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Reduced expression of the complement receptor type 2 (CR2, CD21) by synovial fluid B and T lymphocytes

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SUMMARY

The expression of CR2 (CD21) by synovial B and T lymphocytes of patients suffering from various forms of arthritis was analysed with cytofluorometry and with reverse transcriptase-polymerase chain reaction. CR2 (CD21) cell surface protein was detected in normal quantities on peripheral B cells, but was almost absent on synovial B lymphocytes of the same patients. This reduction was most severe in patients with rheumatoid arthritis, but also observed in all other cases. CR2 (CD21) did not reappear after *in vitro* culture. CR2 (CD21) mRNA was also strongly reduced in synovial B and T lymphocytes. Synovial fluid B lymphocytes were larger than peripheral blood B lymphocytes, while T cells from the same patients showed no size differences. We conclude that synovial fluid B lymphocytes have undergone an irreversible step towards terminal differentiation. The presence or absence of CR2 (CD21) mRNA in peripheral *versus* synovial T cells indicates that CR2 (CD21) is also differentially expressed by T lymphocytes.

Keywords rheumatoid arthritis CR2 (CD21) synovial lymphocytes complement receptor type 2

INTRODUCTION

Rheumatoid arthritis (RA) has a prevalence of about 1% in the human population and is accompanied by an increased mortality [1,2]. The disease is characterized by signs of local inflammation, chronic cartilage and joint destruction and immunological abnormalities such as the occurrence of rheumatoid factors and other autoantibodies. Heavy joint destruction is observed together with changes in the synovial lining layer, which increases in size. This pannus tissue consists of a variety of cell types including fibroblasts, macrophages and lymphocytes which together sustain the chronic cartilage and bone erosion [3]. Most of the cells recruited into the rheumatoid joint are of haematopoietic origin. The increase in cellularity is accompanied by angiogenesis in the synovial tissue, thus increasing delivery of cells and molecules to areas of inflammation [4].

B cells can proliferate and differentiate in the rheumatoid joint. The synovial tissue B lymphocytes undergo selection by antigen in local structures similar to germinal centres [5,6] and display mainly IgG or IgA as antigen-specific receptors [7,8]. Compared with peripheral blood cells the synovial B cell population contains relatively more cells specific for local autoantigens, including collagen type II and rheumatoid factors (RF) and fewer cells specific for foreign antigens [8]. Using a potential polyclonal activation system we showed before that only a small proportion of synovial B cells could be activated to proliferation and differentiation. We speculated that part of the population would not be expanded *in vitro*, because they were already further differentiated *in vivo* [8]. Now we analysed the differentiation state of synovial B cells with antibodies specific for B cell surface proteins and detected a strong reduction in the expression of the complement receptor type 2 (CR2 (CD21)), which is known to disappear when B cells differentiate into plasma cells.

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PATIENTS AND METHODS

Patients and healthy blood donors

Synovial fluid (SF) and peripheral blood (PB) were obtained from patients with various rheumatic diseases (Table 1) treated in the department of Rheumatology and Clinical Immunology at the University Hospital Freiburg. In all instances synovial tapping was therapeutically indicated and patients gave their informed consent. Altogether, we examined lymphocytes from SF and PB from 49 patients with inflammatory joint diseases. Twenty-one patients fulfilled the ACR criteria [2] for RA, 18 were classified as reactive arthritis (ReA), five patients suffered from psoriasis arthritis (PA), one patient had adult onset Still's disease, one ankylosing spondylitis (AS) and three were unclassified. SFL and PBL of all patients were examined for CR2 (CD21) surface expression,

Table 1. Study subjects

Disease	*n**	F/M	Age (years)
Rheumatoid arthritis	21	12/9	53 ± 16
Reactive arthritis	18	4/14	40 ± 16
Psoriasis arthritis	5	2/3	26 ± 8
Still's disease	1	1/0	32
Ankylosing spondylitis	1	0/1	40
Unclassified	3	2/1	29, 69, 80

*The total numbers of patients (*n*), numbers of female and male patients (F/M) and their age (mean ± s.d.) are listed.

but due to the limited number of SFL, not all patients were included in other studies. In addition, PBL were obtained from healthy blood donors (HD) (laboratory personal).

