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Interaction of the terminal complement components C5b–9 with synovial fibroblasts: binding to the membrane surface leads to increased levels in collagenase-specific mRNA

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SUMMARY

The late complement components, apart from their lytic function, are known to trigger the release of various proinflammatory substances from different types of nucleated cells. In the present study, the interaction of C5b–9 with synovial fibroblast cells (SFC) was examined. It was found that incubation of SFC with activated complement components resulted in binding of C5b–9 to the cell membrane; subsequently an increase in abundance of collagenase-specific mRNA was seen, as assessed by Northern blotting. When C8-deficient serum was used as source of complement neither binding of C5b–9 nor an increase in collagenase-specific mRNA could be detected. These findings suggest that C5b–9, which might be generated during rheumatoid inflammation, may contribute to chronic joint destruction by triggering collagenolytic activity.

INTRODUCTION

Degradation of connective tissue is one of the major destructive processes in rheumatoid arthritis. The degradation of extracellular matrix is accomplished by various enzymes, such as collagenase,[1-3] proteoglycanase[4] or kathepsin,[5,6] which are all synthesized by fibroblasts or invading monocytic cells.[2,7,8] With regard to perpetuation of inflammatory processes, mediators stimulating release or the synthesis of the matrix-degrading enzymes appear to be of special interest. Various cytokines that modulate the release of matrix-degrading enzymes of synovial fibroblasts have been identified, e.g. interleukin-1 (IL-1) or tumour necrosis factor-α (TNF-α).[9-11]

In the present study, the effect of activated complement was tested on collagenase mRNA levels in synovial fibroblast cells (SFC). Complement is activated during inflammatory reactions. Aside from the classical activators of the complement cascade, e.g. immune complexes, many other structures such as extracellular matrix,[12] damaged cells[13] or proteolytic enzymes derived from inflammatory cells[14,15] are able to activate the complement cascade. In patients suffering from rheumatoid arthritis, who were positive for rheumatoid factor, low serum levels of complement C4 and C3 were found indicating enhanced complement turnover.[16] In synovial fluid and synovial tissue, complement activation products are found, among them the so-called membrane attack complex C5b–9.[17-19] With an antibody to a neoantigen expressed when C5b–9 is assembled, a positive correlation was found between the deposition of C5b–9 neoantigen and the degree of histopathological inflammation.[18]

C5b–9, aside from its lytic function, also elicits various cell responses, e.g. it causes synthesis and release of eicosanoids from monocytes,[20] granulocytes,[21] platelets,[22] synovial fibroblasts[23] or glomerular mesangial cells[24] or epithelial cells;[25] moreover, it triggers release of oxygen radicals, of IL-1[26] and of collagen type IV.[27]

Since C5b–9 is found in the inflamed joint tissue and since it is known for its stimulatory activity, its effect on collagenase synthesis of cultured synovial fibroblasts was tested. It was found that C5b–9 in doses that did not cause cell death induced the collagenase synthesis as measured by the generation of collagenase-specific mRNA.

MATERIALS AND METHODS

Fibroblasts cultures

Fibroblast cultures were established from synovia obtained from patients suffering from osteoarthritis according to the method of Dayer *et al.*,[28] modified as described in ref. 23. In brief, the lining cell layer was minced and sequentially digested with collagenase (400 U/ml; type Ia, Sigma, München, Germany) and trypsin from bovine pancreas (0·25%; Serva, Heidelberg, Germany) in Dulbecco's minimum essential medium (DMEM; Gibco, Berlin, Germany) for 3 hr at 37°. The cells were then sieved through sterile gauze, spun at 300 *g* for 10 min. After washing in DMEM, containing 1% antibiotic/

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antimycotic (Gibco), they were seeded on 24-well plates (Greiner, Nürtingen, Germany) at a density of 10^5/well in 1 ml DMEM, substituted with 10% foetal calf serum (FCS) and 1% antibiotic/antimycotic. After 24 hr, the nonadherent cells were removed by rinsing with cold phosphate-buffered saline (PBS), pH 7·4. After 7–10 days, the cells reached confluence. For subcultures, they were removed with EDTA–trypsin (Gibco), washed and resuspended as described above. Cells in passages 4 to 7 were used for further experiments.

The cell population, at that time, was homogenous; all cells stained positively for vimentin by indirect immunofluorescence with a vimentin-specific antibody (Dakopatts, Hamburg, Germany) (Fig. 1).

