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Serum levels of soluble Fas/APO-1 (CD95) and its molecular structure in patients with systemic lupus erythematosus (SLE) and other autoimmune diseases

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SUMMARY

There are two major forms of the Fas molecule, membranous Fas and soluble Fas (sFas). To clarify the clinical significance of sFas in autoimmune diseases, we designed a sandwich ELISA to determine serum concentrations of sFas and its molecular structure, and we then analysed the correlation between levels of sFas and laboratory findings in patients with SLE and other autoimmune diseases. The levels of serum sFas were significantly higher in SLE patients than in subjects with other autoimmune diseases and in healthy donors, and the frequency of a positive serum sFas was much greater in SLE patients with high SLE disease activity index scores than in those with low scores. In addition, sFas-positive SLE patients showed a significant difference in various laboratory parameters from sFas-negative SLE patients. Serial measurements of serum sFas levels in SLE patients with active disease revealed that the elevated level of sFas dramatically decreased with improvement in clinical and laboratory findings, following corticosteroid therapy. We propose that the serum level of sFas can serve as an appropriate marker for evaluating SLE disease activity. Serum sFas is heterogeneous with respect to molecular structure, thus several mechanisms are involved in the generation of sFas.

Keywords soluble Fas alternative splicing systemic lupus erythematosus disease activity

INTRODUCTION

While the pathogenesis of autoimmune disease is not fully understood, the breakdown of self-tolerance in the thymus or in the periphery may contribute to related phenomena, and the unusual process of apoptotic cell death at the stage of differentiation of immature lymphocytes or after activation of mature lymphocytes may be related to development of autoimmune diseases [1,2].

Yonehara *et al*. [3] and Trauth *et al*. [4] reported the mouse MoAbs, anti-Fas and anti-APO-1, respectively, both of which induce apoptosis in cells expressing an identical surface molecule, Fas/APO-1 antigen (CD95). Sequence analysis of human Fas cDNA [5,6] revealed that this molecule belongs to the tumour necrosis factor receptor/nerve growth factor receptor family, as do CD40, CD27, OX40, 4-1BB, and CD30 (reviewed in [7]). Subsequent cloning of mouse Fas [8] and Fas ligand (FasL) cDNA [9] revealed *lpr* and *gld* mouse mutations which proved to be defective in Fas antigen [10–14] and FasL [9], respectively. In these mutant mice, in which Fas death signalling is not transduced, activated mature T cells that are normally eliminated at the end of immune responses fail to undergo apoptosis [15,16] and may eventually accumulate in peripheral lymphoid organs as the massive lymphadenopathy phenotype. These findings suggest that Fas and FasL are essential for activation-induced cell death, and that any dysfunction in this system will lead to a breakdown in peripheral tolerance and autoimmune phenomena will ensue.

To explore the role of Fas in development of human SLE, our group and others analysed membranous Fas (mFas) expression by peripheral blood mononuclear cells (PBMC), and found that mFas was up-regulated in T cells [17,18] and in naive/memory T cell subsets [19] from patients with SLE. We further showed that naive $CD4^+$T cells from SLE patients co-expressed Fas and activation antigens, such as CD25 and CD71, indicating an extensive *in vivo* T cell activation [19]. Cheng *et al*. [20] demonstrated that serum levels of soluble Fas (sFas) are elevated in patients with SLE, although no clinical and laboratory data on SLE patients with elevated sFas levels were shown. They added evidence that a Fas–Fc fusion protein with a soluble nature

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evoked autoimmune features in healthy mice. In contrast, Knipping *et al*. [21] and Goel *et al*. [22] recently reported that elevation in sFas levels is a rare feature in SLE and doubted the involvement of sFas in the etiopathogenesis of SLE. To clarify this apparent discrepancy, we designed a sandwich ELISA system to determine serum sFas levels and to characterize the molecular structure of sFas. We measured sFas levels in sera from a larger number of patients with SLE and other autoimmune diseases, and analysed the relationship between sFas levels and laboratory findings in SLE.