Preparation of mononuclear cells

PB and SF samples from patients and HD were diluted 1:2 in PBS and mononuclear cells (MNC) purified by Ficoll–Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden) according to standard protocols.

Cytofluorometry

Anti-CR2 (CD21) MoAb BL13–FITC was purchased from

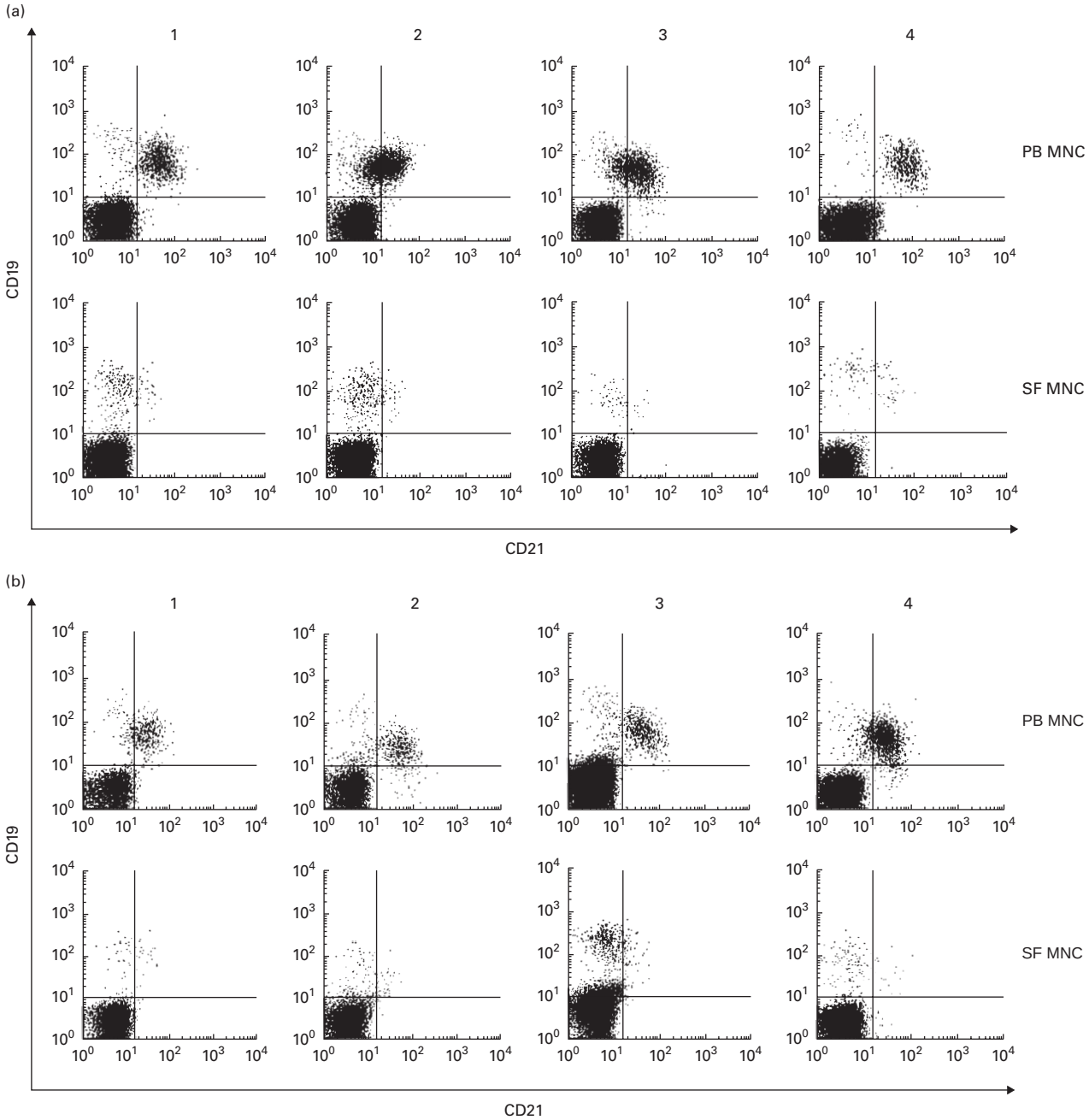

Fig. 1. Expression of CR2 (CD21) on the surface of peripheral blood (PB) and synovial fluid (SF) B lymphocytes from patients with rheumatoid arthritis (RA) or reactive arthritis (ReA). PB mononuclear cells (MNC) and SF MNC were stained with anti-CD19–PE and anti-CR2 (CD21)–FITC MoAbs. Depicted are eight representative examples of lymphocytes from RA patients (a) and patients with ReA (b).

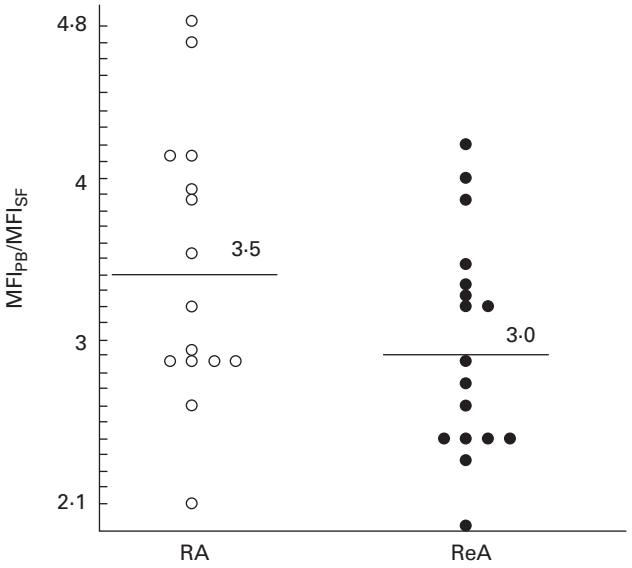

Fig. 2. Ratios of the mean fluorescence intensities (MFI) of CR2 (CD21) on CD19$^+$ peripheral blood (PB) and synovial fluid (SF) B lymphocytes. Individual values are given for patients with rheumatoid arthritis (RA; ●) or reactive arthritis (ReA; ○). MFI averages of CR2 (CD21) expression (black bars) on PB B lymphocytes were 3·5 ± 0·8 (RA) and 3·0 ± 0·7 (ReA).

