observed the same decreased apoptotic rate. T cells of both patients and controls had similar cell-surface expression of CD95, confirming that this resistance to AICD was not due to lack of expression of Fas receptor, and suggesting that the Fas signal transduction pathway was impaired in the AICD-resistant T cells of SSc patients.

Rapid cell death of immune cells in vivo results from exposure to glucocorticoids. DEX is a synthetic glucocorticoid that has been shown to induce apoptosis in hybridoma T cells as well as in normal T cells, including thymocytes and peripheral lymphocytes (lymph node and spleen)[40]. In our study, the results after DEX cultures were similar to those observed after PHA stimulation. It is well known that DEX induces apoptosis of lymphocytes via interaction with the glucocorticoid receptor, endonuclease activation, Ca^{++} mobilization, and caspase activation[41]. Generally, DEX-induced apoptosis in both natural killer cells and T cells is inhibited by interleukin 2 (IL-2) and IL-4[42]. Several studies have confirmed an increased production of IL-4 in SSc, suggesting a role for the Th2 subset during the disease[3,4,7]. Thus, it is possible that activated T cells producing large amounts of IL-4 can counteract the apoptotic effect of DEX. Further, it has been shown that CD25-positive T cells, like activated T cells in SSc[9], express higher levels of glucocorticoid receptor and Bcl-2, and for this reason are more resistant to Dex-mediated cell death than CD25-negative T cells[43].

We evaluated the different ability of T cell subsets to undergo apoptosis. No differences were observed in the percentages of apoptotic cells in the different subsets from patients and controls, under all conditions. To evaluate γ/δ T cells, which represent a small subset of circulating T lymphocytes, we selected cells using magnetic immunobeads and expanded the samples by allogenic stimulation with irradiated (3000 rad) RPMI-8866 cells. After 10 days of coculture we evaluated apoptotic death of stimulated and expanded γ/δ T cells by double-staining with both annexin V and anti-γ/δ TCR antibody. Our data showed an increased survival of total γ/δ T cells in SSc patients after allogenic stimulation, compared to healthy controls. Contrasting results have been reported concerning the apoptosis of the γ/δ lineage. Human

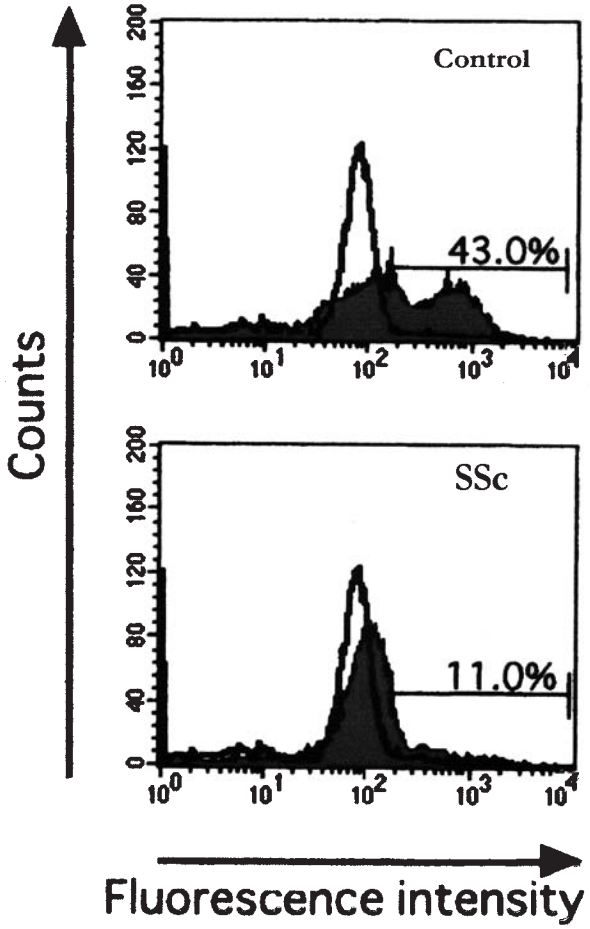

Figure 7. Activation of caspase-3, after anti-Fas treatment, using the PhiPhiLux-G2D2 cell-permeable fluorescent substrate. Data illustrated here are representative of several experiments. A significant decrease in the mean fluorescence displaying caspase-3 activity, after treatment by agonistic anti-Fas antibody, can be observed in the SSc patient plot compared to control.

