Concentrations of glycosaminoglycans in synovial fluids and their relation with immunological and inflammatory mediators in rheumatoid arthritis

A Bensouyad, A P Hollander, B Dularay, A E Bedwell, R A Cooper, C W Hutton, P A Dieppe, C J Elson

Abstract

The dimethylmethylen blue assay showed higher concentrations of glycosaminoglycans in many synovial fluids from patients with rheumatoid arthritis (RA) than in autologous sera or sera or synovial fluids from normal subjects. These results were taken to suggest that the glycosaminoglycans in RA synovial fluid were abnormally raised and derived from cartilage. To discover what stimulated such glycosaminoglycan release in RA joints relations were sought between synovial fluid concentrations of glycosaminoglycans and immunological and inflammatory mediators. It was shown that RA synovial fluid glycosaminoglycan concentrations correlated with synovial fluid C3d concentrations but not with synovial fluid rheumatoid factor concentrations, polymorphonuclear leucocyte numbers, myeloperoxidase concentrations, or the ability of the synovial fluids to release free radicals from normal polymorphonuclear leucocytes. A correlation was found between synovial fluid C3d and interleukin 1 concentrations as judged by both lymphocyte activating factor activity and immunoassay, but no significant correlation was detected between interleukin 1 and glycosaminoglycan concentrations. It is suggested that in the rheumatoid joint locally produced cytokines, in addition to interleukin 1, together stimulate glycosaminoglycan release from cartilage and render it vulnerable to attack by other processes.

Several hypotheses have been advanced to explain the pathogenesis of inflammation and joint damage in rheumatoid arthritis (RA). For example, it has been suggested that immune complexes in synovial fluid activate complement thereby attracting polymorphonuclear leucocytes into the joint space. Phagocytosis of the complexes triggers the release of polymorphonuclear leucocyte lysosomal enzymes and free radicals into the joint space and these may cause joint damage. This scheme is supported by the finding of IgG aggregates, activated complement components, high numbers of polymorphonuclear leucocytes, polymorphonuclear leucocyte lysosomal enzymes, and products of free radical action in RA synovial fluids. Polymorphonuclear leucocyte derived enzymes have been detected in arthritic cartilage from experimental animals and free radicals have been shown to degrade cartilage matrix in vitro. Another hypothesis suggests that local release of cytokines occurs in the joint and these stimulate chondrocytes and osteoclasts to break down cartilage and bone respectively. The original impetus for this theory came from the discovery by Fell and Jubb that a factor from synovial membrane promoted cartilage degradation. More recently, cytokines, such as interleukin 1 and tumour necrosis factor, were shown to stimulate chondrocytes to degrade cartilage matrix and to stimulate osteoclasts to resorb bone in vitro, and these cytokines have been detected in RA synovial fluids. A third hypothesis suggests that sensitised T lymphocytes react with antigen(s) in the joint and liberate lymphokines. Some of these would directly or indirectly (by stimulating other cells to produce cytokines) stimulate chondrocytes and osteoclasts to break down cartilage and bone respectively. Other lymphokines may activate phagocytic cells enabling them to bind collagen and modify the local environment so that their extracellular enzymes can initiate collagen degradation. There is unequivocal experimental evidence from animal models that T cell reactions with antigens on or in the joint initiate arthritis. In addition, T lymphocytes are prominent in rheumatoid synovial membranes, and T lymphocytes already activated in vitro have been identified in a number of laboratories, including our own.

From the observations summarised above it is apparent that a number of immune and inflammatory reactions are taking place in the joints of patients with RA. It remains to be established which (if any) of these reactions, either alone or in concert, causes joint damage. Our study attempts to approach this problem. Firstly, as degrading cartilage releases glycosaminoglycans in vitro, concentrations of glycosaminoglycan in synovial fluids were measured. Next, the concentrations of a number of putatively destructive immunological and inflammatory mediators were measured and correlations sought between their concentrations and glycosaminoglycan concentrations.