PATIENTS AND METHODS

Patients and sera

Serum samples were obtained from 203 Japanese patients with various autoimmune diseases and who were followed in the Department of Medicine II, Hokkaido University School of Medicine. Included were 77 patients with SLE (67 women, 10 men), 60 with rheumatoid arthritis (RA) (49 women, 11 men), 19 with systemic sclerosis (SSc) (all women), 13 with polymyositis/dermatomyositis (PM/DM) (10 women, three men), and 34 with primary Sjögren's syndrome (SS) (all women). Control sera were obtained from 40 healthy donors (18 women, 22 men). All patients fulfilled the standard criteria for each autoimmune disease [23–27]. Using the SLE Disease Activity Index (SLEDAI) score [28], 77 SLE patients were divided into two groups according to McLaughlin *et al*. [29] and to Knipping *et al*. [21], 49 for an inactive stage (SLEDAI score $\leqslant 9$) and 28 for an active stage (SLEDAI score $\geqslant 10$). Laboratory assessments included complete blood cell count, erythrocyte sedimentation rate (ESR), and serum levels of C-reactive protein (CRP), IgG, IgA, IgM, C3, C4, CH_{50}, rheumatoid factor (RF), anti-nuclear antibody (ANA), and anti-DNA antibody. Individual serum samples were divided into aliquots and stored at -70°C until analysed.

Antibodies

Five anti-human Fas MoAbs and a polyclonal antibody were used. DX2 (mouse IgG1) and DX3 (mouse IgG1) have been described in detail elsewhere [30]. CH11 (mouse IgM) [3], UB2 (mouse IgG1) [31] and ZB4 (mouse IgG1) [31] were purchased from MBL Co., Ltd. (Nagoya, Japan). A polyclonal antibody (named anti-FasC) was raised against the synthetic peptide corresponding to the C-terminal sequence (KDITSDSENSNFRNEIQS) of human Fas [5]. The peptide was synthesized with cysteine for its N-terminal and conjugated to maleimide-activated keyhole limpet haemocyanin (KLH; Pierce, Rockford, IL). New Zealand White rabbits were immunized with 0·5 ml of conjugates (0·5 mg of the peptide) emulsified with Freund's complete adjuvant (FCA). Conjugates emulsified with Freund's incomplete adjuvant (FIA) were injected every 2 weeks as a booster. Anti-peptide antibody titre was serially measured by ELISA using peptide–bovine serum albumin (BSA) conjugates as a coating antigen. Anti-peptide-specific antibody was eluted from the peptide column to which hyperimmune serum had been applied. Cross-reactivity of affinity-purified antibody with mouse immunoglobulin was further eliminated by passing twice through the mouse immunoglobulin column.

Measurement of sFas

Serum concentrations of sFas were measured by sandwich ELISA. Briefly, each well of microtitre plates (Sumilon, MS-8496F: Sumitomo Bakelite Co., Tokyo, Japan) was coated with 50 μl of capture MoAb (10 μg/ml) for 3 h at 37°C, followed by blocking of free sites with 100 μl of Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan) for 2 h at 37°C. Test sera (50 μl) were added neat in duplicates and incubated for 3 h at 37°C, followed by 50 μl of biotinylated detector MoAb (10 μg/ml) for 3 h at 37°C, and by 50 μl of streptavidin-conjugated alkaline phosphatase (Zymed Labs, Inc., South San Francisco, CA) for 1 h at 37°C. The wells were finally incubated with 100 μl of diethanolamine buffer pH 9·8 containing *p*-nitrophenylphosphate (1 mg/ml) for 1 h at room temperature. Five washes with 0·05% Tween-20–PBS pH 7·4 were performed between steps. Affinity-purified recombinant human sFas or predetermined culture supernatant from COS-7 cells transfected with sFas cDNA-inserted expression vector served as standards. In some control experiments, sFas standards were quantified in the presence of recombinant human sFasL [32].

Statistical analysis

Data were analysed using a Macintosh computer and a statistical software package (StatView; Abacus Concepts, Inc., Berkeley, CA). The statistical significance of differences between groups was determined using the Mann–Whitney *U*-test. The positive incidence of sFas was compared using Fisher's exact test. Spearman rank correlation coefficients (r_s) were also calculated. $P < 0{\cdot}05$ was considered to have statistical significance.

