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A Tissue-Culture Model of Cartilage Breakdown in Rheumatoid Arthritis

QUANTITATIVE ASPECTS OF PROTEOGLYCAN RELEASE

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1. The destruction of articular cartilage in human rheumatoid and other arthritides is the result of diverse mechanical, inflammatory and local cellular factors. A tissue-culture model for studying cartilage–synovial interactions that may be involved in the final common pathway of joint destruction is described. 2. Matrix breakdown was studied *in vitro* by using bovine nasal-cartilage discs cultivated in contact with synovium. Synovia were obtained from human and animal sources. Human tissue came from patients with 'classical' rheumatoid arthritis, and animal tissue from rabbits with antigen-induced arthritis. 3. Cartilage discs increased their proteoglycan content 2–3-fold during 8 days in culture. Proteoglycan was also released into culture medium, approx. 70% arising from cartilage breakdown. 4. Synovial explants from human rheumatoid and rabbit antigen-induced arthritis produced equivalent stimulation of proteoglycan release. After an initial lag phase, the breakdown rate rose abruptly to a maximum, resulting in a 2-fold increase of proteoglycan accumulation in culture medium after 8–10 days. 5. High-molecular-weight products shed into culture media were characterized chromatographically and by differential enzymic digestion. Proteoglycan–chondroitin sulphate accounted for 90% of the released polyanion, and its partial degradation in the presence of synovial explants was consistent with limited proteolytic cleavage. 6. Rheumatoid synovium applied to dead cartilage increased the basal rate of proteoglycan release. Living cartilage was capable of more extensive autolysis, even in the absence of synovium. However, optimal proteoglycan release required the interaction of living synovium with live cartilage. These findings support the view that a significant component of cartilage breakdown may be chondrocyte-mediated.

The breakdown of articular cartilage in rheumatoid and certain other arthritides is associated with a chronic hypertrophic synovitis. Rheumatoid pannus, often prominent in advanced stages, clearly has invasive properties. This is thought to be related to the large array of hydrolytic enzymes capable of degrading matrix (Barrett, 1975), and breakdown of both proteoglycan and collagen at the pannus–cartilage junction have been described (Kobayashi & Ziff, 1975; Harris *et al.*, 1970, 1977). It has been proposed that some prior alteration adversely affects the matrix of cartilage, permitting synovial ingrowth (Gardner, 1972). The presence of diverse cellular elements in the diseased joint, including those in synovium, synovial fluid and cartilage, provide a complex setting for the interactions that ultimately result in cartilage destruction. Recent evidence points to the conclusion that cartilage does not serve simply as an inert substrate, but that a significant component of the degradative process requires chondrocyte participation (Fell & Jubb, 1977).

We have attempted to reproduce *in vitro* a model of the confrontation of synovium and cartilage, which occurs in the diseased joint. The release of cartilage proteoglycans into culture medium has provided a convenient bioassay of the degradative capacity of synovial explants. It is anticipated that the assay of new pharmacologic agents or techniques (Sledge *et al.*, 1977) in this system will assist in devising appropriate and rational therapy.

Part of this work was presented at the Twenty-Fourth Orthopaedic Research Society Meeting at Dallas, TX, U.S.A., held on 21–23 February 1978.

Materials and Methods

Materials

Chemicals were analytical grade or the best grade commercially available. Tissue-culture media, buffered salt solutions and antibiotics were from Gibco (Grand Island, NY, U.S.A.) and foetal bovine serum from Flow Laboratories (Rockville, MD, U.S.A.). Alcian Blue 8GX from Allied Chemical Co. (Morristown, NJ, U.S.A.) was prepared fresh and clarified by centrifugation immediately before use. Papain (EC 3.4.22.2) (twice-crystallized), bovine testicular hyaluronidase type I (EC 3.2.1.35) and chondroitin sulphate mixed isomers (from whale and shark cartilage), grade III, were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hyaluronoglucosidase (Ohya & Kaneko, 1970) from *Streptomyces hyalurolyticus* was purchased from Miles Biochemicals (Elkhart, IN, U.S.A.). Guanidinium chloride (Absolute grade) was from Research Plus Laboratories (Denville, NJ, U.S.A.). Sepharose 4B and Blue Dextran 2000 were from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.) and [^{3}H]water from Packard Instrument Co. (Downer's Grove, IL, U.S.A.). Carrier-free $Na_2{}^{35}SO_4$ and Aquasol were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.).