γ/δ T cell clones have also been shown to readily undergo apoptosis if stimulated simultaneously by anti-TCR monoclonal antibody and by exogenous IL-2, leading some investigators to propose that the induction of programmed cell death upon repeated mitogenic stimulation might serve as a regulatory mechanism[44,45]. In some infections, γ/δ T cells are preferentially activated before undergoing apoptosis via Fas/FasL interaction[46]. In contrast, it has been shown that CD2 engagement in the presence of IL-12 provides a signal to γ/δ T cells that renders them resistant to AICD, reserving their cytotoxic activity[47]. Recently, increased levels of IL-12 have been observed in sera and in PBMC supernatants from patients with SSc, and this increase may be related to the activation of Th1 cells[48], supporting the possibility that this mechanism may operate in our patients. In this fashion, Vδ1-positive T cells, which display a strongly cytotoxic activity and produce Th1 cytokines[10,11], could be preferentially expanded during SSc, as confirmed in previous reports[8,9]. These data reflect observations in autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjögren's syndrome in which increased survival of autoreactive lymphocytes has been described[49-51].

Our results are not consistent with those reported by Stummvoll, *et al*[52], who did not find a decreased apoptotic rate in PBMC of patients with SSc. The reason for this discrepancy is unclear. It could be related to both a shorter duration of disease and to the prevalence of the diffuse subtype in our patients.

Of note, sFas levels were significantly increased in our patients with SSc, both in sera and in culture supernatants. This soluble form, derived by alternative splicing from the same mRNA as the membrane-associated form, seems to play a pivotal role in cell signaling. Indeed, sFas was able to downregulate the number of membrane-associated receptors and specifically inhibit ligand-receptor association in the extracellular space, preventing the proapoptotic effect of membrane-associated Fas activation[53]. Thus, it may be possible that in our patients, the increased levels of sFas alter T lymphocytes development, increasing their rate of survival and the proliferative response to (auto)antigens. Increased levels of sFas have been described in SSc[54] and several other autoimmune diseases such as SLE, polymyositis/dermatomyositis, mixed connective tissue disease, and Sjögren's syndrome[55,56].

Human FasL consists of 281 amino acids and is a member of the TNF family. Fas ligand molecules can be cut by matrix metalloproteinases from membranes to become a soluble sFasL. FasL can induce apoptosis of cells when it binds to Fas expressed on the cell surface, and acts as a special receptor of FasL. Similarly, as a ligand of Fas, FasL has many important functions. sFasL was increased significantly in the serum of patients with activated SLE and rheumatoid arthritis (RA), and in some autoimmune diseases the serum level of sFasL was dramatically decreased after treatment in some patients[56,57]. Recently, some reports describe that FasL and sFasL play different roles in apoptosis. Generally, membrane-bound FasL, or sFasL, is fully capable of transducing apoptosis Fas-mediated signals, because the membrane-bound FasL possessed both Fas-focusing and signal-transducing functions, while sFasL is known to be deficient in transducing signals upon engagement with membrane Fas[58,59]. Further, in some cases, sFasL was found to be involved in the deletion of potentially hazardous peripheral memory cells, suggesting a possible protective role in autoimmunity[60]. We were unable to confirm increased levels of sFasL observed in other autoimmune diseases, because the levels of sFasL were significantly lower compared to controls. Due to the complexity of and contrasting roles played by sFasL in apoptosis regulation, its function in this disease is still unclear.