Patients and methods

Patients

All patients (120 women, 56 men) fulfilled revised criteria for the diagnosis of RA. Their mean age was 60 (range 36–78) years and their mean disease duration 12 years. One hundred and fifty one (86%) were seropositive by latex agglutination. They were being treated with a variety of analgesics, anti-inflammatory drugs, and slow acting antirheumatic drugs. Synovial fluids were obtained from the knee joint when
arthrocentesis was indicated for therapeutic reasons. Samples were immediately mixed with citrate (10% v/v) and divided into two portions: the first was used to measure the total leukocyte count and the second was centrifuged within two hours of collection. A portion of the cell pellet was applied to a slide, air dried, and stained with Giemsa for differential leukocyte counts. The cell free supernate was stored in aliquots at −70°C, apart from 2–3 ml which was mixed with ethylenediaminetetra-acetic acid (EDTA) before storage at −70°C. Serum samples were collected and separated from whole venous blood within one hour of arthrocentesis and stored in aliquots at −70°C. Samples of synovial fluid were also obtained from 28 patients with osteoarthritis, nine of whom had radiographic or synovial fluid evidence, or both, of crystal deposits. These samples were processed and stored in an identical way to the RA fluids.

Normal synovial fluid samples were obtained from the knee joints of young volunteers without signs and symptoms of joint disease as described previously. Normal serum was obtained from other volunteers in the age range 30–70.

**GLYCOAMINOLIGAN assay**

Estimation of sulphated glycosaminoglycan in synovial fluid and serum was carried out by the colorimetric method of Farndale et al., with some modifications. Synovial fluid and serum samples were diluted to 10% (v/v) and standard whale chondroitin sulphate (Sigma) was diluted to give a range of concentrations, using sodium phosphate buffer (50 mM, pH 7.0). Aliquots (100 μl) of the samples and standards were transferred to polystyrene tubes (LP4, Luckham) and to each was added 0–13 units of papain (Sigma) in 100 μl of sodium phosphate buffer containing 2 mM N-acetylcycteine (Sigma). The tubes were capped, vortex-mixed, and then incubated at 65°C for two hours. The papain was inactivated by addition of 50 μl of iodosacetic acid (Sigma; 20 mM in 2·5 M NaCl) to each tube. 1,9-Dimethylmethylen blue was prepared fresh for each experiment by dissolving 16 mg of the dye (Serva, FRG), 3·0 g glycine (BDH), and 2·37 g NaCl (BDH) in deionised water (final volume 1 litre). The pH was adjusted to 3·0 with concentrated hydrochloric acid. 1,9-Dimethylmethylen blue (2·5 ml) was added to each sample tube in turn and the absorbance at 525 nm read after 15 seconds against a dimethylmethylen blue/phosphate buffer blank. Preliminary experiments showed that a few synovial fluids may produce interference at 525 nm by absorbance which is independent of the dimethylmethylen blue. Therefore every synovial fluid sample was assayed in duplicate with 1,9-dimethylmethylen blue and also in duplicate with 2·5 ml phosphate buffer alone (against a phosphate buffer blank), so that absorbance values could be corrected where necessary. Estimates of the concentration of glycosaminoglycan in each synovial fluid were made using the whale chondroitin sulphate standard curve.

**ESTIMATION OF TOTAL C3d**

C3d was measured by the double decker rocket immunoelectrophoresis method of Brandslund et al., with some modifications. The samples were electrophoresed through two precipitating antibody gels; the first contained 40 μl/ml anti-C3c (Dakopatts, Copenhagen), thus precipitating C3 and C3c but allowing C3d to migrate into the second gel. This second gel contained anti-C3d (20 μl/ml of gel), but polyethylene glycol was not added. Polyclonal antihuman C3d was prepared at the South West Regional Blood Transfusion Centre. Volumes of synovial fluid (3 μl) and five dilutions of a standard serum (prepared from normal human serum incubated at room temperature for 10 days to allow the complete conversion of C3 to C3c and C3d) were applied to the gel. The results are expressed as a percentage of the amount of C3d in the standard serum.

**ESTIMATION OF IMMUNOGLOBULIN BOUND BY ANTI-C3d**

This estimation was performed as described previously. Essentially, polyethylene glycol (2·5%) precipitates of synovial fluids were incubated on the wells of plates which had been coated with monoclonal IgM antihuman C3d. After washing, the amount of IgG attached to the plates was estimated by measuring the uptake of 125I labelled monoclonal antihuman IgG. Counts obtained from control wells which had been blocked and incubated with anti-human IgG alone were subtracted from each sample count and the results expressed as a percentage of the total radioactivity bound to each well.

**RHEUMATOID FACTOR (RF) ASSAYS**

These radioimmunoassays were performed as described previously. Whole human IgG (for IgM RF assays) or human IgG Fe (for IgRF assays) was used as antigen, and 125I labelled monoclonal anti-IgM and anti-IgG (CH2) were the indicators for detecting uptake of IgM and IgG respectively. Results were expressed as a percentage of the total labelled antibody bound by test samples.