RESULTS

sFas ELISA

We first attempted to determine the combination of anti-human Fas MoAb which could serve for a sandwich ELISA. Figure 1 shows data on a representative experiment using biotinylated DX2 as the detector MoAb and others as the capture MoAb. In this combination, only DX3 MoAb did not inhibit binding of DX2 to sFas. These results suggested that DX2 recognizes the same or a nearby epitope on Fas molecule as do CH11, UB2, and ZB4. We therefore adopted the combination of MoAbs DX3 and DX2 in our ELISA

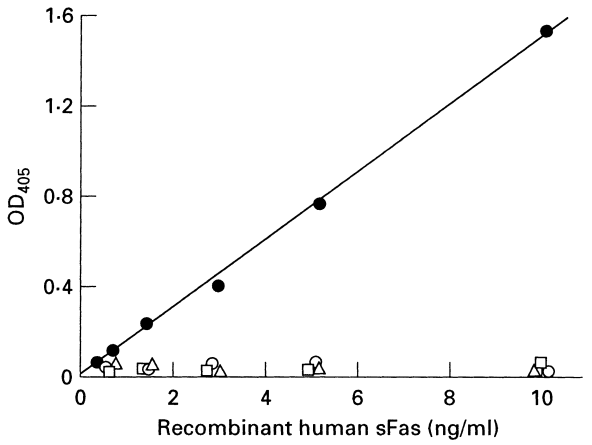

Fig. 1. Selection of anti-Fas MoAb combination used for a sandwich ELISA. Serially diluted recombinant human soluble Fas (sFas) was placed on the microtitre plates precoated with anti-Fas MoAbs DX3 (●), CH11 (○), UB2 (□), or ZB4 (△) as capture antibody and detected by biotinylated DX2 anti-Fas MoAb.

for sFas measurements. To determine whether sFasL interferes with the recognition of sFas by DX3 and DX2, we quantified sFas standards in the presence of an equal amount to 16-fold excess of sFasL. As interference was never observed in this range of sFasL tested, the possibility of underestimation of sFas levels in the sera containing sFas and sFasL was excluded (Fig. 2).

Serum sFas levels in patients with autoimmune diseases

Serum concentrations of sFas in 203 patients with various autoimmune diseases and in 40 healthy donors were determined, using the ELISA described above. Figure 3 shows the distribution of individual concentrations of serum sFas we examined. In healthy controls, serum sFas levels (mean ± s.d.) were 0·22 ± 0·25 ng/ml and were independent of age and gender. In patient groups, SLE sera exhibited higher levels of sFas, as follows: SLE, 0·87 ± 1·54 ng/ml; RA, 0·38 ± 0·81 ng/ml; SSc, 0·24 ± 0·27 ng/ml; DM/PM, 0·30 ± 0·24 ng/ml; SS, 0·31 ± 0·37 ng/ml. In the Mann–Whitney *U*-test, the levels of sFas in patients with SLE were significantly elevated compared with findings in healthy controls ($P < 0·001$), patients with RA ($P < 0·001$), patients with SSc ($P = 0·006$), and patients with SS ($P = 0·004$). Moreover, SLE patients at an active stage of the disease (SLEDAI score $\geqslant 10$) had higher levels of serum sFas than did those in an inactive stage (SLEDAI score $\leqslant 9$) (1·58 ± 2·36 ng/ml *versus* 0·46 ± 0·40 ng/ml; $P = 0·011$) (Fig. 4).

Since the levels of sFas showed a wide distribution in SLE patients, and in some patients sFas levels were below detectable limits (~ 0·1 ng/ml) by our ELISA, we determined a cutoff of 0·97 ng/ml (mean ± 3 s.d. of controls) separating sFas-positive from that in sFas-negative patients. In this manner, the frequency of sFas-positive patients in all disease groups could be determined. The positive frequency was greater in patients with active SLE (39·3%) than in healthy controls (2·5%) ($P < 0·001$), patients with inactive SLE (8·2%) ($P = 0·002$), RA (11·7%) ($P = 0·005$), SSc (0%) ($P = 0·001$), PM/DM (0%) ($P = 0·008$), and SS (2·9%) ($P < 0·001$), assessed by Fisher's exact test.

