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Decreased levels of serum soluble complement receptor-II (CR2/CD21) in patients with rheumatoid arthritis

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***Objective*. The soluble cluster of differentiation 21 (sCD21) represents the extracellular portion of the CD21 glycoprotein and is released by shedding from cell surfaces into plasma. Soluble CD21 binds complement fragments and activates monocytes through binding to membrane CD23. Elevated levels of sCD21 are found during Epstein–Barr virus EBV infections, B-cell lymphoma and other lymphoblastoid tumours. The present study was undertaken to investigate levels of sCD21 in rheumatoid arthritis.**

***Methods*. A specific enzyme-linked immunoassay was developed using sCD21, biochemically purified to homogeneity from human plasma as a standard for the determination of sCD21 concentration in patient sera. Peripheral blood B and T lymphocytes were isolated from healthy donors and rheumatoid arthritis patients and cultured, and supernatants were analysed for CD21 shedding.**

***Results*. The normal values of serum sCD21 in healthy individuals between 20 and 40 yr of age ranged from 100 to 477 ng/ml (median 292 ng/ml), decreasing with age but not differing with gender. In rheumatoid arthritis patients, sCD21 levels ranged from 50 to 300 ng/ml (median 182 ng/ml), did not differ with age and were independent of rheumatoid factor.**

***Conclusions*. In contrast to healthy donors, patients with rheumatoid arthritis have significantly lower sCD21 levels ($P < 0.0001$), independently of the age of the patients. Sorted B cells from rheumatoid arthritis patients released amounts of CD21 comparable with those of normal controls. Possible causes and consequences of the findings are discussed.**

KEY WORDS: Rheumatoid arthritis, CD21, Complement, Shedding, Soluble CD21

Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease that predominantly affects the joints. Diagnosis is based on symptoms related to synovial inflammation, the presence of rheumatoid factor (RF) and radiological signs of joint damage [1]. The aetiology of RA is still unknown. In the extremely complex pathophysiology, innate immunity may play an important role [2]. RF has been established as a useful serological marker for diagnosis, and a variety of other serum parameters, e.g. acute-phase proteins such as C-reactive protein and fibrinogen, indicate disease activity. Recently, a combination of RF and antibodies to cyclic citrullinated peptide was reported as a possible diagnostic tool to differentiate between RA and undifferentiated polyarthritis [3]. Markers of increased connective tissue turnover and certain soluble membrane-bound proteins, such as soluble interleukin (IL)-2 receptor, soluble CD4, IL-6, α-1-antitrypsin and complement activation products, are elevated in RA [4].

Complement receptor II (CD21) is the receptor for C3d fragments on immune complexes and Epstein–Barr virus (EBV). In B lymphocytes, CD21 amplifies the signal through the B-cell receptor [5]. CD21 is shed from the surface of the cell and is found circulating in plasma. The clinical significance of sCD21 is shown by higher levels in sera of patients with certain B lymphomas, EBV infection and other lymphoblastoid tumors [6]. Soluble CD21 activates monocytes through binding to membrane CD23. As sCD21 could potentially bind to its ligands in plasma, the amount of sCD21 in circulation could be a modulator of immunity [7].

We have previously shown a reduction of surface CD21 in synovial lymphocytes compared with peripheral blood lymphocytes and activated T cells [8, 9] and have recently reported the isolation and characterization of sCD21 from human plasma [10]. Here we investigated if and to what extent sCD21 levels might be influenced by autoimmune RA. Using purified sCD21 from human plasma as standard, we established an enzyme-linked immunosorbent assay (ELISA) for the quantification of sCD21 in serum. Our data demonstrate that levels of sCD21 in healthy individuals decrease with age, independently of gender ($n = 235$). In sera of patients with RA ($n = 220$), sCD21 was significantly reduced ($P < 0.0001$). The reduction of sCD21 observed in RA is independent of both age and the presence of RF. Studying isolated lymphocytes, we determined that shedding of the sCD21 protein from RA B lymphocytes is not impaired.

