Clin Exp Immunol 1996; **104**:460–466

Characterization of a soluble form of CD58 in synovial fluid of patients with rheumatoid arthritis (RA)

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(Accepted for publication 14 February 1996)

SUMMARY

Reduced levels of a soluble form of the adhesion receptor and CD2 ligand CD58 (sCD58) were previously described in RA patients. In order to understand the biological significance of this finding we biochemically characterized sCD58 in RA and asked how well sCD58 binds to CD2. sCD58 concentrations were measured in serum and synovial fluid (SF) samples of RA patients by two ELISAs, one detecting domain 1 of CD58 (CD58-D1), and the other one the complete molecule (CD58-D1 + D2). Small amounts of split sCD58-D1 were found in most RA sera, but not SF. In addition, split sCD58-D2 was detected in SF by affinity chromatography, SDS–PAGE, and Western blotting. Gel filtration gave similar peaks at 95–125 kD for RA sera, SF, and normal serum. Binding of SF-sCD58 to the $CD2^+$ Jurkat variant JBB1 or recombinant CD2 was stronger than urinary sCD58 and reached binding of oligomeric recombinant CD58 at low concentrations. In conclusion, sCD58-split products were found in RA sera and SF. At concentrations as they occur *in vivo*, SF-sCD58 binds to CD2 much more strongly than urinary sCD58. It is conceivable that locally released sCD58 blocks the CD2/CD58 interaction under physiological conditions. Insufficient release of sCD58, e.g. in synovitis, might result in T cell accumulation and perpetuation of inflammation.

Keywords rheumatoid arthritis soluble adhesion molecules CD2 CD58 LFA-3

INTRODUCTION

Rheumatoid synovitis is histologically characterized by mononuclear cell infiltrates consisting mainly of T lymphocytes, macrophage-like synoviocytes, and plasma cells. It is generally believed that activated T cells (CD4, CD45RO, $CD29^+$) drive this inflammatory process [1,2]. In order to reach the joint and to interact locally with B cells and synoviocytes T cells express on their membranes so called adhesion molecules (AM). These AM are required not only for physical adhesion but also for cell activation [3–7]. To this end, several receptor ligand interactions have been described mediating T cell, B cell, monocyte, and/or neutrophil activation, e.g. CD40 ligand/CD40, CD28/CD80 (previously B7.1), CD2/CD58 (previously LFA-3), LFA-1/intercellular adhesion molecule-1 (ICAM-1) (CD54), and VLA-4/vascular cell adhesion molecule-1 (VCAM-1) [7–15]. While the receptor/ligand pairs LFA-1/ICAM-1 and VLA-4/VCAM-1 are mainly involved in stimulation of resting T cells, CD28/CD80 and CD2/CD58 have been shown to stimulate activated T cells [9]. Previous reports suggest that the CD2/CD58 interaction is involved in most T cell interactions with other cell types, e.g. B cell, monocyte, and neutrophil activation as well as T cell/endothelial cell interactions [8,12–14]. Since activation processes of these cell types are involved in the immunopathogenesis of RA, it is conceivable that this receptor ligand pair is involved in rheumatoid synovitis. This notion is further supported by the finding that both the T cell marker CD2 and its ligand CD58 are up-regulated on synovial fluid (SF) mononuclear cells [16,17].

Both CD2 and CD58 are heavily glycosylated proteins and belong to the immunoglobulin supergene family. While CD2 is only expressed on T cells, natural killer (NK) cells, and thymocytes, the CD58 molecule is expressed on most human cell types [18]. We previously characterized the CD2/CD58 interaction using recombinant variants of CD58 and a panel of MoAbs directed at different epitopes of CD58 [19,20]. Both molecules, CD2 and CD58, have two extracellular immunoglobulin-like domains. While CD2/CD58 binding is mediated via the N-terminal part of each domain 1 (D1), domain 2 (D2) connects D1 to the membrane [19–23].

We previously described a naturally occurring soluble form of CD58 (sCD58) in human body fluids. Purified sCD58 from human urine binds to CD2 and blocks CD2/CD58 at high concentrations [24]. Compared with healthy controls or patients with spondylarthropathies, sCD58 levels were found to be significantly reduced in sera of patients with RA [25]. Similar to other serum proteins, sCD58 levels in SF were found to be 20–30% lower than serum levels [26–31]. The reduction of serum sCD58 correlated

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significantly with clinical and laboratory parameters of disease activity [25]. In order to understand the biological significance of this reduction, we examined whether sCD58 from sera and SF of RA patients exists as a complete, potentially functional molecule, and to what extent SF-sCD58 binds to CD2.