Complement preparations

For generation of activated C5b6–9, a 'deviated lysis' system was used[29] with zymosan as complement activator. In brief, zymosan (Sigma) was boiled in 0·9% NaCl, washed repeatedly and suspended to a final concentration of 4 mg/ml. For activation equal volumes of zymosan (Z) and normal human serum (NHS) were incubated for 10 min at 37°. Because activated Z-NHS is very unstable, it was prepared just prior to use.

A serum deficient in C8 (C8def) was obtained from a patient genetically deficient in the C8-β-chain.[30] For the use of isolated terminal complement components, C5, C6, C7 and C9 were isolated from human plasma according to Hammer *et al.*,[31] C8 as described by Steckel.[32] Activated C5b6 was generated from isolated C5 and C6 by cleavage with a zymosan-bound C3/C5 convertase and subsequently purified as described by Yamamoto and Gewurz.[33]

The activity of the complement components as well as of Z-NHS was tested in a haemolytic assay with chicken erythrocytes (E) as target cells. Contamination of the isolated proteins by endotoxin was tested with a commercially available limulus assay. The following concentrations were found: C5b5, C8 and C9 60–600 ng/ml; C7 6–60 ng/ml).

Haemolytic assay

To test the lytic activity of Z-NHS, NHS (0·1 ml) and zymosan were incubated at 37°. After various times EDTA was added (to a final concentration of 0·01 M), then washed E [10^7 in 0·1 ml veronal-buffered saline (VBS), pH 7·4, containing 0·01 M EDTA] were added. After incubation for 60 min at 37°, the E and zymosan were removed by centrifugation; lysis was determined by measuring haemoglobin release photometrically at 412 nm. The lysis was calculated as hit/cell (z) as described in ref. 34. To test activity of the single components, 0·1 ml E were incubated with C5b6 (1–5 μg) in 0·1 ml for 5 min, then C7 (1 μg in 0·1 ml) was added, and C8 and C9 (1 μg in 0·1 ml each) after another 5 min. Lysis was measured after 60 min incubation as described previously.

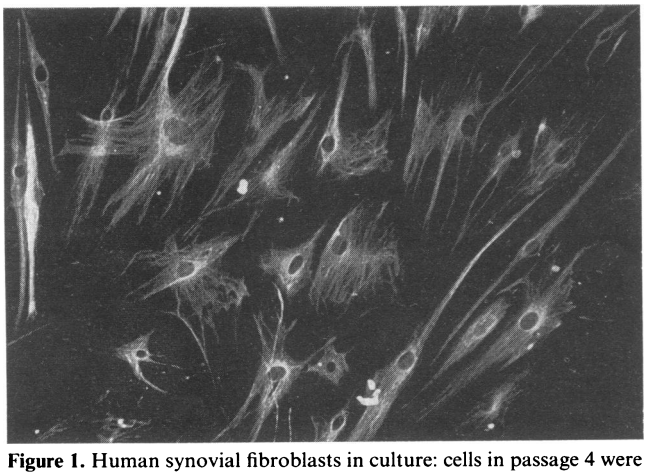

Figure 1. Human synovial fibroblasts in culture: cells in passage 4 were used for indirect immunofluorescence (original magnification × 250). All cells reacted positively with antibody to vimentin.

Visualization of membrane-bound C5b–9

Fibroblasts, cultivated on cover slips near to confluence were incubated with Z-NHS at 37°. After various times, the cells were fixed with methanol (−20°) for 1 min. After washing, an antibody to C9 (Dako, Hamburg, Germany) raised in rabbit, was added, diluted in Tris-buffered saline (TBS), containing 1% bovine serum albumin (BSA). Binding of anti-C9 was visualized using anti-rabbit IgG and alkaline phosphate–anti-alkaline phosphatase complex (APAAP) (Dako).

Cell ELISA for detection of membrane-bound C5b–9

The cell ELISA was carried out as described in ref. 35 with minor modifications. In brief, SFC were plated in 96-well plates to near confluence. Z-NHS was added and the cells were fixed at various times with glutaraldehyde (1% in PBS). Following incubation in PBS containing 1% BSA, a monoclonal antibody (mAb) to C5b–9 neoantigen[36] purchased from Diatec (Oslo, Norway) was added. Binding of the antibody was tested by alkaline phosphatase-conjugated anti-mouse IgG (Dianova, Hamburg, Germany). All samples were run as triplicates; SFC, which had not been exposed to Z-NHS, served as 'negative' controls.