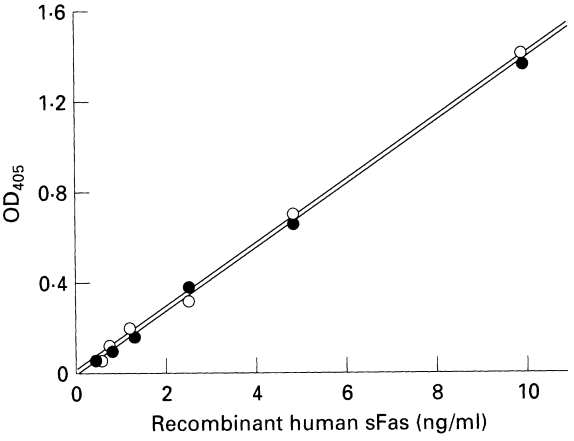

Fig. 2. Effect of recombinant human soluble Fas ligand (sFasL) on sFas measurement. sFas standards were quantified alone (●) or in the presence (○) of recombinant human sFasL (10 ng/ml) by the DX3/DX2 sandwich ELISA.

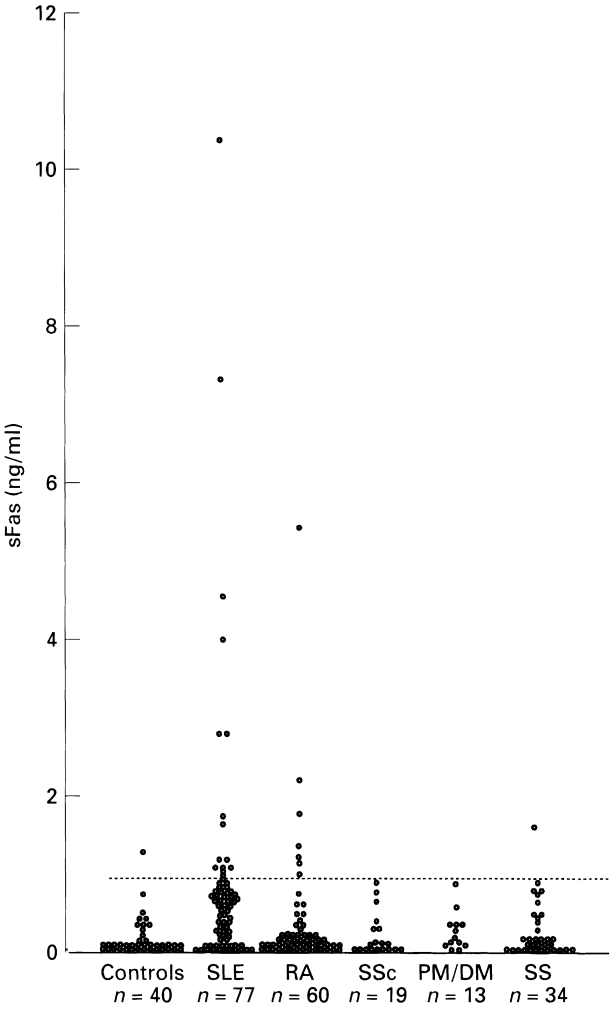

Fig. 3. Serum levels of soluble Fas (sFas) in patients with autoimmune diseases. Sera were randomly obtained from patients as indicated and healthy controls and assayed for sFas levels by the sandwich ELISA using DX3 and DX2 anti-Fas MoAbs. The horizontal dotted line denotes mean + 3 s.d. (confidence interval 99%) of controls. Subjects with sFas levels over this cutoff (0·97 ng/ml) were considered to be sFas-positive. RA, Rheumatoid arthritis; SSc, systemic sclerosis; PM/DM, polymyositis/dermatomyositis; SS, Sjörgen's syndrome.

Correlation between levels of sFas and laboratory parameters in patients with SLE

A significant positive or negative correlation was observed in SLE patients between serum levels of sFas and some laboratory parameters, including leucocyte counts ($r_s = -0·342$, $P = 0·006$), C3 levels ($r_s = -0·245$, $P = 0·033$), CH_{50} levels ($r_s = -0·241$, $P = 0·036$), anti-DNA antibody levels ($r_s = 0·292$, $P = 0·012$), IgA levels ($r_s = 0·332$, $P = 0·044$) and SLEDAI scores ($r_s = 0·240$, $P = 0·037$), by the Spearman rank correlation test. To characterize SLE patients with significant sFas levels, a comparison was made between data on sFas-positive and -negative SLE patients. Similar results were obtained with differences between laboratory findings in the two patient groups. As shown in Table 1, sFas-positive SLE patients had a significant decrease in leucocyte counts and serum levels of C3 and C4, and a significant elevation in ANA and anti-DNA levels and SLEDAI scores.