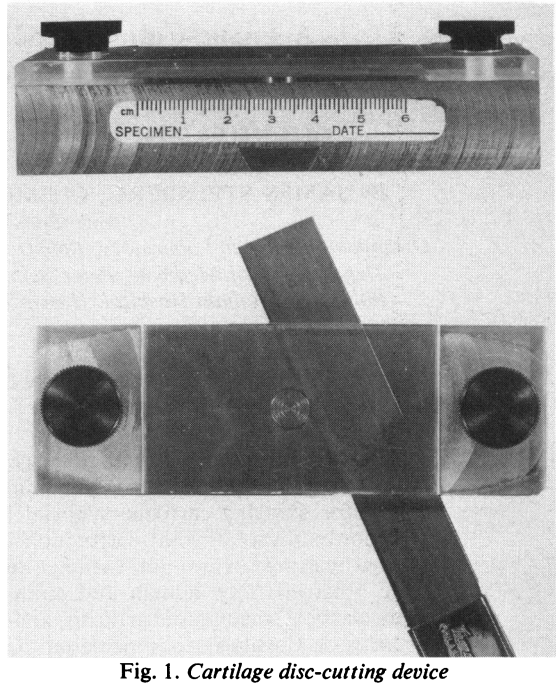

Fig. 1. *Cartilage disc-cutting device*

Tissues

Bovine snouts obtained fresh at the time of slaughter were removed immediately to the laboratory. The nasal septum was excised and thoroughly scrubbed with a Betadine (1-ethenyl-2-pyrrolidinone homopolymer compound with iodine)/soap solution. The mucosa and perichondrium were stripped off aseptically and the cartilagenous septum was again washed with Betadine/soap followed by immersion in 5% (v/v) Betadine solution for 1 h. The remainder of the procedure was performed in a laminar-flow hood. Final washing was completed by repeatedly dipping in Gey's balanced salt solution. The cartilage sheet was laid on a sterile towel supported on a firm surface. Uniform 8 mm cores were removed with a metal cork-borer. Top and bottom surfaces were shaved off with a scalpel, discarded, and the remaining tissue plug was placed in Gey's balanced salt holding solution. When a sufficient number of cores had been obtained, each was sectioned into several 1 mm-thick discs. The use of cartilage discs for organ-culture experiments was based on the work of Dingle (1976). The cutting device (Fig. 1), designed to our specifications (Cintor Division, Codman and Shurtleff, Randolph, MA, U.S.A.) to feed a cartilage plug automatically on to a cutting surface, consists of a no. 303 stainless-steel block with a central well (9 mm × 15 mm) holding a spring-loaded stainless-steel platform on which a tissue plug is placed. The Plexiglass top has been machined on its undersurface so that when completely assembled and secured by the two knurled thumbscrews, a cutting slot 1.25 mm high is formed between the top and the base. The cartilage plugs were cut with a Stadie–Riggs blade fitted with a handle, and the discs were held in a Petri dish containing Gey's balanced salt solution before being placed in organ culture. Discs produced in this manner were highly uniform, generally ranging from 60 to 70 mg, depending on the particular cartilage source. In each of three experiments, blotted wet weights obtained on 40–60 replicates at the end of a 10-day culture period were found to vary over an S.E. range of 2.1–2.6%. Although only the relatively avascular portion of each septum was used, it was recognized that bovine nasal cartilage is penetrated by microscopic vascular channels, and to that extent not all discs are pure cartilage.

Human synovia were obtained from patients with 'classical' rheumatoid arthritis at the time of surgery for total knee replacement or synovectomy. Tissue was removed to the laboratory where it was placed in a Petri dish containing 0.9% NaCl and stripped of its fibrous capsule and surrounding fat. Synovium was finely minced with a scalpel in preparation for organ culture. Rabbit synovia were obtained from the knee joints of animals with antigen-induced arthritis 6–8 weeks after intra-articular challenge with ovalbumin. This chronic condition, very similar to

rheumatoid arthritis, was produced as described previously (Sledge *et al.*, 1977).