PATIENTS AND METHODS

Patients

Serum samples were obtained from 16 patients with RA. SF samples were obtained from four of these patients and 14 other patients with RA and major knee effusions (20–50 ml). All RA patients fulfilled the 1987 revised criteria for RA by the American College of Rheumatology [32]. Among a total of 30 RA patients there were 12 male and 18 female patients with a mean age of 55·9 years (range 21–77 years). All patients were seen at the Division of Rheumatology at the Medical School of Hannover as either in- or out-patients.

Serum as well as SF samples were collected and centrifuged for 20 min at 2500 ***g***. All samples were stored at –80°C until analysis.

MoAbs, recombinant proteins, and cell lines

MoAbs directed at different epitopes of human CD58 were AICD58.1 (epitope 4 of domain 1, IgG2a), AICD58.5 (epitope 5 of domain 2, IgG2a), AICD58.6 (epitope 1 of domain 1, IgG2a), AICD58.16 (epitope 6 of domain 2, IgG1), and TS2/9 (epitope 1 of domain 1, IgG1) [20]. All AICD58 MoAbs as well as the anti-CD2 hybridoma 8E5 ($T11_{1b}$, IgG2a) were kindly provided by S. C. Meuer (University of Heidelberg, Germany). The hybridoma TS2/9, and the anti-CD2 hybridoma OKT11 (IgG1) were obtained from ATCC (Rockville, MD). Recombinant CD58 and recombinant CD2 were expressed in the baculovirus system (transfer vector pACC5) and rCD58 was purified as previously described employing affinity chromatography [19]. Virus supernatants were also gifts from S. C. Meuer.

For binding studies the Jurkat cell line was subcloned and one clone with highest CD2 and lowest CD58 expression was selected (JBB1 cell line). The JBB1 and Daudi cell line (ATCC) were cultured in RPMI plus 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin, and 2% glutamine at 37°C and 5% CO_2.

sCD58 assay (for domain 1)

Soluble CD58 was measured by a sandwich ELISA using the anti-CD58 MoAb TS2/9 as coating antibody, and the anti-CD58 MoAb AICD58.1 or the anti-HLA class I MoAb W6/32 (IgG2a, ATCC) as detecting MoAb. The assay was performed essentially as previously described [24]. Briefly, a 96-well flat-bottomed microtitre plate was coated with 100 μl of MoAb TS2/9 (5 μg/ml) overnight in a cold room. After two washes with PBS–0·1% Tween the plates were blocked with PBS–1% bovine serum albumin (BSA) for 1 h followed by washing twice and antigen incubation using non-diluted serum, SF, or dilutions of recombinant CD58. After 2 h and washing five times, plates were incubated with 5 μg/ml MoAb AICD58.1 or W6/32 for 1 h. Finally, after washing three times the wells were incubated for 1 h with goat anti-mouse IgG2a coupled to alkaline phosphatase (AP), again washed three times and developed at room temperature using *p*-nitrophenyl-phosphate (PNPP) (Sigma, Munich, Germany). The reaction was stopped after 15 min by the addition of 3 M NaOH, and absorbance was determined at 405 nm using a Titertek ELISA reader. Non-specific reactions were defined as absorption more than twice the background absorption in the TS2/9-W6/32 ELISA. These samples were excluded from the study. Intra-assay variation for sCD58 was between 3% and 6·4%, interassay variation was between 2·8% and 7·6%. Soluble CD58 levels of seven SF samples treated with 50 U/ml hyaluronidase (Sigma) at 37°C were identical to mock treated samples. For further sCD58 measurements of SF no hyaluronidase treatment was performed.

sCD58 assay (domains 1 and 2)

The complete CD58 molecule was measured by a sandwich-type enzyme linked immunoassay as described above using the anti-CD58 MoAb AICD58.16 as coating antibody and AICD58.6 as second antibody.