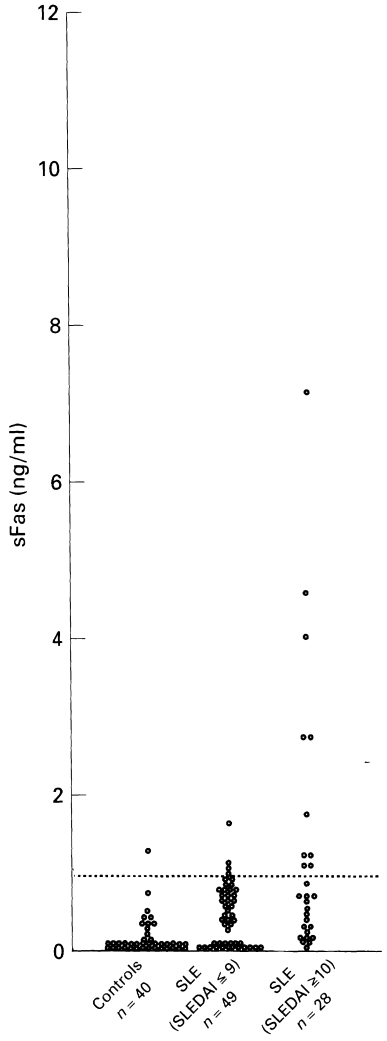

Fig. 4. Serum levels of soluble Fas (sFas) in inactive and active SLE. SLE patients were divided into two groups, active SLE with higher ($\geqslant$ 10) SLE Disease Activity Index (SLEDAI) score or inactive SLE with lower ($\leqslant$ 9) SLEDAI score. The dotted line indicates mean + 3 s.d. of controls.

Correlation between clinical course and levels of sFas in the same patient

We also evaluated the level of sFas serially during the clinical course of active SLE. Figure 5 shows representative data on one patient with active SLE. In this patient, who had been maintained on a low dose of prednisolone, serum sFas levels were significantly elevated during the active stage and decreased dramatically with clinical improvement following ingestion of a high dosage of prednisolone. The decrease in sFas levels closely correlated with that in anti-DNA antibody titre and SLEDAI score, and with the recovery of complement levels.

Characterization of serum sFas in SLE patients

Anti-human Fas MoAbs DX3 and DX2 recognize a different epitope on the extracellular region of the Fas molecule. Therefore, our assay system for sFas can detect any form of sFas which retains an extracellular portion with two epitopes recognized by DX3 and DX2. To characterize the molecular structure of serum sFas, SLE sera were analysed for sFas derived from alternatively spliced Fas mRNA, as described by Cheng *et al*. [20]. Since this spliced form of Fas lacks the transmembrane region but retains the cytoplasmic portion, rabbit anti-FasC with biotinylation was used as detector antibody. As shown in Fig. 6, there was a strong correlation ($r_s = 0{\cdot}571$, $P < 0{\cdot}001$) between sFas levels obtained by two different detector antibodies. In some sera, however, the levels of sFas detected by anti-FasC were negligible, despite significant levels of sFas measured by the DX3-DX2 MoAb system.

Table 1. Clinical activity, laboratory parameters, and prednisolone dosage in sFas-negative and -positive patients with SLE

	sFas-negative ($n = 62$)	sFas-positive ($n = 15$)	P*
SLEDAI score	6·6 ± 6·5	12·1 ± 6·8	0·004
ESR (mm/h)	37·0 ± 37·8	60·0 ± 40·1	NS
CRP (mg/dl)	0·54 ± 1·47	0·36 ± 0·20	NS
Leucocyte (/μl)	6173 ± 3149	3857 ± 2049	0·004
Lymphocyte (/μl)	983 ± 491	626 ± 302	NS
Platelet ($\times 10^{-4}$/μl)	19·6 ± 8·1	16·5 ± 9·0	NS
Haemoglobin (g/dl)	12·9 ± 1·4	11·0 ± 3·3	0·004
IgG (mg/dl)	1740 ± 1177	2338 ± 953	NS
IgM (mg/dl)	141 ± 76	191 ± 117	NS
IgA (mg/dl)	246 ± 124	332 ± 198	NS
C3 (mg/dl)	45·2 ± 17·1	33·3 ± 9·8	0·011
C4 (mg/dl)	18·1 ± 12·1	11·2 ± 6·0	0·042
CH_{50} (U/ml)	27·8 ± 12·5	23·6 ± 12·1	NS
ANA (10×2^m)	4·5 ± 2·0	5·8 ± 1·3	0·026
Anti-DNA (U/ml)	57·8 ± 150·5	357·4 ± 787·4	< 0·001
RF (U/ml)	26·8 ± 28·4	23·5 ± 5·3	NS
Prednisolone (mg/day)	9·6 ± 7·7	8·1 ± 15·9	NS