Immunotech (Hamburg, Germany), anti-CR2 (CD21) MoAb BU33-FITC from Harlan Sera-lab (Belton, UK) and CD19–PE from Dakopatts (Glostrup, Denmark). Anti CD4–PE and anti-CD8–FITC antibodies were purchased from Dakopatts and Medac (Hamburg, Germany), respectively. Surface expression of CR2 (CD21) and CD19 was measured by cytofluorometry using a FACScan (Becton Dickinson, Mountain View, CA). Scatter profiles were used to gate for lymphocytes. For quantitative comparisons of CR2 (CD21) expression we gated on CD19$^+$ cells. All data were analyzed using Cell Quest software (Becton Dickinson).

Cell cultures

MNC from PB or SF were cultured *in vitro* for up to 48 h in standard medium with 5% fetal calf serum (FCS).

Preparation of RNA and cDNA synthesis

Single-cell suspensions were washed in ice-cold PBS and taken up in denaturing solution at 10^7 cells/ml. Denaturing solution contained 4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7, 0·1 M 2-mercaptoethanol (2-ME), 0·5% sodium lauroyl sarcosinate. The cells were passed ten times through a 20 × $1^{1/2}$ gauge needle and kept on ice for 15 min to allow complete solubilization and denaturation of proteins. The RNA was prepared by phenol extraction and isopropanol precipitation. To synthesize cDNA 10 μg of RNA were incubated with Superscript II (GIBCO BRL, Eggenstein, Germany) for 1 h at 42°C. Each sample was checked with GAPDH-specific oligonucleotides for successful cDNA synthesis.