Partial purification of sCD58 from SF of patients with RA and normal urine

RA SF samples ($n = 18$) (see above) without non-specific binding in the sCD58-D1 ELISA were pooled (400 ml) and treated with 1 μg/ml hyaluronidase at 37°C for 1 h. Sequential chromatography was used for partial purification of sCD58 employing a 100-ml G25 precolumn, 1·5 ml protein G Sepharose (Pharmacia, Freiburg, Germany) column, and finally a column of 2 ml cyanogen bromide (CnBr)-Sepharose (Pharmacia) coupled to the anti-CD58 MoAb TS2/9 (4 mg/ml). The third column was sequentially washed with 20 column volumes of PBS at a flow rate of 60 ml/h, 20 column volumes of 20 mM glycine HCl, 0·25 M NaCl pH 11, at a flow rate of 30 ml/h, 20 column volumes of 20 mM glycine HCl, 0·25 M NaCl pH 9 at 30 ml/h, and finally eluted with five column volumes of 0·1 M glycine HCl, 0·25 M NaCl pH 3, at a flow rate of 20 ml/h. The collected fractions were immediately neutralized by addition of 0·1 volume of 1 M Tris–HCl pH 7·5, pooled, dialysed against 15 mM NH_4CO_3, and lyophilized.

The pH of normal urine was adjusted to 7·5 using 10 N NaOH and centrifuged at 2000 ***g*** for 30 min. The partial purification was performed as for SF.

Molecular weight determination by gel filtration

Analytical molecular weight determination of sCD58 was performed employing a 350-ml Sepharyl 300 column (Pharmacia). Briefly, 6 ml of filtered SF or serum (0·45 μm) were loaded onto the column at a flow rate of 1 ml/min. Proteins were separated and eluted with 50 mM Tris, 0·5 M NaCl pH 7·6, at a flow rate of 2 ml/min. Fractions (2·5 ml) were analysed for sCD58-D1 by ELISA as above. Molecular weight was determined using a calibration curve.

SDS–PAGE and Western blotting

Lyophilized sCD58 was reconstituted with PBS and subjected to SDS–PAGE (10% polyacrylamide) under reducing conditions. Proteins were then blotted onto nitrocellulose sheets using a semidry blotting chamber (BioRad, Munich, Germany) according to the manufacturer's instructions. sCD58 was visualized as previously described using the anti-CD58 MoAbs AICD58.1 for D1 or AICD58.5 for D2 at 100 μg/ml [24].

CD2 binding assays

Binding of sCD58 to CD2 on the Jurkat variant JBB1 was tested as previously described [24]. Briefly, 4×10^5 JBB1 or Daudi cells were incubated simultaneously with dilutions of purified sCD58 or recombinant CD58, and 100 μg/ml AICD58.16 for 1 h at 4°C.