Cryopreservation

The feasibility of preparing a reservoir of uniform cartilage discs and preserving them for future use was examined. Freshly prepared discs were placed directly into separate polypropylene vials containing 2ml of freezing solution, consisting of complete culture medium (see below) supplemented with 10% (v/v) glycerol and 0.25% dimethyl sulphoxide. Discs chilled and equilibrated for 16h at 4°C were transferred to a freezing unit (Cryo-Med, Mt. Clemens, MI, U.S.A.; Programmable Freezing System no. 900) and taken down to the temperature of liquid N_2 by a standard cryogenic technique (Shannon & Macy, 1973) at an initial rate of −1°C/min. Preserved discs were restored as needed by rapid thawing to 37°C, followed by several changes of fresh culture medium to eliminate glycerol and dimethyl sulphoxide.

Organ culture

Each bovine nasal septum provided a large number of highly uniform cartilage discs, permitting adequate statistical replication. Individual organ cultures were established in 10mm×35mm plastic Petri dishes (Falcon no. 3001), each holding a shallow table of wire-mesh stainless steel on which was placed a quarter-circle of cellulose acetate filter (Millipore Filter Corp., Bedford, MA, U.S.A.; type SM, 25mm, 5μm pore size). Cartilage was placed on the membrane, and 2ml of Dulbecco's modified Eagle's medium (Smith *et al.*, 1960), containing 4.5mg of glucose/ml, 10% (v/v) foetal-calf serum, penicillin/streptomycin (100i.u./ml and 100μg/ml respectively) plus 5μg of amphotericin B/ml was added. Cultures were placed in a modified McIntosh–Fildes Chambre (Baird and Tatlock, Chadwell Heath, Essex, U.K.), flushed for 20min with CO_2/O_2 (1:19) and incubated at 37°C. Media were changed at 2-day intervals. As required for individual experiments, approx. 10mg portions of freshly minced synovium were transferred with a scalpel blade and applied to the edge of the cartilage in an overlapping fashion as illustrated in Fig. 2. In one experiment, cartilage discs were radioactively labelled by preincubation in modified ($MgCl_2$ replacing $MgSO_4$) complete Dulbecco's modified Eagle's medium containing $^{35}SO_4^{2-}$(10μCi/ml). After 24h, radioactive medium was decanted, discs were washed with several changes of phosphate-buffered saline (Dulbecco & Vogt, 1954) and established as individual organ cultures as described above. After a 24h 'chase' period, several discs were removed as zero-time samples. In the remainder, media were changed, and freshly prepared synovial mince was applied to one half of the cultures, which were then incubated for 8 days. At the end of the culture period, synovium was discarded; 2-day media collections from each culture and cartilage discs were held at −20°C for analysis.

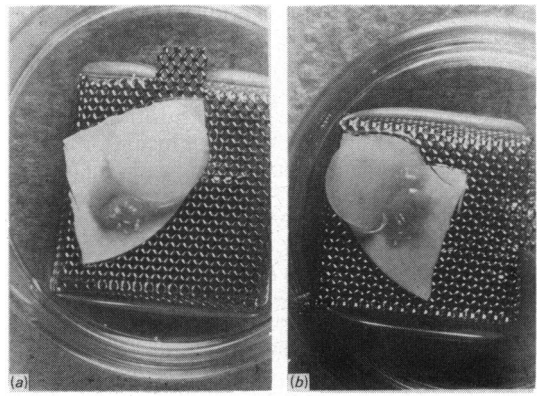

Fig. 2. *Co-cultivation of bovine nasal cartilage disc and human rheumatoid synovium at (a) day 0 and (b) day 8*

Analytical methods

Proteoglycan was measured by a modification (Dingle *et al.*, 1975) of the procedure of Whiteman (1973). Portions of culture medium, cartilage digest (see below) or column effluent were made to react with 0.05% Alcian Blue dye in 50mM-sodium acetate buffer, pH5.8, containing 200mM-$MgCl_2$. Ethanol-washed complexes were solubilized in 2% (w/v) sodium dodecyl sulphate/50mM-sodium acetate buffer, pH5.8; A_{620} was measured to determine proteoglycan, with chondroitin sulphate as a standard. Hyaluronate released from synovium into culture medium was excluded under these conditions (Scott & Dorling, 1965), and in preliminary experiments the contribution of proteoglycan from synovium incubated alone was always less than 5% of the total.