Reverse transcriptase-polymerase chain reaction

To analyse the expression of CR2 (CD21) mRNA we performed reverse transcriptase-polymerase chain reaction (RT-PCR) as described elsewhere [9]. In brief, a region surrounding the transmembrane domain was amplified under the following optimized PCR conditions: 95°C 20 min, 57°C 60 min, 72°C 60 min, 30 cycles, 0·1 U Taq polymerase/reaction. The primers were 5′ to 3′: GGA ACC TGG AGC CAA CCT GCC (21S2761) and CTG GGC TCC CAT CTT TAC CAT (21R3360).

RESULTS

B and T lymphocytes in SF and blood of rheumatic patients

SF samples of all patients examined contained 0·25–0·5% CD19$^+$ B cells within the lymphocyte gate, while CD4$^+$ or CD8$^+$ T cells amounted to > 90%. The percentages of B lymphocytes in the PB of these patients were comparable to those of HD (1–11%).

Reduced surface expression of CR2 (CD21) on B lymphocytes from SF

To study the differentiation stage of SF B lymphocytes in RA patients, we analysed several cell surface markers, using specific MoAbs and flow cytometry analysis. For most, no difference between PB and SF B lymphocytes was detected (not shown). In contrast, the expression of CR2 (CD21) was clearly diminished on SF B lymphocytes compared with PB B lymphocytes taken from the same patient at the same time (Fig. 1). Following these early observations, a total of 49 patients entering the clinics with various diagnoses (Table 1) and requiring synovial tapping were examined for the cell surface expression of CR2 (CD21) on their SF and PB B lymphocytes. In all cases the surface expression of CR2 (CD21) was reduced or even absent on SF B lymphocytes, irrespective of the type of arthritis, the age or gender of the patient, and the type of treatment (Fig. 1a,b). T lymphocytes from both compartments studied at the same time did not express detectable amounts of CR2 (CD21) on their surface (not shown).

Quantitative comparison of the reduction in CR2 (CD21) surface expression

The degree of CR2 (CD21) reduction differed from patient to patient (Fig. 1). For a quantitative comparison we therefore measured the means of the fluorescence intensities (MFI) of the CR2 (CD21) expression in PB and SF B lymphocytes, and calculated the ratios between these values (MFI$_{PB}$/MFI$_{SF}$). These ratios allowed the comparison of data from different patients as well as from different dates. On average, MFI ratios were slightly higher in RA patients (3·5 ± 0·8 (mean ± s.d.); n = 15) than in non-RA patients (3·0 ± 0·7; 17 cases of ReA), without being statistically significant (Fig. 2).

Stability of low CR2 (CD21) expression by synovial B lymphocytes

SF, especially when derived from the inflamed joint, differs in its composition from PB, both in quality and quantity of various soluble factors, including enzymes and cytokines. The reduction of CR2 (CD21) on synovial B lymphocytes therefore could be due to a site-specific effect brought about by the microenvironment of the inflamed synovium, e.g. digestion of parts of the molecule by a protease. In this case, removal of SF could be expected to result in re-appearance of CR2 (CD21) on the B cell surface. However, culturing synovial and peripheral B lymphocytes for 2 days under standard tissue culture conditions, i.e. in medium containing no SF, did not change their expression of CR2 (CD21) (Fig. 3). Also,