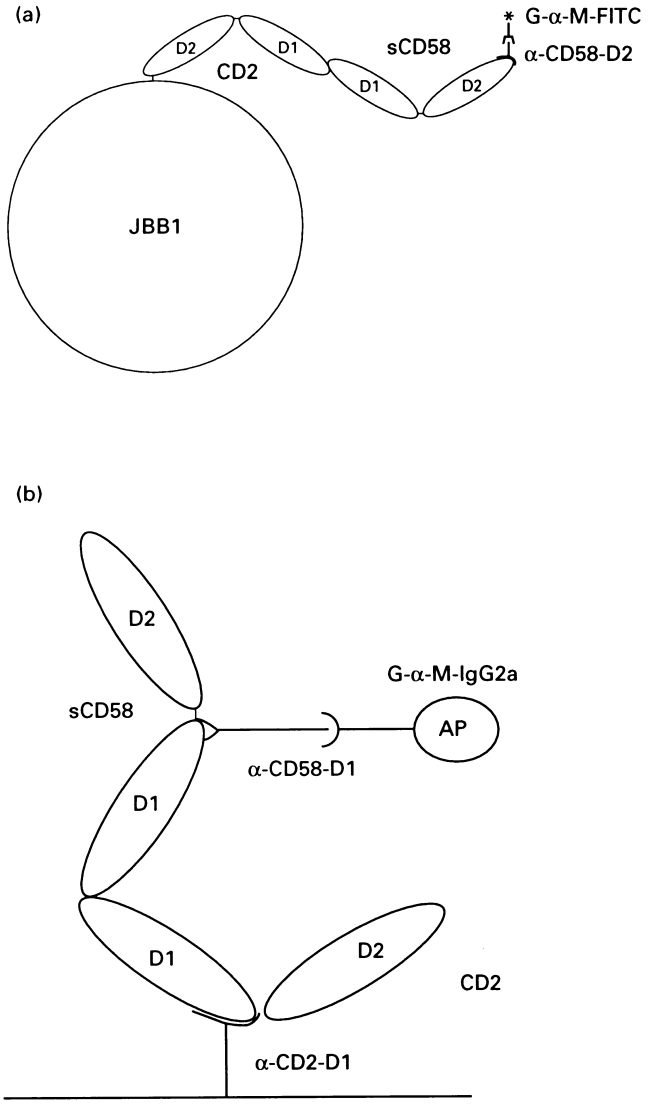

Fig. 1. Assay systems for the CD2/CD58 interaction. (a) Soluble CD58 (sCD58) binds to the $CD2^+$ Jurkat variant JBB1. Binding is detected by anti-CD58-D2 MoAb that does not interfere with the binding site and goat anti-mouse coupled FITC (G-α-M-FITC). (b) Anti-CD2 MoAb is coated into a microtitre plate followed by recombinant CD2. Binding of sCD58 to rCD2 is detected by anti-CD58-D1 MoAb not interfering with the CD2 binding and a secondary goat anti-mouse antibody coupled to alkaline phosphatase (G-α-M-IgG2a-AP).

Subsequently, cells were washed twice with PBS and incubated with FITC-labelled IgG1-specific goat anti-mouse antibody (Dianova, Hamburg, Germany) at a 1:20 dilution in PBS (Fig. 1a). After two washes with PBS, labelled cells were resuspended in 200 μl PBS containing 1% formalin, and fluorescence was determined using a FACScan flow cytometer.

Binding of sCD58 to recombinant CD2 was performed using a 'double' sandwich ELISA using the anti-CD2 MoAb OKT11 (IgG1, ATCC) as coating antibody and AICD58.1 or 8E5 as detecting MoAb at 5 μg/ml (Fig. 1b). Briefly, 96-well flat-bottomed plates were coated with 5 μg/ml OKT11, washed and blocked as in the sCD58 ELISAs (see above). Subsequently, wells were incubated with saturating concentrations of recombinant CD2 (about 2 μg/ml, 100 μl/well) or PBS/BSA 0·1% followed by washing and a second blocking step. Thereafter, serial dilutions of either recombinant CD2, recombinant CD58, partially purified urinary sCD58, or partially purified SF sCD58 were added. A 2-h incubation was followed by washing four times and adding the 8E5 MoAb or the AICD58.1 MoAb at 5 μg/ml for 1 h. The final steps using goat anti-mouse IgG2a-AP and PNPP were as described above.

RESULTS

Molecular structure of sCD58 from SF and serum of RA patients as defined by different ELISAs

sCD58 was measured in parallel for sCD58-D1 and sCD58-D1 + D2 in 16 serum samples and 18 SF samples of patients with RA. sCD58-D1 serum concentrations were significantly higher than sCD58-D1 + D2 levels (15·1 $\pm$ 0·9 ng/ml and

12 ± 0·7 ng/ml, respectively; $P = 0{\cdot}02$) (Fig. 2a). A strong correlation was found between sCD58-D1 and sCD58-D1 + D2 ($r = 0{\cdot}75$, $P < 0{\cdot}001$; Fig. 2b). In contrast, equal amounts of sCD58-D1 and sCD58-D1 + D2 were found in SF samples of RA patients (13·6 ± 1 ng/ml and 12·6 ± 0·8 ng/ml, respectively) (Fig. 3a), and the correlation was again significant ($r = 0{\cdot}57$, $P = 0{\cdot}015$; Fig. 3b). These data suggest that small amounts of split D1 occur in sera of RA patients, but no split D1 was detected in SF samples. Since CD58-D2 cannot be measured by these ELISAs, split CD58-D2 may still exist in SF of RA patients.

Biochemical characterization of sCD58 from SF and serum of RA patients

SF (400 ml) was pooled for purification and further analysis of SF-sCD58. SF was digested with hyaluronidase for 1 h and sequentially passed over two precolumns (G25 and Protein G) and finally over an anti-CD58 affinity column using MoAb TS2/9. The elution profile is shown in Fig. 4. After dialysis and lyophilization the purified sCD58 was subjected to SDS–PAGE under reducing conditions, and Western blotting was performed using the MoAb AICD58.1 directed at CD58-D1, and AICD58.5 directed at CD58-D2. Interestingly, no split products of CD58-D1 were detected, though split CD58-D2 (20–30 kD) was found in addition to the normal band at 40–65 kD (Fig. 5). Such split CD58-D2 or CD58-D1 was not detected in purified urinary sCD58 (data not shown). In order to analyse further the molecular structure of natural sCD58 in RA serum and SF, three serum and six SF samples were subjected to gel filtration followed by detection of sCD58 with the CD58-D1 ELISA. As shown in Table 1, sCD58 was detected in RA serum, SF, and normal serum in a similar range of 35–150 kD, with peaks at 95–125 kD.