Isolation of RNA and Northern blotting

Total cellular RNA was extracted from fibroblasts by the acid–guanidinium–phenol–chloroform method. Briefly, cells were washed once with PBS, pH 7·4, and lysed by addition of 125 μl/well guanidin thiocyanate solution (GTC 4 M, 0·5% *N*-laurolylsarcosine, 25 mM citric acid, 0·1 M β-mercaptoethanol). All subsequent steps were carried out according to the method described by Chomczinsky and Sacchi.[37] Seven micrograms total RNA per lane was electrophoresed on a 1% agarose gel in MOPS buffer (0·02 M morpholinopropane sulphonic acid, 0·005 M sodium acetate, 0·001 M EDTA), containing 2·2 M formaldehyde, transferred to nylon membrane (GENE-SCREEN, NEN, Dreieich, Germany) by capillary blotting in 10 × SSC (NaCl 1·5 M, sodium citrate 0·15 M, pH 7·0), and cross-linked by ultraviolet (UV) illumination at 254 nm for 2 min.

Hybridization of Northern blots was performed according to Thomas.[38] Prehybridization (3 hr) and hybridization (14 hr) were performed using 50% formamide, 5 × SSC, 50 mM sodium phosphate pH 6·5, 4 × Denhardt's solution (Sigma), 0·5 mg/ml yeast RNA (Boehringer, Mannheim, Germany), and 1% SDS at 42°. After hybridization, the membranes were washed extensively in 0·2% SSC/1% SDS at 42°. Autoradiography and relative quantitation densitometry was performed according to standard procedures. Collagenase-specific cDNA, a 2 kilobase (kb) insert in an sp 64 vector, was a kind gift from Drs P. Angel and B. Stein, (Kernforschungszentrum, Karlsruhe, Germany).[39]

*Sal*I linearized vectors, containing the insert in both orientations were labelled with an sp6 transcription kit (Boehringer). For labelling of hu β-actin cDNA, a gift from Dr B. Heckl-Östereicher (Institut für Immunologie, Heidelberg, Germany), the random priming kit from Boehringer was used.

RESULTS

Binding of C5b–9 to cultured fibroblasts

In a first set of experiments, the binding of the terminal components to fibroblasts was tested. To activate the complement system, NHS was incubated with zymosan for various times at 37°. Then activation was stopped by the addition of EDTA. The generation of activated terminal components was tested as lytic activity towards E. Maximal lytic activity was seen 10 min after addition of zymosan, but prolonged incubation resulted in a loss of lytic activity (Fig. 2). The data are in accordance with the observation that during a brief incubation time, aside from the C3/C5 convertases, C5b6 is also generated which, together with C7, C8 and C9, forms a C5b–9 complex on the membrane. Prolonged incubation of C5b6 with C7–9 in the absence of a membrane results in a fluid phase formed C5b6 complex that has lost its membrane-binding capacity.[29] When lytically active Z-NHS was added to cultured fibroblasts, binding of C5b–9 could be demonstrated as described above (Fig. 3). When lytically inactive C5b–9 was added, i.e. generated by prolonged incubation of NHS with zymosan, no binding of C5b–9 was seen (data not shown).

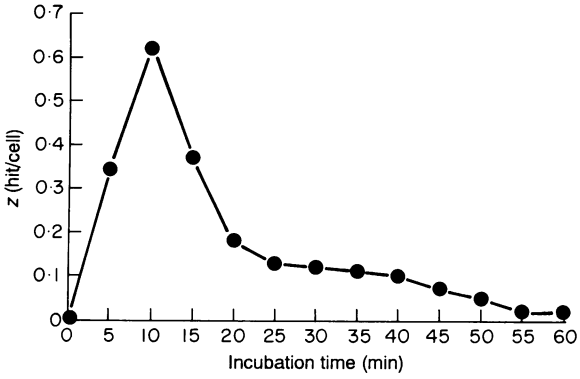

Figure 2. 'Deviated lysis': serum was incubated with zymosan (4 mg/ml) for various times (*x*-axis) and then added to chicken erythrocytes and suspended in EDTA 0·01 M. Haemolysis was measured after 60 min and calculated as hit/cell (*z*) (*y*-axis).