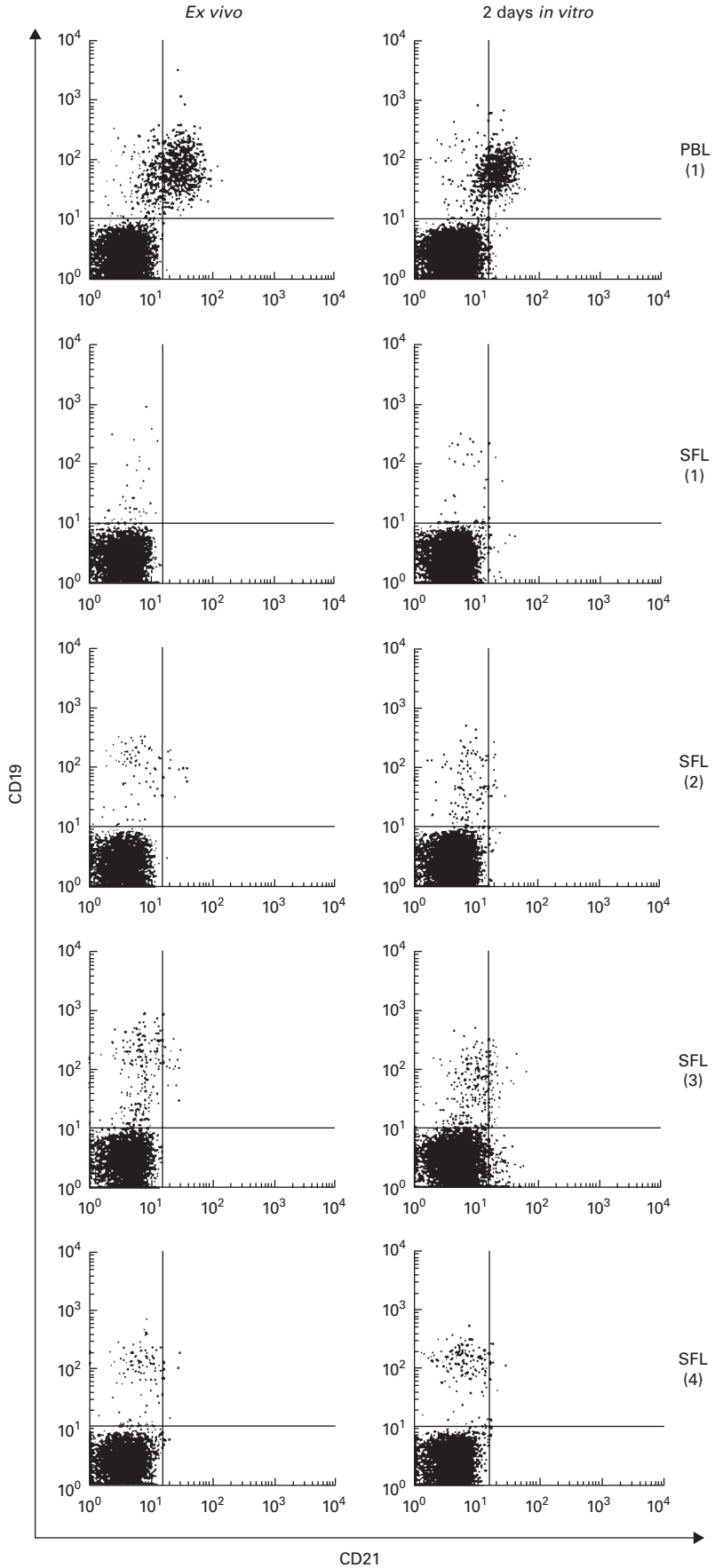

Fig. 3. See next page for caption.

the addition of IL-2 or IL-4 did not change their phenotype (data not shown).