For enzymic digestion, culture media were freeze-dried and reconstituted in a decreased volume of water. Samples containing 600–800μg of proteoglycan were incubated in a total volume of 2ml as follows. (*a*) Papain digestion: 0.1M-sodium phosphate buffer, pH6, 5mM-Na_2EDTA, 5mM-cysteine, 0.2ml of papain suspension (25mg/ml), 65°C, 18h; (*b*) bovine testicular hyaluronidase digestion: 0.15M-NaCl, 0.2M-sodium acetate buffer, pH5, 1500 turbidity-reducing units of hyaluronidase, 37°C, 18h; (*c*) *Streptomyces* hyaluronidase digestion: 0.15M-NaCl, 0.2M-sodium acetate buffer, pH5, 20 turbidity-reducing units of hyaluronidase, 65°C, 18h. Cartilage discs were prepared for analysis by thorough digestion with papain as above, except that two 24h incubations were made with 0.5ml of papain suspension/disc in a final volume of 5ml.

Gel-filtration chromatography was performed at room temperature on a column (1.5cm×28cm) of Sepharose 4B previously calibrated with Blue Dextran (V_0), [^{3}H]water (V_t), and chondroitin sulphate (V_{cs}) to mark the elution position of glycosaminoglycan. Samples of culture media or enzymic digests were loaded on the column, and 1ml fractions were eluted with 0.15M-NaCl, 20mM-sodium acetate buffer, pH5.8, and 0.02% NaN_3 at a flow rate of 10ml/h. In dissociative runs the column was re-equilibrated and samples eluted with 4M-guanidinium chloride, 20mM-sodium acetate buffer, pH5.8. Effluent fractions dried *in vacuo* were assayed for glycosaminoglycan by the Alcian Blue method; in a preliminary experiment the presence of high concentrations of guanidinium chloride (2–4M) was found not to interfere with the assay.

All ^{35}S radioactivity counting was done in a Packard Tri-Carb liquid-scintillation spectrometer, model no. 3375. Samples of medium, papain-digested cartilage or resolubilized Alcian Blue complexes were counted for radioactivity in Aquasol at an efficiency of 92%; no corrections were made for colour quench.

Results

Typical cartilage-disc rheumatoid synovium co-cultures are shown in Fig. 2. Such cultures could be readily maintained for at least 2 weeks, but because of a gradual decline in $^{35}SO_4^{2-}$ incorporation into cartilage in older cultures (J. Steinberg & C. B. Sledge, unpublished work), incubations were generally terminated at 8–10 days. Not infrequently at that time considerable erosion of the disc was evident immediately adjacent to the synovial explant (Fig. 2*b*). In one experiment the loss of cartilage mass was assessed by the change in wet weights during prolonged culture. Disc weights declined from an initial 80.84± 4.49mg (mean±S.E.M., $n=5$) to a value of 69.85± 1.80mg ($n=40$) over a 10-day period ($t=2.48$, $0.01<P<0.02$). This was not routinely done, however, because preliminary weighing of cartilage samples was wasteful of discs, and the method was inadequately sensitive to distinguish minor differences between treatment groups. The extent of breakdown could be readily quantified by the Alcian Blue method. Fig. 3 illustrates the characteristic time course of such an experiment. The addition of human rheumatoid synovium to one half the cultures on day 2 had very little immediate effect. After an initial 2-day lag, proteoglycan rapidly accumulated in such cultures, so that by the end of the experiment there was a 2.5-fold increase compared with the basal release from discs alone. The pattern of proteoglycan release in similar cultures containing rabbit antigen-induced arthritis synovium strongly resembled their human rheumatoid counterpart.

Pooled data from a number of experiments are shown in Fig. 4, in which proteoglycan accumulation is expressed as the treatment/control (T/C) ratio. Proteoglycan release was only modestly stimulated by the second day after addition of synovium, reflecting the initial lag noted in most experiments. There was an eventual doubling by the tenth day in complete cultures. It is noteworthy that synovia from humans with rheumatoid arthritis and rabbits with a related form of experimental antigen-induced arthritis both followed essentially the same time course.