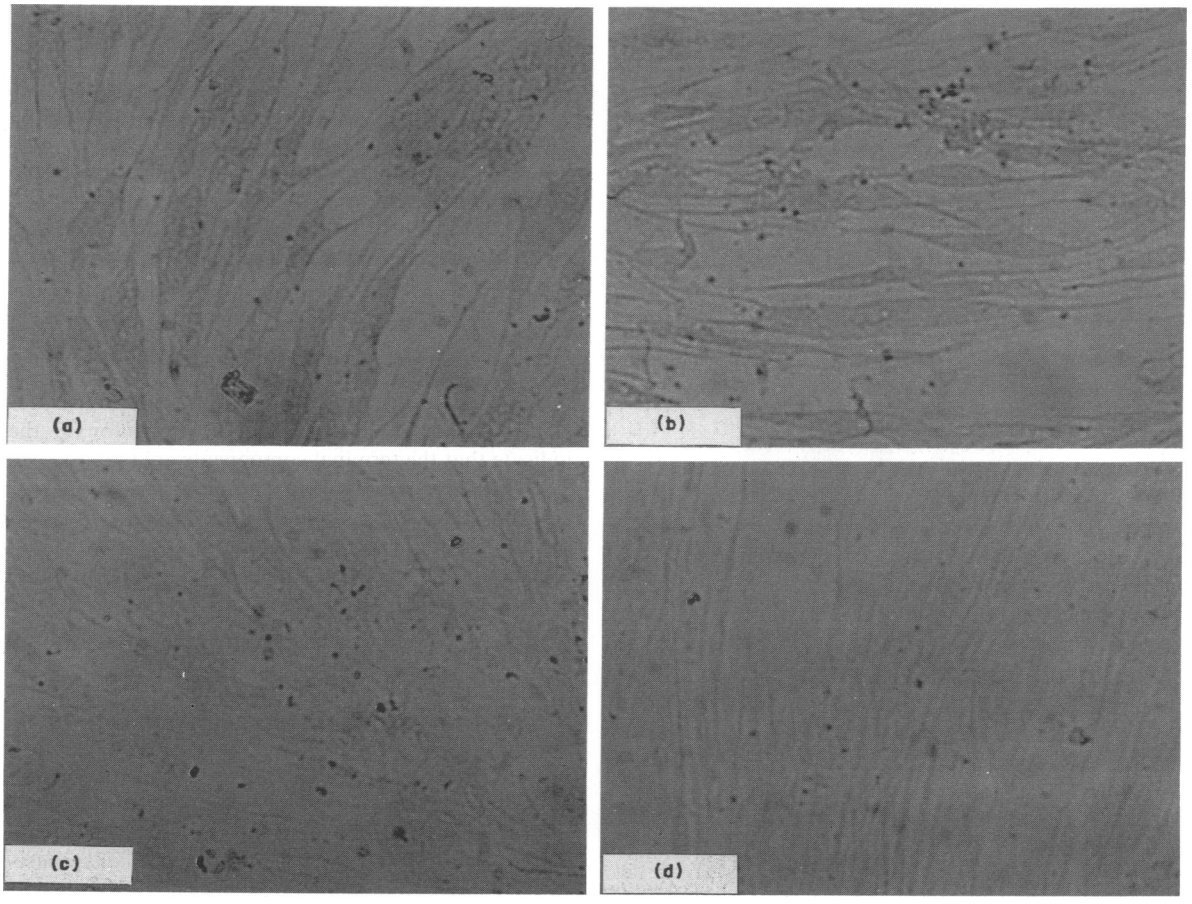

Figure 3. Surface binding of C5b–9 to membranes of synovial fibroblasts: SFC were incubated with zymosan-activated complement for the times indicated; membrane-associated C5b–9 was detected by binding of anti-C9 and visualized by an alkaline phosphate–anti-alkaline phosphatase complex. Maximal staining was observed between 5 and 20 min after addition of the activated serum; within 60 min the amount of membrane-bound C5b–9 declined; after 6 hr C5b–9 was no longer detectable. Original magnifications: (a) × 200; (b) × 200; (c) × 100; (d) × 100.