Analysis of CR2 (CD21) mRNA

Recently we published that peripheral blood $CD4^+$ and $CD8^+$ T cells express amounts of CR2 (CD21) mRNA similar to $CD19^+$ B lymphocytes [10]. In contrast, CR2 (CD21) protein could only be detected on the surface of B lymphocytes, but not on the surface of T lymphocytes [10]. To compare the expression of CR2 (CD21) mRNA in SF- and PB-MNC, these cells were isolated from five patients with different forms of arthritis, mRNA and cDNA prepared and subjected to RT-PCR using CR2 (CD21)-specific primers. Interestingly, CR2 (CD21) mRNA was strongly reduced in the mRNA derived from SF MNC compared with the mRNA derived from PB MNC from the same patient (Fig. 4). As most SF MNC are T lymphocytes and only few B lymphocytes, the effect seen is probably due to a reduction in the expression of T lymphocyte-derived CR2 (CD21) mRNA. In addition, B lymphocytes may also be affected. At this point we did not however attempt to separate these populations in order to prove the involvement of both types of lymphocytes.

Advanced differentiation state of SF B lymphocytes

Is the loss of CR2 (CD21) due to plasma cell differentiation? CR2 (CD21) is differentially expressed during B cell development, it appears, when pre-B cells develop into mature B cells, which stay CR2 $(CD21)^+$. When mature B cells encounter antigen they differentiate into B cell blasts and further into antibody-secreting plasma cells which no longer express CR2 (CD21) [11]. Plasma cells can easily be discriminated from other types of B cells by their distinct morphology and size. Therefore, we analysed these parameters for SF and PB B lymphocytes (gated as $CD19^+$ cells) by comparison of their forward scatter profiles. This analysis comprised 19 patients of our panel. In all cases we found a strong difference between the $CD19^+$ cells from both compartments. Patients' SF B lymphocytes were significantly enlarged, while PB B lymphocytes were smaller (Fig. 5a) and indistinguishable in their forward scatter profile from PB B lymphocytes derived from HD. Forward scatter analysis of $CD4^+$ and $CD8^+$ T cells did not display such enlargement (Fig. 5b). Thus $CD19^+$ B cells in the synovium represent a phenotype which could be best described as B cell blasts.

DISCUSSION

We have shown that the CR2 (CD21) glycoprotein is strongly reduced on synovial B cells of arthritis patients while still being present on the PB B cells of the same patients. Moreover, this reduction in surface CR2 (CD21) glycoprotein is accompanied by the reduction of CR2 (CD21) mRNA as revealed by RT-PCR analysis. Recently we have shown that normal PB $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells express similar amounts of CR2 (CD21) mRNA, although T cells are lacking CR2 (CD21) surface expression [11]. Because B cells are a minor fraction of the synovial lymphocytes ($< 0{\cdot}5\%$), the reduction of the CR2 (CD21) mRNA can only be explained by the lack of these transcripts in synovial T cells as well. The small B cell numbers did not allow to use quantitative PCR or Northern blotting to discriminate between the CR2 (CD21) mRNA content in B and T cells in our samples. Therefore we can not formally conclude that CR2 (CD21) mRNA is also reduced in the synovial B cells.

Expression of CR2 (CD21) on B cells is found only on mature B cells, but not on immature or on plasma B cells [10]. CR2 (CD21) plays a pivotal role in the activation and proliferation of B cells and is a prerequisite for T-dependent immune responses. Engagement of CR2 (CD21) with the B cell receptor lowers the threshold required for activation of B cells by antigen and is involved in T-dependent immunity [12–18]. According to these results one can suppose that SF B cells would need high-affinity antigen to be further activated via the B cell receptor, because the CR2 (CD21) co-receptor is lacking. Alternatively, synovial B cells might have undergone antigen-dependent selection and are prone to terminal differentiation.

Recent studies in mice have demonstrated that CR2/CR1 (CD21/CD35) is involved in the induction of anergy and peripheral tolerance. In anti-hen-egg lysozyme (HEL) immunoglobulin/sHEL double transgenic mice crossed to CR2 (CD21)-deficient mice the induction of anergy to HEL-binding self-reactive B cells required expression of CR2/CR1 (CD21/CD35). These mice showed a strong reduction in tolerance as the HEL-specific B cells matured and accumulated in the peripheral lymphoid compartment. In further experiments CR2 (CD21)/35-deficient mice were crossed into Fas-deficient lpr/lpr mice developing spontaneously a mild form of lupus-like disease. The deficiency of CR2/CR1 (CD21/CD35) in lpr/lpr mice resulted in increased lymphadenopathy, antinuclear and anti-dsDNA autoantibodies, increased renal immunoglobulin deposits and glomerulonephritis [19,20].