Materials and methods

Human serum, cells, antibodies and reagents

Human serum was collected from healthy blood donors and patients with RA, diagnosed according to the American College of Rheumatology classification criteria [1]. In all instances patients gave informed consent. The monoclonal anti-CD21 antibodies BU32 [immunoglobulin (Ig) G1] and THB5 (IgG2a) were grown in serum-free hybridoma medium (Invitrogen, Karlsruhe, Germany)

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at 37°C in 7.5% CO_2 and purified by affinity chromatography with protein G Sepharose (Amersham, Freiburg, Germany). Biotinylated BU32 was purchased from Chemikon (Hofheim, Germany).

Purification of soluble CD21

Soluble CD21 from human plasma was purified to homogeneity by affinity chromatography and density gradient centrifugation as described before [10]. Using purified sCD21 as standard, we developed a sandwich ELISA for the determination of sCD21 in the sera of healthy and diseased individuals.

Quantification of soluble CD21 from human serum by ELISA

A sandwich ELISA was performed to measure sCD21 levels in human serum. The monoclonal antibodies THB5 and biotinylated BU32 were used as capture antibody and revealing antibody respectively. Titration was performed with purified sCD21 [10] and a standard curve was plotted. Briefly, THB5 was coated onto an ELISA plate (TPP, Trassadingen, Switzerland) at a concentration of 5 μg/ml in coating buffer (0.1 M Na_2HPO_4/NaH_2PO_4, pH 9.0) for 12–15 h at 4°C. After two washes with phosphate-buffered saline containing 0.1% Tween 20 (PBST), the plate was blocked with 1% milk powder in PBST for 2 h. The serum samples at appropriate dilutions were added to the plates along with the standard in triplicates. The plates were incubated at 4°C for 12–15 h, washed twice with PBST and then incubated for 2 h with BU32-biotin. After two washes, streptavidin coupled to horseradish peroxidase was added and H_2O_2 /*o*-phenylenediamine as substrate/colouring agent. The enzyme reaction was quantified by taking the optical density at 492 nm in an ELISA reader (Anthos Microsystem, Krefeld, Germany) and sCD21 concentrations were calculated by extrapolating from the standard graph.

Isolation, sorting and culture of peripheral blood lymphocytes

Lymphocytes were purified by Ficoll density gradient centrifugation of fresh human blood. B and T cells were enriched by magnetic sorting as previously described [9]. The relative purity of the cells was determined by cytometric analysis using anti-CD4-PE/anti-CD8-FITC as markers for T cells and anti-CD19-PE/anti-CD21-FITC as markers for B cells. Cells were stained in microtitre plates and loaded into a FACScan (Becton Dickinson, Heidelberg, Germany) using a self-constructed loader as previously described [11]. B and T cells (1×10^7 per ml) were cultured for 36 h in Iscove's DMEM medium (Invitrogen) supplemented with 10% fetal calf serum, 1000 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 7.5% CO_2. Soluble CD21 concentrations in cell culture supernatants were then analysed by ELISA.

Statistics

Statistical calculations and graphical illustrations were performed using Instat/Prism software. The Kruskal–Wallis test was used for non-parametric analysis of variance (ANOVA) and the Mann–Whitney test to obtain non-parametric two-tail *P* values.