Data are expressed as means ± s.d.
*P values were determined using the Mann–Whitney U-test.

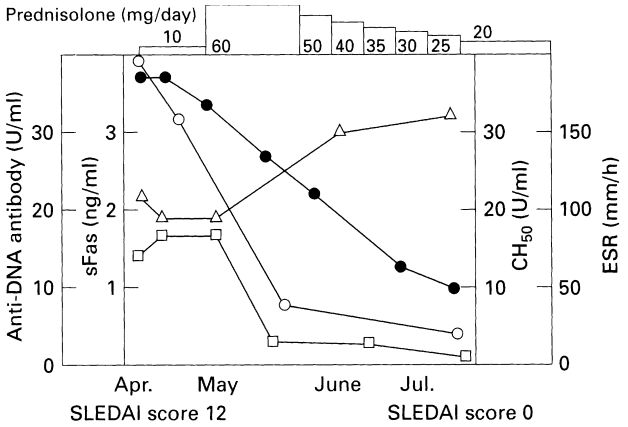

Fig. 5. Clinical course of a representative patient with active SLE. Serum level of soluble Fas (sFas) (●) was dramatically decreased after a high dose of prednisolone, in parallel with a reduction in anti-DNA antibody level (○), erythrocyte sedimentation rate (ESR) (□) and SLE Disease Activity Index (SLEDAI) score, and with elevation of the CH_{50} level (△).

DISCUSSION

In the present study, we designed an ELISA system to measure the levels of sFas and to characterize sFas in sera from patients with autoimmune diseases (Figs. 1 and 2). We found here that (i) the occurrence of sFas is more characteristic of active SLE than of other autoimmune diseases examined (Figs. 3 and 4); (ii) some laboratory parameters correlated significantly with serum sFas levels in SLE patients; (iii) sFas-positive patients with SLE are significantly different from sFas-negative patients, in laboratory findings as well as clinical activity (Table 1). It is noteworthy that most of these parameters indicating statistical significance are often taken as important indicators for lupus activity and therapeutic effectiveness. One example is serial sera from the same individual, where a highly significant correlation between sFas levels and clinical and laboratory findings was obtained during the course following standard corticosteroid therapy (Fig. 5). Thus, serial determination of sFas levels may provide an additional laboratory indicator of SLE disease activity.

Our work confirms and much extends observations of Cheng *et al*. [20], but presents a remarkable contrast with those of Knipping *et al*. [21] and Goel *et al*. [22] who recently reported no elevation in serum levels of sFas in SLE patients. First of all, differences in anti-Fas antibodies used in each sandwich ELISA system may account for this apparent discrepancy. The detection limit of our ELISA ($\sim 0{\cdot}1$ ng/ml) is the most sensitive among those described in the literature. Our anti-Fas MoAb combination was carefully selected, and the sFas level was not affected by the presence of sFasL in the range tested. FasL gene expression is up-regulated in PBMC from SLE patients (our unpublished observations). sFasL seems to be rapidly generated by matrix metalloproteinases from the cell surface of activated T cells [32]. Taken together with *in vivo* T cell activation in SLE [19], these findings strongly suggest that sFas may be complexed with sFasL in SLE sera and therefore will not be detected by anti-Fas MoAb which recognizes the Fas epitope hidden by the binding with FasL. The accessibility of anti-Fas MoAb to sFas/sFasL complex should be confirmed preliminarily, as described here.