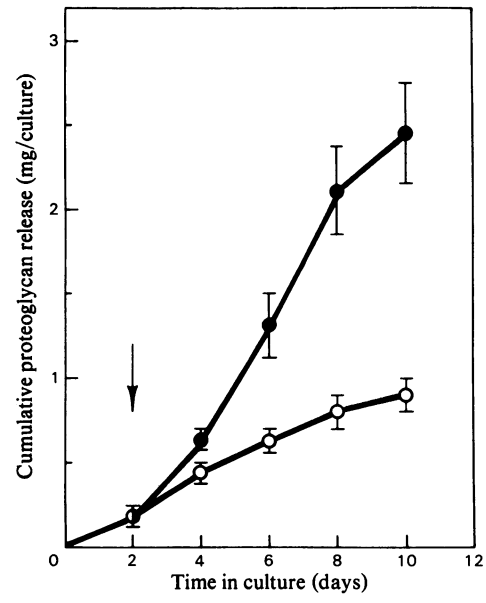

Fig. 3. *Stimulation of proteoglycan release from bovine nasal cartilage discs by human rheumatoid synovium*
Replicate cartilage discs were established in organ culture at zero time, and minced synovium was added to one half at the arrow. Media were harvested at 2-day intervals and individually analysed by the Alcian Blue method. ○, Cartilage alone; ●, cartilage plus synovium. Values are means±S.E.M. ($n=6$).

Nature of proteoglycan-release phenomenon

The cartilage disc in organ culture is a viable, biosynthetically active tissue. Of the new proteoglycan formed during the culture period, some might be released directly into culture medium without prior incorporation into matrix (metabolic 'shunting'). Therefore, despite the obvious erosion in most co-cultures and the demonstrable loss of cartilage weight in some experiments, to use the medium proteoglycan content as an index of cartilage breakdown, it was necessary (*a*) to confirm that breakdown does indeed occur, and (*b*) to arrive at an estimate of the extent to which breakdown contributes to the medium proteoglycan pool. The experiment illustrated in Fig. 5

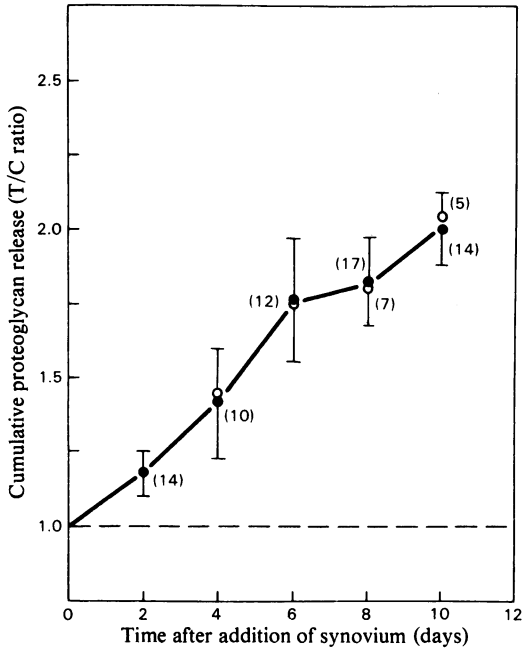

Fig. 4. *Proteoglycan release in human and experimental arthritis*

Data were pooled from several experiments in which either human rheumatoid (●) or rabbit antigen-induced arthritis (○) synovium were used. Individual data were expressed as (cartilage+synovium)/cartilage alone (T/C) ratio. Values are means ± S.E.M., with the numbers of experiments in parentheses.

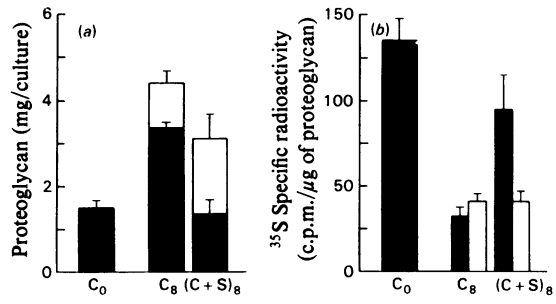

Fig. 5. *Effect of human rheumatoid synovium on proteoglycan synthesis and distribution*

(*a*) Proteoglycan content of discs and media. (*b*) ^{35}S specific radioactivities of discs and media. Cartilage discs prelabelled for 24h in ^{35}S medium were washed, established in organ culture and 'chased' for 24h. Five discs were removed as zero-time samples (C_0), minced synovium was added to one half of the remainder, and incubation continued for an additional 8 days. Media harvested at 2-day intervals and cartilage discs collected at the end of the experiment were analysed for their proteoglycan content and total ^{35}S radioactivity. Medium values are expressed as the accumulation during culture. C_0, cartilage removed at zero time; C_8, cartilage incubated in the absence of synovium for 8 days; $(C+S)_8$, cartilage/synovium co-cultures incubated for 8 days; ■, cartilage; □, medium. Values are means ± S.E.M. for five cultures in each group.

provides information on both points. $^{35}SO_4^{2-}$-prelabelled discs were established and maintained in organ culture for 8 days as described under 'Organ culture'.