Results

Serum soluble CD21 levels in healthy volunteers

We analysed sCD21 levels in sera of 235 healthy volunteers of different ages. Soluble CD21 concentration was highest between 1 and 20 yr of age and was significantly lower in older individuals

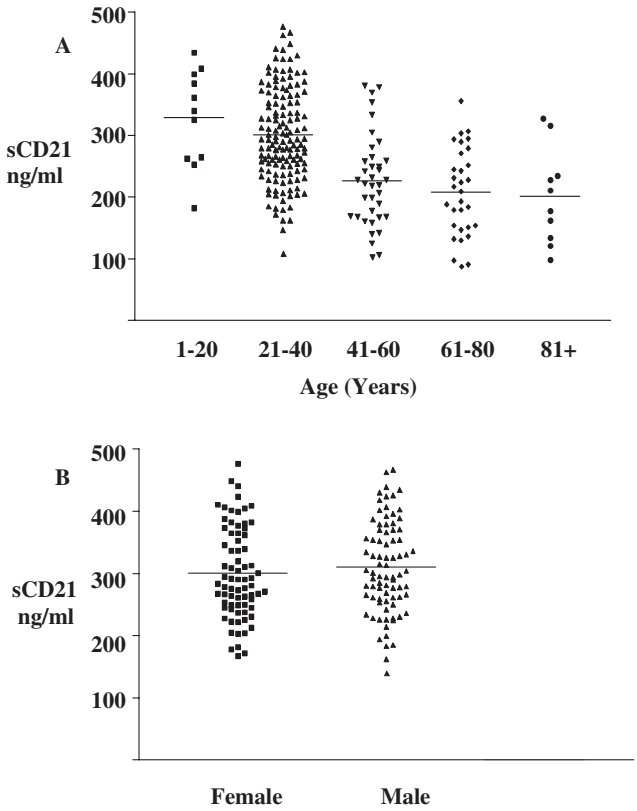

FIG. 1. Serum soluble CD21 concentration decreases with age and is independent of gender. Sera were obtained from healthy volunteers and tested for sCD21 concentration by ELISA (see Materials and methods). The data are grouped according to age in panel A and sex in panel B. Sample numbers in panel A were as follows: ■, 1–20 yr, $n = 11$; ▲, 20–40 yr, $n = 143$; ▼, 40–60 yr, $n = 38$; ◆, 60–80 yr, $n = 32$; ●, 80+ yr, $n = 11$. Sample numbers in panel B were as follows: ■, females, $n = 76$; ▲, males, $n = 84$. Kruskal–Wallis test for non-parametric ANOVA was performed to derive the statistical significances. For variation with age and gender, $P < 0.0001$ and $P = 0.3014$ respectively.

($P < 0.0001$). The normal range of sCD21 in healthy individuals 20–40 yr of age was 100–477 ng/ml with a median of 292 ng/ml (Fig. 1A). The median values of sCD21 of other age groups were as follows: 1–20 yr, 340 ng/ml; 40–60 yr, 222 ng/ml; 60–80 yr, 209 ng/ml; ≥80 yr, 195 ng/ml. While the serum levels of sCD21 were not significantly different between genders (Fig. 1B), an age-dependent decrease was found in both male and female control groups, with high significance using the non-parametric ANOVA ($P = 0.002$ for males and 0.005 for females; data not shown).

Serum soluble CD21 concentrations in RA

Serum samples of 220 confirmed RA patients were tested for sCD21 concentrations by ELISA and compared with those of controls (Fig. 2A). The sCD21 concentrations were found to be significantly lower in RA patients than in normal controls: from 50 to 300 ng/ml (median 182 ng/ml). In order to make sure that the observed reduction in sCD21 levels was not due to old age of the patients, we compared sCD21 concentrations in RA with those in age-matched normal controls (Fig. 2A), revealing a statistically highly significant reduction ($P < 0.0001$). Unlike sCD21 concentrations of controls, those of RA patients showed no statistically significant reduction with age ($P = 0.0732$, data not shown). We thus conclude that the reduction in serum sCD21 is associated with