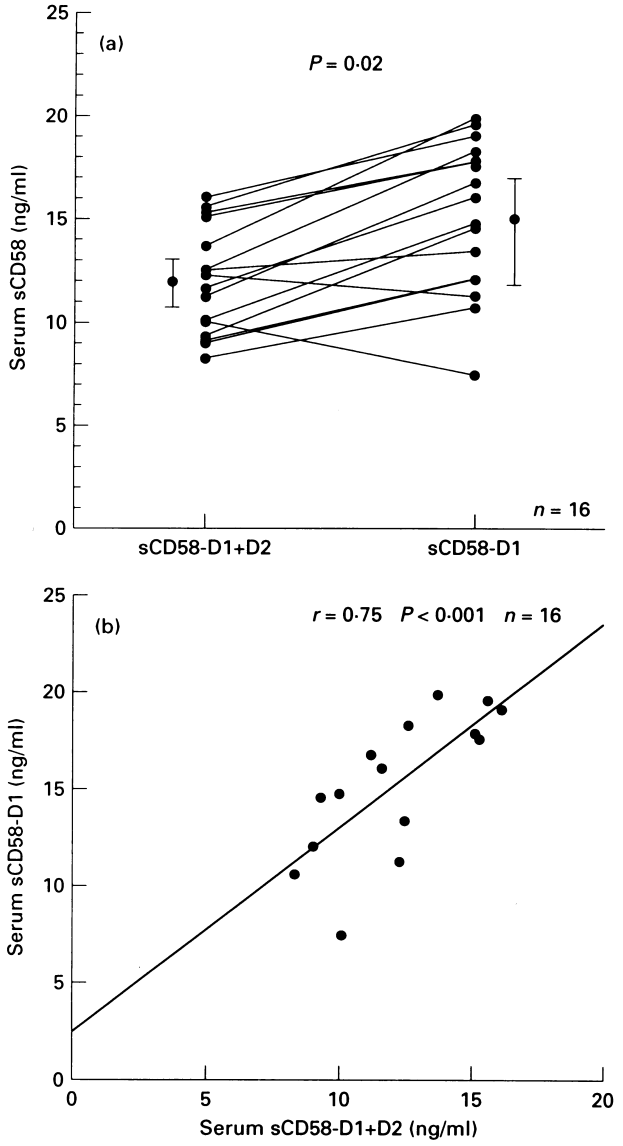

Fig. 2. Increased soluble CD58 domain 1 (sCD58-D1) in serum of patients with RA. (a) sCD58 measured by an ELISA directed at the complete CD58 molecule (sCD58-D1 + D2) or directed at sCD58-D1. Indicated are means (●) ± 2 s.e.m. (thin bars). (b) Correlation of sCD58-D1 and -D1 + D2 serum levels of RA patients.