**MYELOPEROXIDASE ASSAY**

Synovial fluid digested with bovine hyaluronidase (100 U/ml; Sigma) was assayed for myeloperoxidase by an adaptation of the method of Suzuki. The enzyme activity was measured spectrophotometrically using the substrate tetramethylbenzidine in the presence of hydrogen peroxide and the results expressed as the change in absorbance at 655 nm/5 min.

**STIMULATION OF POLYMORPHONUCLEAR LEUCOCYTE FREE RADICAL RELEASE BY SYNOVIAL FLUID**

Superoxide generation was measured by superoxide dismutase inhibitable reduction of cytochrome c using a recording double beam spectrophotometer. The sample and
Glycosaminoglycan concentrations and immunological/inflammatory mediators

Glycosaminoglycan concentrations and synovial fluid (SF) sulphate. Of rheumatoid arthritis the scintillation liquid and the accordingly, if IN MEASUREMENT OF Biotechnology/Laboratory Cistron of enzyme were calculated for interleukin assayed for interleukin 1β by a monospecific enzyme linked immunosorbent assay (ELISA; Cistron Biotechnology/Laboratory Impex) according to the manufacturer's instructions.

LYMPHOCYTE ACTivating factor activity
This was performed as described by Gery et al,31 with some modifications. Triplicate cultures were prepared in 96-well microtitre plates (Nunc); each well contained 2.5 x 10⁶ mouse thymocytes in 200 µl of RPMI 1640 medium containing 4 µg/ml phytohaemagglutinin (Wellcome) and 10% dilutions of hyaluronidase treated synovial fluid. Control wells contained thymocytes alone or thymocytes and phytohaemagglutinin. As a positive control, thymocytes were cultured in medium containing 10 ng/ml recombinant interleukin 1 (Roussel, UCLAF). After incubation for 48 hours at 37°C the thymocytes were pulsed with 37 kBq/well of [3H]thymidine (Amersham) for the last six hours and harvested with an automatic cell harvester. Thymidine uptake was measured in a liquid scintillation counter. Data were expressed as the mean (SD) of triplicate cultures.

IMMUNOASSAY FOR interleukin 1β
Hyaluronidase treated synovial fluids were assayed for interleukin 1β by a monospecific enzyme linked immunosorbent assay (ELISA; Cistron Biotechnology/Laboratory Impex) according to the manufacturer's instructions.

Results
MEASUREMENT OF GLYcosaminoglycan Concentrations in Synovial Fluid
Preliminary experiments were carried out to determine if RA synovial fluid contained inhibitors which would affect the detection of glycosaminoglycans. Accordingly, standard concentrations of chondroitin sulphate were prepared in the presence and absence of 10% RA synovial fluid and the absorbance at 525 nm measured after addition of 1,9-dimethylmethylen blue. Figure 1 shows that the standard curve over the dose range used was linear, and synovial fluid did not alter the slope of the curve. Similar results were obtained with three other synovial fluids. In other experiments synovial fluids at different dilutions were assayed for glycosaminoglycan concentrations. The table shows that virtually the same values were obtained for the glycosaminoglycan concentration/ml of whole synovial fluid over the range of dilutions tested.

The glycosaminoglycan concentrations in synovial fluids and serum samples from normal individuals and patients with RA and other arthritides were measured and the results are expressed in fig 2. The mean glycosaminoglycan concentration in normal synovial fluid was 56 (range 10–150) µg/ml, in normal sera was 85 (range 70–100) µg/ml, and in RA sera was 84 (range 50–125) µg/ml. By contrast, many of the values obtained for glycosaminoglycan concentrations in RA synovial fluids (mean 158, range 20–490 µg/ml) and osteoarthritis synovial fluids

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**Table 1:** Effect of synovial fluid dilution on estimation of glycosaminoglycan concentrations

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<th>Synovial fluid dilution</th>
<th>Mean (SD)</th>
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<td>1/100</td>
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*µg/ml whole synovial fluid.

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Figure 1: Effect of rheumatoid arthritis synovial fluid (SF) on the measurement of chondroitin sulphate.