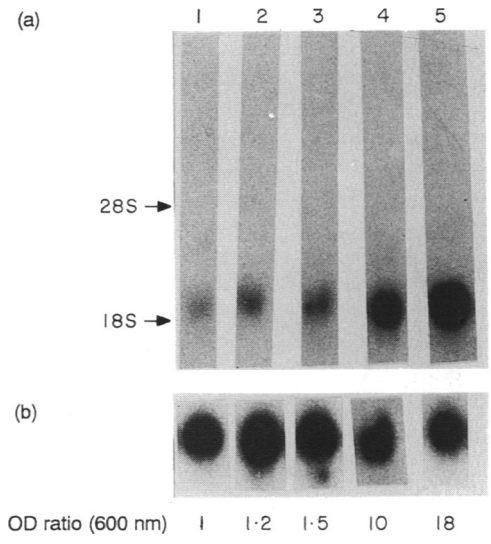

Figure 4. Induction of collagenase-specific mRNA depends on complete C5b–9 assembly: 10 μg of total RNA was separated by electrophoresis and transferred to nylon membranes. Northern blot analysis was performed with collagenase-specific mRNA (a) and β-actin-specific mRNA (b). Values indicate the collagenase mRNA/β-actin mRNA ratio, as calculated from densitometric evaluation of the autoradiographs. Ratio of medium control (SFC that had been incubated with culture medium alone) served as the internal reference value. RNA was extracted from cells cultured for 15 hr either with medium alone (lane 1), in the presence of lytically inactive Z-NHS (lane 2), C8-deficient Z-NHS (lane 3), lytically active Z-NHS (lane 4) or PMA (1 nM) (lane 5).

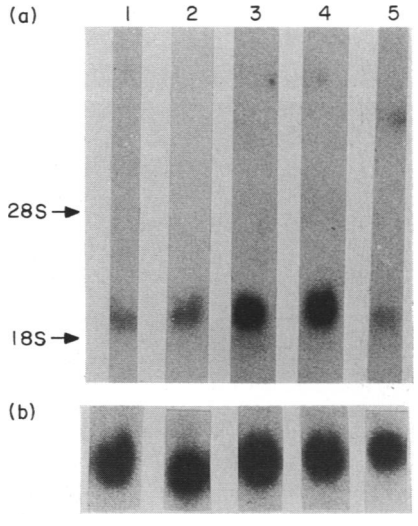

Figure 5. Kinetic studies of collagenase gene transcription induced by C5b–9: 10 μg of total RNA was separated by electrophoresis and transferred to nylon membranes. Northern blot analysis was performed for collagenase-specific mRNA (a) and β-actin-specific mRNA (b). Cells were incubated with lytically active Z-NHS for 30 min (lane 1), 4 hr (lane 2), 12 hr (lane 3), 24 hr (lane 4) and 48 hr (lane 6).

Active C5b–9, even though haemolytically active, did not cause fibroblast killing. There is evidence that nucleated cells actively defend themselves against the complement attack, e.g by inserting or by shedding membrane-bound complexes.[40,41] Therefore, it was tested whether fibroblasts would also remove C5b–9 from the surface. Fibroblasts were incubated with lytically active Z-NHS at 37°. After various times, the cells were fixed in methanol and binding of C5b–9 was tested by the use of an antibody against C9. Binding of anti-C9 which reached a maximum at 10–20 min (Fig. 3) and gradually disappeared with prolonged incubation time (Fig. 3c–d). Since anti-C9 does not react with untreated SFC before complement binding it is assumed that it recognized the membrane-associated C5b–9 complex. To verify the data, a cell ELISA was performed. Adherent SFC were incubated with Z-NHS as described previously, and membrane-associated C5b–9 was then detected by using a mAb with specificity for the C5b–9 'neoantigen'. The antibody reacted optimally with SFC that had been incubated with Z-NHS for 15 min. Thereafter the reactivity disappeared with time; after 1 hr, about 40% of the reactivity had disappeared; after 4 hr reactivity was no longer seen.

Increase in collagenase-specific mRNA

To assess synthesis of collagenase mRNA, fibroblasts were incubated with lytically active Z-NHS for 15 hr. Then total RNA was extracted and collagenase-specific mRNA was identified by Northern blotting as a band in the area of 1·9–2 kb (Figs 4, 5). When fibroblasts were incubated with medium or with zymosan, only a weak band was seen, most probably reflecting a low level baseline transcription of collagenase gene under culture conditions.

When cells were incubated with lytically active Z-NHS an increase in abundance of collagenase-specific mRNA was seen (Fig. 4).