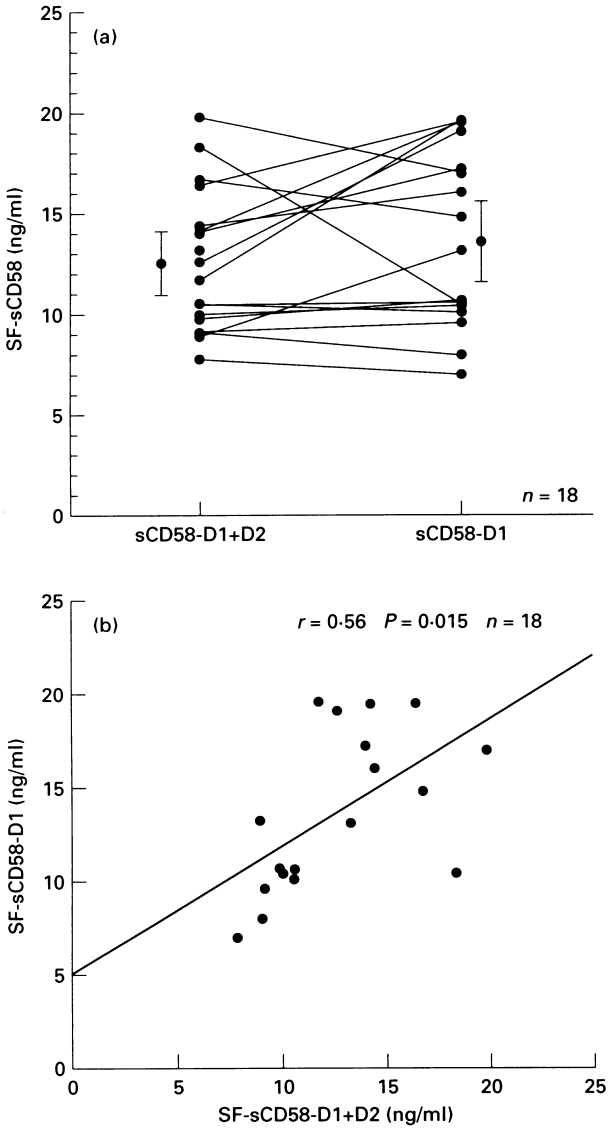

Fig. 3. Equal amounts of soluble CD58 molecules in synovial fluid (SF) of patients with RA. (a) sCD58 measured by an ELISA directed at the complete CD58 molecule (sCD58-D1 + D2) or directed at sCD58 domain 1 (sCD58-D1). Indicated are means (●) ± 2 s.e.m. (thin bars). (b) Correlation of sCD58-D1 and -D1 + D2 SF levels of RA patients.

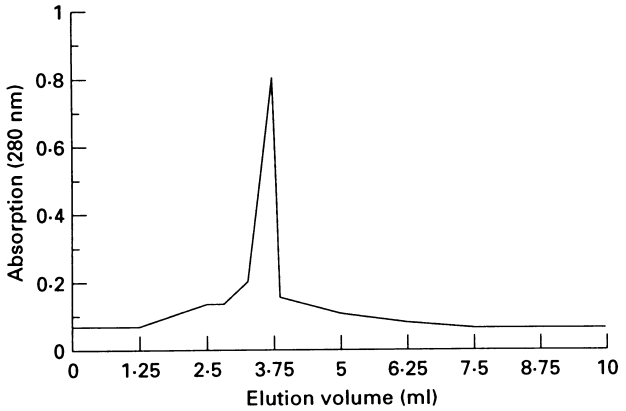

Fig. 4. Elution profile of synovial fluid (SF) sCD58 from 400 ml of SF from RA patients, partially purified by affinity chromatography employing an anti-CD58 affinity column.

Binding of SF-sCD58 of RA patients to CD2

Since the biochemical characterization of SF-sCD58 of RA patients revealed that split products of CD58 are present in RA serum (sCD58-D1) and in SF of RA patients (sCD58-D2), we examined whether these split products have any functional consequence for binding of sCD58 to CD2. As shown in Fig. 6a, partially purified SF-sCD58 bound significantly better to recombinant CD2 than urinary sCD58. At low concentrations (2·5–75 ng/ml) CD2 binding of SF-sCD58 was equal to recombinant CD58; however, at high concentrations rCD58 bound better to CD2 than SF-sCD58. These results were essentially confirmed using the Jurkat subclone JBB1 with high expression of CD2 (Fig. 6b). Binding of sCD58 to CD2 was blocked by the anti-CD2 MoAb 8E5 directed at the $T11_{1b}$ epitope of CD2 (data not shown).

DISCUSSION

We identified small amounts of sCD58-split products in sera and SF of patients with RA, but not in sera or urine from healthy controls. Under non-denaturing conditions serum and SF-sCD58 from RA patients were found to have similar molecular weights to the previously described normal urinary sCD58 [24]. Surprisingly, binding of partially purified SF-sCD58 to CD2 was found to be much stronger than urinary sCD58.

In the past, serum proteins have been shown to occur in SF depending on their molecular size and depending on pathological changes in the synovial membrane [26–31]. Accordingly, protein SF/serum ratios can be predicted from the molecular weight for different types of joint disease. The predicted SF/serum ratio for the described sCD58 here (95–125 kD by gel filtration) would be 0·8 for RA. Indeed, we previously found a SF/serum ratio of 0·8 for sCD58 from RA patients with a high correlation for paired SF/serum samples [25]. These data suggest that SF-sCD58 is part of a serum protein filtrate occurring in SF similar to other serum proteins. As a consequence, the molecular structure of sCD58 should be similar for SF-sCD58 and serum sCD58. Our ELISA data show that split sCD58-D1 exists frequently in RA sera, but uncommonly in SF. However, the largest part of sCD58 both in sera and SF exists as a complete CD58 molecule (D1 + D2), and the molecular weight of serum and SF-sCD58 was found to be 95–125 kD under non-denaturing and 40–65 kD under denaturing conditions (Western blotting), as previously reported for urinary and serum sCD58 of healthy controls [24]. Therefore, the molecular structure of most sCD58 in RA is indeed similar for serum and SF. The different molecular weights under denaturating (SDS–PAGE) and non-denaturating conditions (gel filtration) suggest a non-covalently associated homotrimer conformation or an association with another protein [24]. Although small amounts of sCD58-split products are a unique feature of RA patients, they are probably of no functional relevance since even purified CD58-D1 (the CD2-binding domain) binds only very weakly to CD2 (S. C. Meuer, personal communication). These split