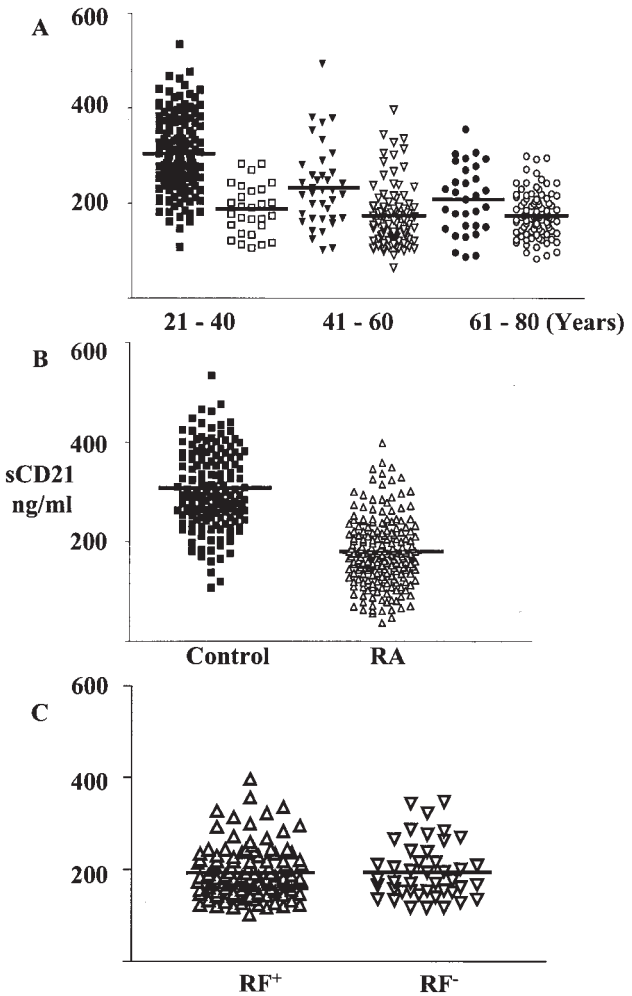

FIG. 2. Serum soluble CD21 concentration is significantly reduced in RA independently of the presence of RF. Soluble CD21 concentrations were measured by ELISA from confirmed RA patients and compared with those of healthy controls. The controls are the same individuals as shown in Fig. 1 and their data are used again here for comparison. (A) Age independence of sCD21 levels in RA patients. Age of RA patients (open symbols, □, 21–40 yr, ▽, 41–60 yr, ○, 61–80 yr) compared with the corresponding age group of healthy controls (solid symbols, ■, ▼, ●). $P < 0.0001$. (B) RA, △ ($n = 220$) compared with controls, ■ ($n = 235$). $P < 0.0001$ for the comparison between control and RA subjects. (C) Comparison of RF^+ (△, $n = 113$) with RF^- (▽, $n = 46$) RA patients ($P = 0.6336$).

the disease itself and not only leads to lower levels of sCD21 but also eliminates differences between age groups. We were further interested to know if the presence of RF correlates to sCD21 levels. Comparison of sCD21 concentrations of RF^+ and RF^- patients did not demonstrate significant differences between the two groups ($P = 0.6336$) (Fig. 2C).

B lymphocytes from RA patients and controls shed similar amounts of CD21

Because RA is characterized by autoimmune reactions, lymphocyte proliferation and complement activation, we presumed that the reduction in sCD21 could be due to defective shedding of CD21 from lymphocytes. To test this hypothesis we isolated peripheral blood lymphocytes from two healthy individuals and two RA patients and sorted B and T lymphocytes for further culture. The purity of sorted lymphocytes was tested by cytometric analysis (Fig. 3A and B). Supernatants were collected after 36 h of incubation and tested for sCD21 by ELISA (Fig. 3C). B cells from controls and RA patients shed similar amounts of CD21 into the supernatant. T cells from the control and RA patients shed very little or undetectable amounts of CD21 molecules. We therefore conclude that the shedding of CD21 protein by peripheral B cells is normal in RA and shedding by T cells is at least not elevated to the extent of yielding detectable amounts of sCD21.