Quantitative proteoglycan data are presented in Fig. 5(*a*). There was net synthesis of proteoglycan both in the absence (C_8) and in the presence [$(C+S)_8$] of rheumatoid synovium, with a 3-fold ($P<0.001$) and 2-fold ($P<0.005$) increase respectively. In control cultures (C_8), the proteoglycan content of the disc itself more than doubled ($P<0.001$ versus C_0), and accounted for 77% of the total. Synovium-containing cultures [$(C+S)_8$] synthesized less proteoglycan overall ($P<0.05$ versus C_8) and, in contrast with the control cultures, cartilage proteoglycan content remained constant over the 8-day period. On the other hand there was significantly more proteoglycan in their media (mean difference 670 μg; $t=2.55$; $P<0.05$), representing a larger fraction of the total (58 versus 23% in 8-day co-cultures and control cultures respectively).

In Fig. 5(*b*), specific-radioactivity data from the same cultures are depicted. The high specific radioactivity of cartilage at the start of the experiment (C_0) was strikingly decreased in control cultures (C_8; $P<0.001$), consistent with the synthesis *de novo* and retention of proteoglycan in cartilage (see Fig. 5*a*). Similarly, in synovium-containing cultures [$(C+S)_8$] cartilage specific radioactivity was also decreased ($P<0.05$ versus C_0), but to a lesser extent ($P<0.05$ versus C_8). Medium specific radioactivities from both sets of cultures were not detectably different.

Thus (*a*) proteoglycan biosynthesis occurred in all cultures and was significantly decreased in the presence of synovium. Overall decrease was estimated at 45%:

Net proteoglycan gain in control cultures

$\Delta_1 = C_8 - C_0$ (μg)

Net proteoglycan gain in co-cultures

$\Delta_2 = (C+S)_8 - C_0$ (μg)

Percentage decrease of synthesis in co-cultures:

$$\frac{\Delta_1 - \Delta_2}{\Delta_1} \times 100 = \frac{2900-1600}{2900} \times 100 = 45\%$$

Even in synovium-containing cultures in which there was no apparent change in cartilage proteoglycan content, some of the newly synthesized material was

incorporated in the disc, as evidenced by a decline in its specific radioactivity. Therefore cartilage breakdown must have occurred, with release of products into the culture medium. (*b*) Medium proteoglycan, as well as being derived from cartilage breakdown, could have arisen in part from metabolic 'shunting'. Such molecules would be unlabelled, decreasing the medium specific radioactivity accordingly. Because specific radioactivity was not significantly altered, preferential metabolic 'shunting' was not an important contribution to medium proteoglycan in synovium-containing cultures. Their greater proteoglycan content (Fig. 5*a*) thus largely reflected increased breakdown. Moreover, because all ^{35}S radioactivity in zero-time cartilage (C_0) was in macromolecules (demonstrated by Sepharose 4B chromatography of papain-digested discs; results not shown), the appearance of small molecular forms of $^{35}SO_4^{2-}$ provided an additional index of breakdown. On the basis of Alcian Blue-soluble radioactivity, a minimum estimate of the contribution was 65 and 73% of the medium radioactivity in $(C+S)_8$ and C_8 cultures respectively.

Cartilage–synovial interactions

Cartilage discs maintained in organ culture released proteoglycan into their surrounding media. The addition of rheumatoid or antigen-induced arthritis synovia stimulated this process. It seemed unlikely that cartilage served merely as an inert substrate in such co-cultures. Experiments were therefore designed to assess the cell-mediated contribution to breakdown by each of the components of the system.

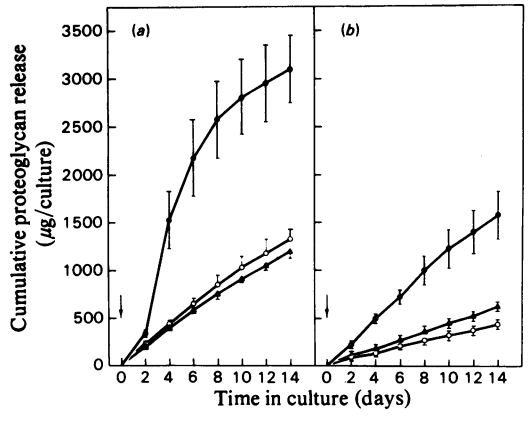

Fig. 6. *Cartilage–synovium interactions: accumulation of proteoglycan in culture medium*

Replicate bovine nasal cartilage discs were established at zero time either as (*a*) live ($n = 12$) or (*b*) dead ($n = 12$) cultures. Human rheumatoid synovium was added at the arrow to both groups either as a live or prekilled preparation. Tissues were killed by immersion in a 100°C water bath for 4 min. ○, Cartilage alone; ●, cartilage plus live synovium; ▲, cartilage plus dead synovium. Values are means ± S.E.M. ($n = 4$).