To account for the CR2 (CD21) deficiency on B cells from the SF of patients with arthritis, two mechanisms can be considered. First, CR2 (CD21) can be down-regulated upon contact with immune complexes, autoantibodies or by proteases. Second, the synovial B cells might represent a specific step in B cell differentiation towards the generation of plasma cells and/or memory B cells. With respect to the first possibility, immune complexes bearing C3 fragments are present in the SF [21] of patients suffering from RA. These immune complexes may interact via C3d with CR2 (CD21) and cause the removal of CR2 (CD21) from the cell surface. Autoantibodies directed against CR2 (CD21) have been observed in the SF [22]. Alternatively CR2 (CD21) may be shed from the surface by means of a cell-bound or soluble protease. Proteolytic activities which shed CR2 (CD21) and lead to a soluble form of the molecule have been demonstrated [23,24]. When we cultured synovial B cells devoid of CR2 (CD21) for 2 days in the absence of SF and growth factors, the CR2 $(CD21)^-$ phenotype remained stable. This suggests that the reduction of CR2 (CD21) on SF B cells is not a transient phenotype mediated by immune complexes, autoantibodies or soluble proteases.

Studies of the ontogeny of B cells showed that terminal differentiation is accompanied by the loss of CR2 (CD21) expression [10]. The possibility that synovial B cells are more differentiated is supported by our findings that synovial B cells are

Fig. 3. (See previous page.) Stability of CR2 (CD21) expression by synovial and peripheral B lymphocytes *in vitro*. Peripheral blood (PB) mononuclear cells (MNC) and synovial fluid (SF) MNC of rheumatoid arthritis (RA) patients were analysed directly after isolation, or after *in vitro* culture for 48 h in medium containing 5% fetal calf serum.

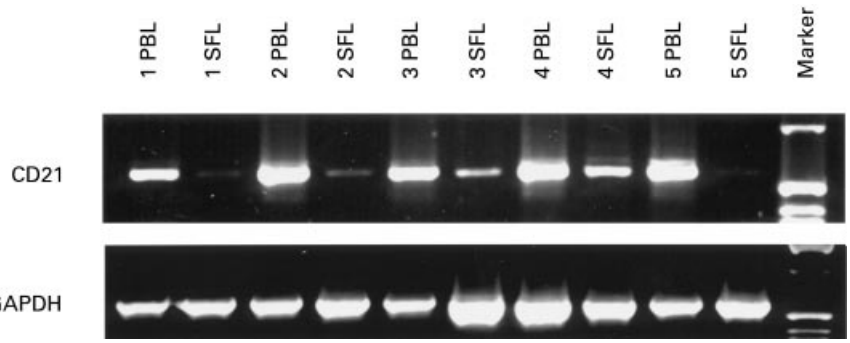

Fig. 4. Reduction of CR2 (CD21) mRNA in synovial but not peripheral blood lymphocytes of the same patients. Equal amounts of RNA were reverse transcribed into cDNA and used to amplify the transmembrane region of CR2 (CD21). GAPDH primers were used to show that equal amounts of cDNA were used for each polymerase chain reaction. The patients suffered from rheumatoid arthritis (lanes 1 and 5), reactive arthritis (lane 2), psoriasis arthritis (lane 3) or unclassified polyarthritis (lane 4).

enlarged compared with peripheral blood B cells from the same patients. Furthermore synovial B cells have been shown to express several different immunoglobulin isotypes [8,25,26]. Immunoglobulin class switch recombination characterizes B cells, which have encountered antigen and have been selected in germinal centres [27,28]. B cells enter the synovium through vessels in the pannus tissue. In order to reach the articular lumen they have to cross this tissue. Analysis of the V gene repertoire of SF B cells has suggested that they are selected by antigen because of the restricted repertoire of the V elements and common length of CDR3 regions they use [29], and more recently a germinal centre type of reaction has been demonstrated for B cells of the pannus tissue [5]. The differentiation of mature B cells towards plasma cells might have as many steps as the differentiation of precursor B cells into mature cells [30,31]. It should be possible to define these steps of plasma cell differentiation by differential gene expression. The lack of CR2 (CD21) and the enlargement of the B cells, while CD19 is still present, might represent such a step towards terminal differentiation.