Table 1. Molecular weight of sCD58 by gel filtration.

Sample	No.	Peak (kD)	Range (kD)
Serum, normal		95	45–115
Serum, RA	1	105	40–150
	2	100	35–115
	3	95	30–110
SF, RA	1	102	48–102
	2	125	60–200
	3	115	55–115
	4	100	41–112
	5	94	43–140
	6	98	43–143

SF, Synovial fluid.

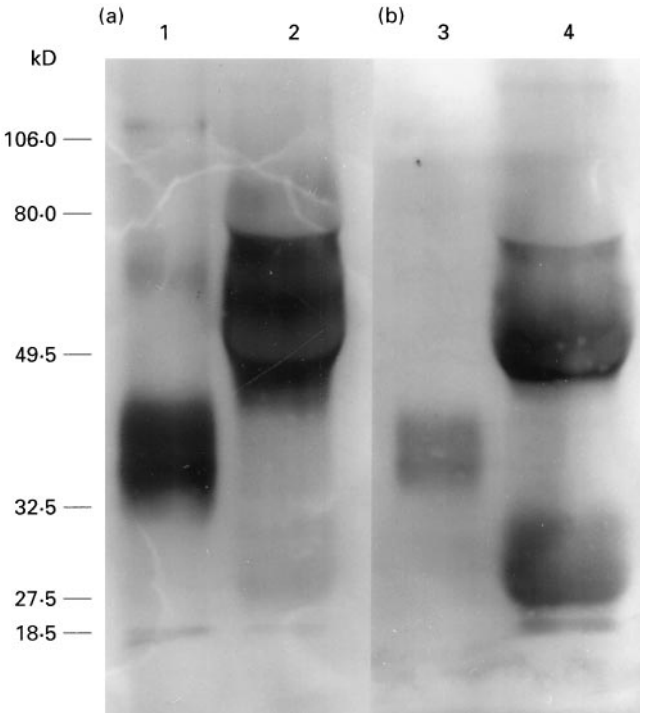

Fig. 5. Split sCD58-D2 is present in synovial fluid of RA patients. (a) Recombinant CD58 (lane 1, 150 ng) or partially purified sCD58 from RA synovial fluid (lane 2, 200 ng) were detected using an anti-CD58 domain 1 MoAb (AICD58.1). (b) As in (a) using an anti-CD58 domain 2 MoAb (150 ng recombinant CD58, lane 3; 400 ng sCD58, lane 4, AICD58.5).