Figure 2: Comparison of glycosaminoglycan (GAG) concentrations in rheumatoid arthritis (RA) synovial fluid with those in sera from patients with RA and normal individuals and synovial fluid from patients with other arthritides and normal individuals. OA = osteoarthritis; SF = synovial fluid; S = sera.
(mean 96, range 20–260 µg/ml) were markedly raised. To determine whether glycosaminoglycan concentrations were consistently higher in individual RA synovial fluids than autologous sera the glycosaminoglycan concentrations in paired serum samples and synovial fluids from patients with RA were measured and the results are shown in fig 3.

CORRELATIONS BETWEEN IMMUNOLOGICAL AND INFAMMATORY MARKERS AND GLYCOSAMINOGLYCAN CONCENTRATIONS

As raised RF concentrations are the most consistent abnormality in RA sera and synovial fluids IgM RF concentrations in RA synovial fluid were measured and relations sought between their level and glycosaminoglycan concentrations. Figure 4 shows that no correlation (n=37, r=0.34, NS) existed between glycosaminoglycan and IgM RF concentrations. Similar results were obtained for IgG RF levels and glycosaminoglycan concentrations (n=37, r=0.24, NS).

The possible influence of complement activation and of the complexes which trigger activation was examined. Figure 5 shows the correlation (n=34, r=0.62, p<0.01) between glycosaminoglycan and C3d concentrations, which was confirmed with a second series of RA synovial fluids (n=14, r=0.70, p<0.01). A comparison between the concentrations of IgG aggregates bearing C3d and glycosaminoglycan concentrations showed no correlation (n=52, r=0.27, NS), however.

If polymorphonuclear leucocytes contribute to cartilage degradation in the joint then a relation might be expected between either polymorphonuclear leucocyte numbers or their products and glycosaminoglycan concentrations. We found no correlation between glycosaminoglycan concentrations and synovial fluid polymorphonuclear leucocyte numbers (n=56, r=0.12, NS), however. Similarly, there was no correlation between synovial fluid myeloperoxidase concentrations and glycosaminoglycan concentrations (n=51, r=0.24, NS). The ability of RA synovial fluids to stimulate the release of...
Glycosaminoglycan concentrations and immunological/inflammatory mediators

Figure 6: Relation between lymphocyte activating factor activity and glycosaminoglycan concentrations in rheumatoid arthritis synovial fluid.

free radicals from normal polymorphonuclear leucocytes was measured but only 7/47 were active, and thus this variable too was unrelated to glycosaminoglycan concentrations.

Complement activating immunoglobulin aggregates but not immunoglobulin aggregates alone have been shown to stimulate the release of interleukin 1—a known stimulant of cartilage degradation—from monocytes. These findings prompted us to ask, firstly, if there is a relation between complement activation in RA synovial fluid and synovial fluid interleukin 1 concentrations, and, secondly, if the relation (reported above) between C3d concentrations and glycosaminoglycan concentrations might be due to complement activation stimulating local interleukin 1 production, which in turn stimulates glycosaminoglycan release from cartilage. Figure 6 shows that the levels of synovial fluid lymphocyte activating factor activity and C3d were related (n = 25, r = 0.68, p < 0.01). In addition, synovial fluid interleukin 1β concentrations, as judged by an ELISA, correlated with C3d concentrations (n = 9, r = 0.61, p < 0.05). Finally, a possible relation between synovial fluid glycosaminoglycan concentrations and interleukin 1 concentrations was sought. No significant relation (n = 20, r = 0.42, NS) between immunoreactive interleukin 1β concentrations and glycosaminoglycan was found and no relation (n = 20, r = 0.29, NS) between lymphocyte activating factor activity and glycosaminoglycan concentrations.

Discussion

Dingle and his colleagues and Carroll were the first to report the detection of glycosaminoglycans by the dimethylmethylene blue assay in arthritic synovial fluids. Our results extend their work by showing that many RA synovial fluids have high glycosaminoglycan concentrations as compared with serum samples or synovial fluids from normal individuals. The synovial fluids from normal joints were obtained from young healthy volunteers who were not matched for age and sex with the patient groups. Most had glycosaminoglycan concentrations which were similar to normal serum concentrations, in contrast with the raised synovial fluid, but not serum concentrations, found in RA. Although age of subjects and sample stage could be compounding variables, it seems likely that the high glycosaminoglycan concentrations found in many pathological synovial fluids represent a true variation in cartilage derived products.