Discussion

The activation of different parts of the complement cascade in RA has been known for a long time [5, 12]. One finding among many showed that levels of the CD21 ligand C3d are elevated in RA synovial fluids [13]. The study presented here demonstrates that the levels of sCD21 in the sera of RA patients were significantly lower than in a control group of age-matched healthy individuals. In addition, we examined a small heterogeneous group of patients with other autoimmune diseases ($n = 17$) in whom the serum levels of sCD21 appeared to be comparable to those of the control group of healthy individuals (data not shown). However, because in this heterogeneous group of diseases the number of cases for each disease was low, we cannot conclude that the reduction in sCD21 is specific for RA. It may well be a feature in the pathogenesis of other autoimmune or inflammatory diseases.

Our data indicate that the reduction of sCD21 levels is not due to the age of the patients or related to the presence of RF. There are several possible reasons for this reduction. First, an increased amount of complement activation products (e.g. C3d) could bind to sCD21, leading to increased clearance from plasma. Other immune complex-associated autoimmune disorders show no association with sCD21 levels, suggesting that classical immune complexes might not be involved in this process. However, in RA patients the turnover of C3 (which is about 1 g/l in healthy human sera) might be slightly elevated compared with other diseases. Comparing the amounts of C3 and sCD21 in sera, a small percentage of converted C3 would readily be sufficient to 'neutralize' sCD21. Secondly, the elevated concentrations of protease inhibitors, such as α-1-antitrypsin, could prevent shedding of CD21 from the lymphocyte surface by inhibiting unknown proteases [14]. Thirdly, the release of sCD21 from cells other than lymphocytes or lymphocytes outside the peripheral blood might be impaired during RA.

In RA patients the switched memory B-cell pool is enlarged and $CD38^+$ plasma-cell-like B cells are present in higher numbers [15]. However, the reduction in sCD21 cannot be attributed to peripheral blood B cells alone because peripheral blood B cells from RA patients were shown to produce the same low amounts as those from healthy individuals. This finding suggests that the majority of sCD21 is produced by other cell types or by B cells in different locations, for example in the spleen.

Activation of B cells through either the B-cell receptor or through C3d opsonized immune complexes decreases the density of CD21 on the surface of B cells [16]. Therefore, the decrease in sCD21 levels in plasma may be caused either by activation of B cells (as seen in synovial lymphocytes [8]) or by increased clearance of plasma sCD21 through circulating C3d opsonized immune complexes. Raji B-cells activated by mitogen shed more CD21, in addition to a decrease in surface levels (M. Masilamani, D. Kassahn and H. Illges, unpublished observation), suggesting that the decrease in sCD21 in RA is mainly due to increased clearance of sCD21 through immune complexes. The activated complement fragments in RA may be sufficiently elevated to neutralize the increased shedding of CD21 from activated B cells.