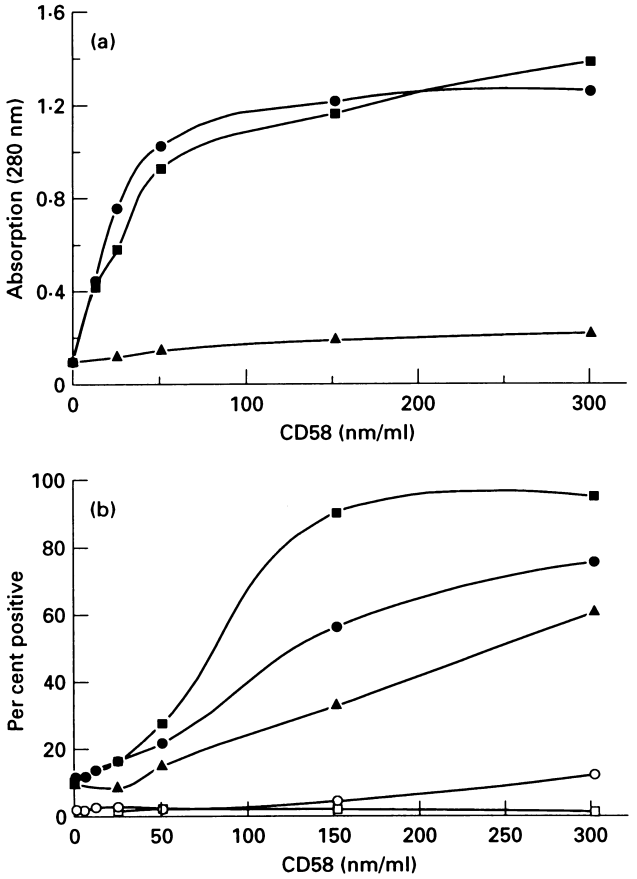

Fig. 6. SF-sCD58 binds better to CD2 than urinary sCD58. (a) Binding of different forms of sCD58 to the recombinant CD2 tested by ELISA. Shown is the absorption at 280 nm using AICD58.1 as detecting MoAb for recombinant CD58 (■), SF-sCD58 (●), and urinary sCD58 (▲). (b) Binding of sCD58 forms to the $CD2^+$ Jurkat variant JBB.1 (closed symbols) or $CD2^-$ Daudi cell MoAb (open symbols) using AICD58.16 (symbols as in (a)).

products are most likely an epiphenomenon of synovitis, where proteinases are released by neutrophils and macrophage-like synoviocytes.

One important factor for adequate binding of CD58 to CD2 is the degree of oligomerization [19,33]. Therefore, monomeric recombinant CD58 binds weakly to CD2 and is easily displaced using anti-CD2 MoAb or $CD58^+$ cells. In contrast, oligomeric CD58 binds strongly even at low concentrations to CD2, blocks cytotoxic reactions by interference with the CD2/CD58 interaction, and blocks the mixed lymphocyte reaction. We found dose-dependent binding of both urinary and SF-sCD58 to CD2 in two diffirent binding assays. Particularly at low concentrations of 25–50 ng/ml, as they occur in vivo, SF-sCD58 bound to the $CD2^+$ cell line JBB1 equally compared with recombinant oligomeric CD58. At this concentration range SF-sCD58 binding to recombinant CD2 was even better in the *in vitro* assay than for recombinant CD58. This increased binding was independent of the detected split products which have previously been shown not to bind effectively to CD2. This increased binding cannot be explained by the molecular size of sCD58, since both SF- and urinary-sCD58 have the same molecular weight. However, it is possible that SF-sCD58 has a different conformation or is differently glycosylated compared with urinary sCD58, resulting in improved binding to CD2. Because N-glycosylation of CD2 has been shown to be required for binding to CD58 [23], the pattern of CD58 glycosylation is probably also important for CD2/CD58 binding. Since the circulation and synovial fluid are directly linked, as discussed above, one can assume that serum sCD58 also binds much more strongly to CD2 than urinary sCD58. This appears to be particularly relevant when trying to understand the physiological and pathophysiological role of sCD58. Although not proven for the *in vivo* situation, sCD58 release can be influenced by cytokine stimulation *in vitro* [24,34]. One can therefore speculate that sCD58 is released locally at high concentrations, giving systemic levels of 10–60 ng/ml. In this model, high local concentrations result in effective binding of sCD58 to CD2, and sCD58 interferes with binding of $CD2^+$ T or NK cells with their ligand, resulting in de-adhesion. If the sCD58 release is not adequate, adhesion predominates over de-adhesion, leading to accumulation of $CD2^+$ cells. This might occur in rheumatoid synovitis, where activated T cells are trapped in the joint and perpetuate the inflammatory process. This notion is supported by our recent finding that rat adjuvant arthritis can be suppressed by anti-CD2 MoAb therapy [35].

In conclusion, we have identified split products of sCD58 in sera and SF of patients with RA. More importantly, partially purified SF-sCD58 was found to bind at low concentrations, as they occur *in vivo*, as strongly to CD2 as oligomeric recombinant CD58. It is conceivable that sCD58 binds *in vivo* to $CD2^+$ T and NK cells, interfering with immunological processes mediated via the CD2/CD58 interaction. Insufficient local release of sCD58 may therefore favour activation processes of monocytes, B cells, and neutropils, and/or lead to accumulation of activated T cells.

ACKNOWLEDGMENTS

We thank S. C. Meuer (Heidelberg, Germany), for providing anti-CD58 hybridomas, and supernatants of recombinant CD2 and recombinant CD58. This work was supported by DFG-grant Ho 1561/1-1 and by an award to J.C.H. from the Kurt-Eberhard Bode foundation.

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