These observations raise the question of how pathological synovial fluid glycosaminoglycan concentrations relate to cartilage degradation in vivo. Normal cartilage consists mainly of collagen and proteoglycans; the latter being complex macromolecules consisting of an extended protein core to which are attached glycosaminoglycan side chains. In normal cartilage proteoglycans turn over slowly and as the degraded molecules are released they are replaced by newly synthesised components. Animal experiments have shown that intra-articular injection of agents, which either inhibit the synthetic capacity of chondrocytes or their production of degradative enzymes, cause a net loss of cartilage and also increased glycosaminoglycan concentrations in synovial fluid, at least in the short term. In view of these findings and because higher concentrations of glycosaminoglycan were found in many RA synovial fluids than in autologous serum samples and normal synovial fluids it seems reasonable to assume that the glycosaminoglycans found in RA synovial fluid derive from cartilage. Whether the high concentrations found in RA synovial fluid result from increased synthesis and release of RA cartilage glycosaminoglycan or from increased degradation of cartilage matrix glycosaminoglycan remains unclear, though degradation seems the most likely. A third possibility—namely, that the process which leads to removal of glycosaminoglycan from the joint space is inhibited in patients with RA, is not supported by animal experiments, which showed that the clearance rate of glycosaminoglycan from normal and arthritic synovial fluids was similar (half lives of 12 hours and 14 hours respectively). As already mentioned (see 'Introduction') a number of processes take place in RA joints which might affect the cartilage. It was hoped to elucidate which (if any) of these processes is important in relation to cartilage degradation by asking if particular initiators or mediators of immunological and inflammatory joint reactions were related to synovial fluid glycosaminoglycan concentrations. The results showed that no correlation exists between glycosaminoglycan concentrations and polymorphonuclear leucocyte numbers or between glycosaminoglycan concentrations and those of synovial fluid myeloperoxidase. Moreover, most RA synovial fluids were unable to stimulate the release of free radicals from normal polymorphonuclear leucocytes. It is thus tempting to speculate that polymorphonuclear leucocyte degranulation and release of free radicals is not important in causing cartilage damage. No significant correlation was found between RA synovial fluid
concentrations of glycosaminoglycan and interleukin 1, however, despite the known ability of this cytokine to stimulate glycosaminoglycan release from animal cartilage. One explanation for these results is that the patients have reached different stages of the disease and different processes may be involved at these different stages. Therefore, non-correlation of glycosaminoglycan concentrations with particular indices may not be evidence for non-causal relations. Alternatively, the power of association between each variable may be too small for a statistically significant relation between interleukin 1 and glycosaminoglycan concentrations to emerge from the small number of patients assayed. Finally, cytokines in addition to interleukin 1 may stimulate glycosaminoglycan release from RA cartilage in vivo. It may be significant that recent work from our laboratories (Hollander, Atkins, Dieppe, and Elson, unpublished) has shown that RA synovial fluids do stimulate glycosaminoglycan release from live human cartilage in vitro, though interleukin 1 at concentrations 20 times those found in the fluid is without effect.

A correlation was found between C3d and glycosaminoglycan concentrations. This result might be due to glycosaminoglycan activating complement either directly or indirectly by reacting with an autoantibody, but to our knowledge there is no evidence for these hypotheses. We also considered the possibility that complement activation is related to the local production of interleukin 1 as it is known that interleukin 1 production may be stimulated by complement activating IgG aggregates32 and by the binding of C5a to macrophages.30 In support of this contention synovial fluid lymphocyte activating factor activity and C3d concentrations were found to be related. It might be argued that the synovial fluid lymphocyte activating factor activity was due to the presence of a cytokine such as interleukin 6 rather than interleukin 1 but a correlation, albeit weaker, was found between interleukin 1 concentrations, as judged by immunosassay, and C3d concentra-
tions in a small group of fluids. Despite these results the explanation for the relation between synovial fluid glycosaminoglycan and C3d concentrations remains uncertain.

In conclusion, it has been shown that some RA synovial fluids contain increased concentrations of glycosaminoglycans, which are thought to be released from cartilage. The results failed to support a role for polymorphonuclear leuco-
cyte derived degradative enzymes or free radicals in glycosaminoglycan release. Similarly, no evidence was found for the involvement of interleukin 1, and it is suggested that glycos-
aminoxylic release is stimulated by a mixture of cytokines. How glycosaminoglycan loss from cartilage is involved in the rheumatoid process remains to be established, but we postulate that the loss occurs over much of the cartilage thereby rendering cartilage more susceptible to attack at the pannus-cartilage junction.

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