In our patients an increased intracellular expression of Bcl-2 protein was shown. Members of the Bcl-2 gene family are able to influence the ability of lymphocytes to undergo apoptosis[61], and lymphocytes express at least 4 Bcl-2-related proteins[62]. In our patients, this overexpression was not restricted, including both α/ß and γ/δ T cells, while higher levels may be

observed in γ/δ T lymphocytes. The increase in Bcl-2 observed in γ/δ T cells, compared with α/ß T cells, may provide SSc γ/δ T lymphocytes with a survival benefit, and consequently could lead to the increased percentage of these cells as described[8-10]. This increased amount of Bcl-2 protein may be observed in SLE and other autoimmune diseases[63-65], and might be responsible for the resistance of T cells to steroid-induced death, as the induction of steroid-induced apoptosis in several cell systems is prevented by expression of Bcl-2[66]. It would be interesting to understand the mechanism by which Bcl-2 expression in our T cells was induced and maintained. Since Bcl-2 is known to be regulated through the action of the common γ-chain of the IL-2 receptor[67], cytokines are obvious candidates, with potential to induce high levels of expression of Bcl-2 in α/ß and γ/δ T cells. In addition to IL-2, cytokines such as IL-4, IL-7, IL-13, and IL-15 may be involved in modulating Bcl-2 expression.

Resistance to apoptosis may also be due to impairment of the signaling cascade downstream of Fas, as shown in caspase-3 or caspase-8 knockout mice[68] and in a human T cell line defective in caspase-3[69]. Caspases are broadly categorized into upstream regulatory caspases and downstream effector caspases. The upstream caspases such as caspase-9 and -8 typically have a long N-terminal prodomain that facilitates interaction with and recruitment of proapoptotic proteins, including other caspases. Downstream caspases, which typically have short prodomains, primarily cleave proteins important for cellular functions, resulting in the execution of cell death[70]. Caspase-3 is thought to be a key apoptotic "executioner" enzyme in mammalian cells because its activation triggers the cascade of enzymatic events that culminates in the death of the cell[71]. Thus, we focused our attention on this particularly important pathway relative to cell proliferation and apoptosis. Downstream of activated caspase-3 are the sequelae of apoptosis, including PARP cleavage and inhibition of DNA repair, DNA fragmentation, and nuclear membrane fragmentation. It is well known that increase or decrease in expression of the components of any signaling pathway is not necessarily probative of pathway activity. Instead, it is the change in the level of activated protein that is physiologically important. Thus, we evaluated the level of activated caspase-3 using a specific test that identifies only the phosphorylated or cleaved proteins and that does not crossreact with the inactive protein. We found a significant decrease ($p < 0.01$) in the mean of fluorescence specific for caspase-3 activity in the lymphocytes of SSc patients, after treatment by agonistic anti-Fas antibody. Whereas Fas crosslinking by agonistic anti-Fas antibody generally results in processing of all the detectable procaspase-3 (p32), only partial processing of the prodomain seems to be present in patients with SSc. Further, our results reflect similar data where a significant reduction in AICD was observed in different settings in which caspase-3 was absent or inhibited[72]. Recently, it was shown that nitric oxide production is markedly increased in patients with early-stage diffuse cutaneous SSc[73], the most representative stage in our patients, and this molecule was able to prevent apoptosis in other cell lines by directly inhibiting caspase-3 activation[74]. Of note, during mycobacterium infection, upon challenge with bacteria, phagocytes acquire the ability to specifically inhibit the apoptosis of γ/δ T cells, which are recruited early into the mycobacterial lesions, and that ability is due to nitric oxide production interfering with the intracellular activation of caspases[75].

Our data indicate that resistance to apoptosis is present in lymphocyte subsets of patients with SSc, both in α/ß and in γ/δ T cells. Several pathways seem to be connected in this setting. The complete significance of this finding is not fully understood, although its potential pathogenetic role has been discussed. Investigations of the intracellular components that mediate cytokine stimuli and genetic control of apoptosis are in progress.

ACKNOWLEDGMENT

The authors are indebted to Federica Sensini for her technical contributions.

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