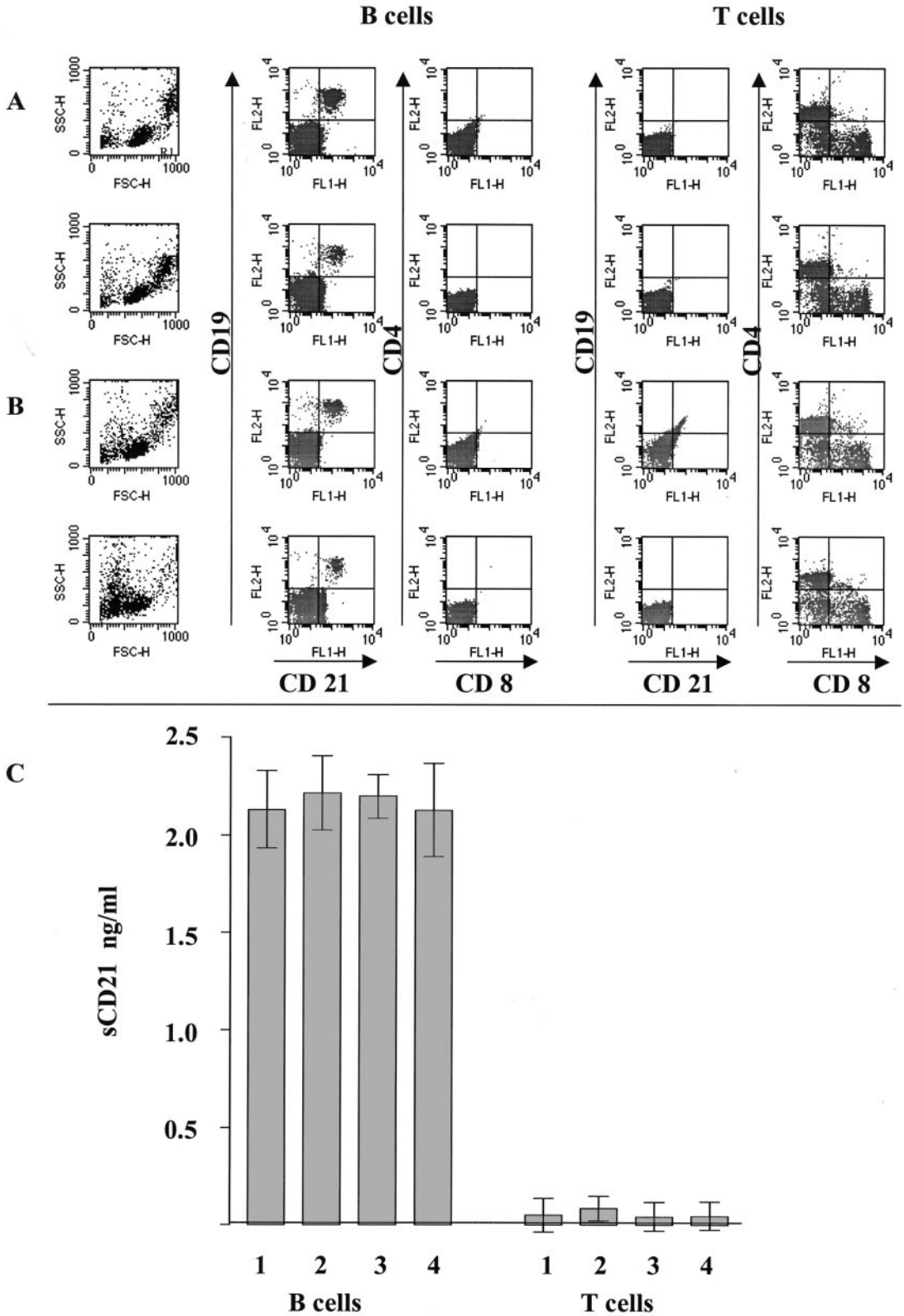

FIG. 3. RA and healthy control lymphocytes shed similar amounts of CD21. Peripheral blood lymphocytes from two healthy controls and two RA patients were obtained under sterile conditions. B and T lymphocytes (10^7 per ml) were sorted negatively by magnetic activated cell sorting using CD3 and CD19 magnetic beads respectively and cultured for 36 h. The purity of sorting from each sample of control B and T cells (A, upper and lower panels) and RA B cells and T cells (B, upper and lower panels) was determined by FACS analysis. Soluble CD21 concentration was estimated in the cell culture supernatants. (C) Amounts of CD21 shed from B and T cells from controls (1 and 2) and RA patients (3 and 4).

In conclusion, the reduction in sCD21 serum level seen in RA underlines the importance of the innate immune system in autoimmune reactions and possibly in the pathogenesis of RA. Experiments using tissues from patients and healthy donors are under way to analyse the mechanisms leading to the reduction in sCD21 in the peripheral blood of RA patients.

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The authors have declared no conflicts of interest.

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