The expression of CR2 (CD21) glycoprotein in T cells might be similarly regulated as during B cell differentiation. CR2 (CD21) has been reported to be expressed on the surface of a subfraction of PB T lymphocytes and on thymocytes. In addition, Epstein–Barr virus (EBV) can infect thymocytes and T cells [32–38]. CR2 (CD21) mRNA was shown to be expressed in $CD4^+$ and $CD8^+$ PB T cells to a similar extent as in B cells [11]. Although our staining protocol failed to detect CR2 (CD21) on the surface of T cells, others have described low levels of CR2 (CD21) on a fraction of PB T cells [32]. Recently Prodinger *et al*. [38] described a new complex of CR2 (CD21) emerging on T cells after CR2 (CD21) ligation. There are currently no other data supporting a functional role of CR2 (CD21) expression in T cells. Our data are the first to describe the reduction of CR2 (CD21) mRNA in T cells and may enable us to investigate a possible function in relation to activation and differentiation processes.

In conclusion, we show that patients suffering from arthritic diseases have reduced CR2 (CD21) expression in synovial but not peripheral blood lymphocytes. We speculate that the reduction of CR2 (CD21) might be involved in the autoimmune-related progression of the arthritic diseases.

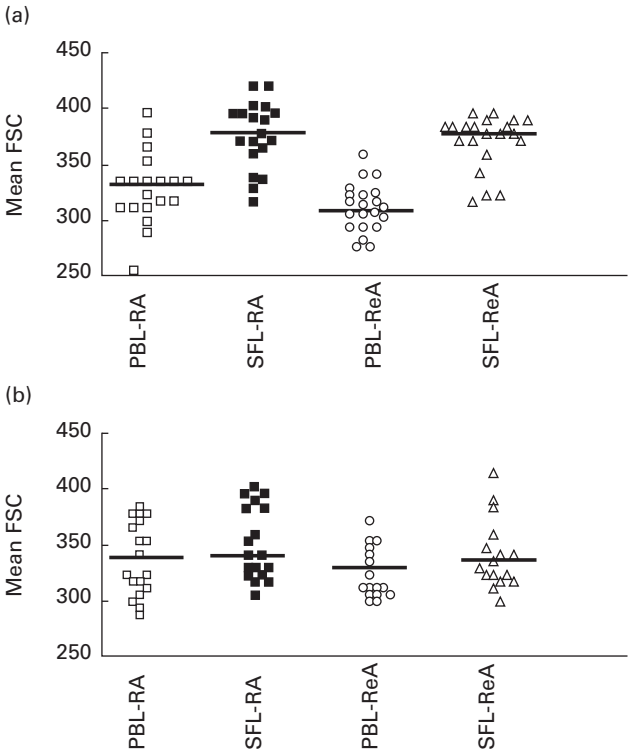

Fig. 5. Size of synovial fluid B and T lymphocytes in comparison with peripheral blood (PB) cells. Mean forward scatters of lymphocytes expressing either CD19 (a) or CD4 or CD8 (b) were calculated. (a) Peripheral blood and synovial B cells from rheumatoid arthritis (RA) patients (□ and ■, respectively) and reactive arthritis (ReA) patients (○ and Δ, respectively) are depicted. (b) Mean forward scatter from RA patients' $CD4^+$ and $CD8^+$ T cells from PB (□ and ○) and synovial fluid (■ and Δ, respectively) display the same average.

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