With C8-deficient serum, i.e. when no C5b–9 complex could be formed, only a small amount of collagenase-specific mRNA was seen (Fig. 4), indicating that C8 was required to obtain an increase in collagenase-specific mRNA. Since formation of the C5b–9 complex is the only function known for C8, these data indicate that the terminal components are required.

When the amounts of collagenase-specific mRNA and β-actin mRNA as internal standard were assessed by densitometry of autoradiographs, the semi-quantitative evaluation showed about a 10-fold increase of collagenase-specific mRNA by C5b–9 (Fig. 4). A comparison with phorbol myristate acetate (PMA) as stimulator showed that the terminal complement complex was about half as efficient (Fig. 4).

In a further experiment, the kinetics of increase in collagenase-specific mRNA was analysed. Fibroblasts were incubated with lytically active Z-NHS ranging from 30 min to 48 hr. Then total mRNA was isolated and collagenase-specific mRNA was assessed by Northern blotting. Twelve hours after the addition of activated complement an increase in collagenase-specific mRNA was seen (Fig. 5). After 24 hr, collagenase-specific mRNA levels were still increasing (Fig. 5), but a decrease to baseline levels was seen by 48 hr (Fig. 5).

DISCUSSION

Exposure of cultivated human synovial fibroblast to activated

complement resulted in an increase in collagenase-specific mRNA. The increase in activity could be attributed to the effect of the terminal complement components since: (a) the zymosan-activated complement used for stimulation triggered the fibroblast only when lytically active C5b–9 was detectable, but not when the lytic activity had decayed, i.e. when SC5b–9 had been formed; (b) in serum deficient in C8 a stimulatory activity could not be generated, ruling out the participation of C3 or C5 split products; and (c) deposition of complement C9 was detectable on synovial fibroblasts, incubated with activated serum. Moreover, C5b–9 neoantigen could be detected by cell ELISA on the SFC surface.

The data are in line with the findings on the lytic attack of complement on innocent bystanders, the so-called 'deviated lysis'.[29] The term 'innocent bystander lysis' or 'deviated lysis' describes lysis of erythrocytes by complement which was activated in the immediate vicinity, but not on the erythrocyte proper. During activation, e.g. on zymosan, a very short-lived complex of C5 and C6 is generated, which can bind C7, C8 and C9. When the activation occurs in the immediate vicinity of a membrane, the terminal components form the complex on the membrane, insert and form a lytic pore. In the absence of a membrane, a fluid-phase C5b–9 complex without lytic activity is formed. In our experiments, by using zymosan as an activator of complement, a lytically active C5b6 complex is formed, that loses its lytic activity after prolonged incubation. It was found that in parallel to the lytic activity, stimulatory activity was also generated which also disappeared with prolonged incubation. Thus it is postulated that similar to 'deviated lysis' after complement activation by zymosan, activated C56 attaches to the fibroblasts and together with C7–C9 forms a membrane-associated complex.

Even though the Z-NHS contained lytically active terminal complement components, there was no evidence for killing of the fibroblasts. These data are also in line with earlier studies on the interaction of complement with nucleated cells. For killing of nucleated cells more than one complement channel is required;[42] the inefficiency of the terminal components was attributed to 'repair processes'.[43] Removal of the C5b–9 by internalization[40] or shedding[41] from complement-attacked cells was shown within 30 min after complement attack. In these experiments a loss of C5b–9 from the fibroblasts was also seen. Together with the relative inefficiency of complement to attack homologous cells[44] this mechanism might contribute to the resistance of fibroblasts to the lytic complement attack.

Even though there is strong evidence for a T-cell-mediated aetiology in rheumatoid arthritis, former studies suggest a participation of complement and its late components in pathogenic mechanisms of joint inflammation and cartilage breakdown.[16,18,19] Potential activators of complement are abundant: immune complexes, exposed collagen or destroyed cells are known activators of the complement system. Moreover, there is evidence that complement is activated in the synovial fluid of rheumatoid arthritis patients; complement split products were found, as well as the terminal complement complex.[18,19] Thus, despite the fact that humoral factors might not be involved in the initiation of rheumatoid arthritis, they may well participate in the 'effector phase', i.e. in tissue destruction. We postulate that C5b–9, by stimulating fibroblasts to release prostaglandin[23] or collagenase mediates tissue destructing processes, thereby contributing to the loss of joint integrity.

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