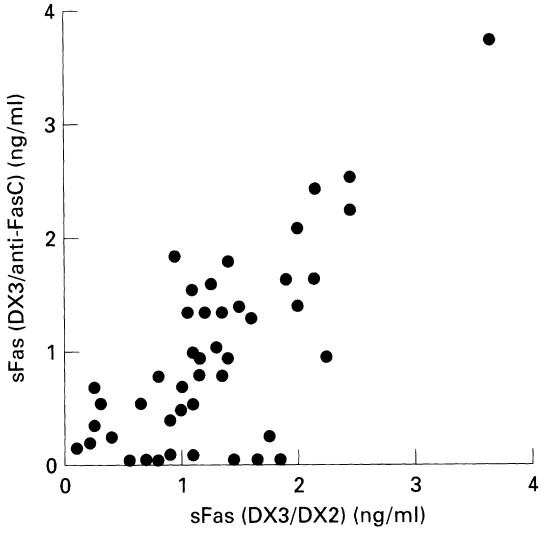

Fig. 6. Correlation between soluble Fas (sFas) levels in SLE sera measured by different detector antibodies. Identical serum samples were assayed for sFas on the same ELISA plate. sFas captured by DX3 MoAb was detected by either DX2 MoAb or anti-Fas C-terminal polyclonal antibody (anti-FasC).

Since isotypes of our two MoAbs are both mouse IgG1, overestimation of sFas levels by bridging the two MoAbs due to RF activity in sera would need to be considered. This is unlikely, because (i) there was no relationship between sFas and RF levels in SLE sera, and (ii) most seropositive RA patients were sFas-negative. Moreover, addition of RA serum with high-titred RF activity did not affect the results of sFas standards (data not shown).

To date, three Fas cDNA variants derived from alternatively spliced mRNA have been isolated and their corresponding sFas molecules are produced by transfected COS-7 cells ([20,33] and this study). However, of these three forms, Fas mRNA transcript lacking transmembrane-encoding exon 6 is most easily detected by reverse transcription-polymerase chain reaction in fresh PBMC from SLE patients and even from healthy donors, whereas the other two transcripts lacking exons 3 and 4, and exons 3, 4 and 6 were hardly recognized in ethidium bromide staining (S. Kobayashi, unpublished data). Most sFas levels in SLE sera measured using DX3/DX2 ELISA highly correlated with those using DX3/anti-FasC ELISA (Fig. 6). Thus we have obtained the first evidence that the major spliced form of sFas is the transmembrane-deleted Fas molecule with the cytoplasmic region retained. However, some SLE sera contained considerable levels of sFas not detected by anti-FasC, suggesting heterogeneity in the sFas molecule. As is the case for other cytokine receptors such as tumour necrosis factor receptor [34] and IL-2 receptor [35], sFas may be derived in part by proteolytic cleavage of mFas.

We have not specified the source of serum sFas in SLE patients. As previously noted in mice [8,36], Fas mRNA is expressed by most tissues, and to a variable extent all tissues expressing Fas may be responsible for the potential sources of any form of sFas. As peripheral blood T cells from SLE patients overexpress mFas [17–19], they are one of the most likely sources of increased levels of sFas. Parenchymal and interstitial cells in tissues subject to autoimmune damage may be another source of sFas. In this respect, sFas in some sera of RA patients probably originated from inflammatory synovial tissues, since mFas is not up-regulated in peripheral blood T cells from RA patients [19]. Analysis of biopsied specimens from SLE patients could contribute additional information about sFas sources.

Accumulating evidence revealed that Fas-mediated apoptosis plays a vital role in the immune system. Examples include elimination of self-reactive, immature T cells by clonal deletion in the thymus [31,37], T cell-mediated cytotoxicity [38], and activation-induced T cell apoptosis [39–41]. As indicated in the case of *lpr* [10–14] and *gld* [9] mouse mutations, and of recent human Fas mutations [42, 43], dysfunction in this apoptosis system is most likely to be involved in the initiation and/or the exacerbation of various immunological disorders. Among its putative mechanisms, sFas would function as an inhibitor of Fas/FasL interactions. sFas, although a chimaeric Fas–Fc fusion protein, has been reported to inhibit cytotoxic T lymphocyte-mediated cytotoxicity *in vitro* [44], and to alter lymphocyte development and proliferation in response to self antigen *in vivo* [20]. These findings strongly indicate that sFas has the potential to modify immune responses associated with Fas/FasL interactions. We are investigating the pathological function of native sFas at concentrations found in patient sera. As demonstrated in the present study,

further characterization of sFas might provide clues for monitoring SLE disease activity and new strategies for immune intervention could be designed.

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