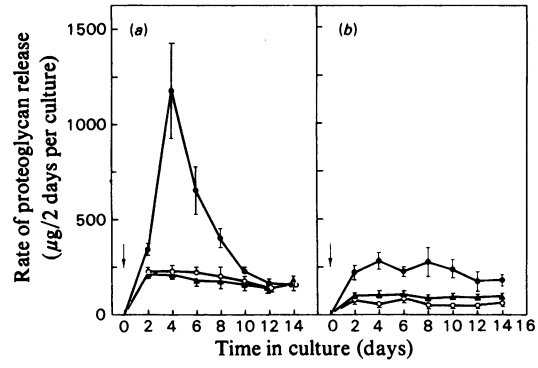

Fig. 7. *Cartilage–synovium interactions: rates of proteoglycan release*

For details, see the legend to Fig. 6. (*a*) Live cartilage; (*b*) dead cartilage.

In the experiment depicted in Fig. 6, cartilage discs were established either as live cultures (Fig. 6*a*) or after preliminary killing by immersion in a 100°C water bath for 4 min (Fig. 6*b*). To both sets of cultures, human rheumatoid synovium was added at zero time, either as a live preparation, or after similar heat-killing. As noted previously, in the completely live system, proteoglycan release was abruptly stimulated after an initial lag (Fig. 6*a*); basal release remained low and was unaltered in the presence of dead synovium. Prekilled cartilage, on the other hand, showed even greater decrease in basal release (Fig. 6*b*), which was also minimally affected by the addition of dead synovium. However, the addition of live synovium significantly restored cartilage breakdown to approx. 50% of that seen in the completely live system. Shown in another way (Fig. 7), the quantity of proteoglycan released per 2-day period (i.e. the rate of breakdown) was abruptly stimulated to a maximum by day 4 in completely live co-cultures (Fig. 7*a*), decreasing rapidly thereafter to basal values by day 10. In comparison, proteoglycan release from dead cartilage (Fig. 7*b*) was greatly decreased, but could be enhanced by the addition of live synovium; the extent of this restoration, however, was only to a value comparable with that achieved by live cartilage alone (compare Fig. 6*a*). These data indicate (*a*) that for cartilage breakdown to proceed optimally both synovial and chondrocyte components must be viable, and (*b*) that the peak interaction between the two (day 4) is more than additive, suggesting a positive-amplification effect.

Freeze–thawing was used as an alternative method of cartilage inactivation. (*a*) Rapidly frozen discs (Table 1) demonstrated a high rate of autolysis, especially in early cultures, exceeding the basal release from live cartilage by 45% on day 2 ($0.05 < P < 0.1$). (*b*) Cryopreserved discs on subsequent restoration proved to be non-viable, as evidenced by their failure to incorporate $^{35}SO_4^{2-}$ (J. Steinberg & C. B. Sledge, unpublished work) and failure to accumulate proteoglycan; the total proteoglycan content of 10-day cultures was not detectably different from the starting value in freshly thawed discs (Table 2). Thus for practical purposes such cartilages were considered simply as frozen–thawed. The rate of autolysis in thawed cartilage was sufficiently high so that little additional stimulation of proteoglycan release was achieved in the presence of synovium. However, autolysis could be significantly decreased by preliminary heat-inactivation, which did not affect the degradative response to added synovium. These data are consistent with the interpretation that spontaneous autolysis was attributable to the liberation and persistence of active degradative enzymes rather than intact cellular metabolic activity, and that heat inactivation, although decreasing basal autolysis, did not significantly affect the susceptibility of extracellular matrix to enzymic attack.

Proteoglycan nature of breakdown products

The molecular nature of the Alcian Blue-precipitable (polyanionic) material shed into the culture medium was assessed by gel-filtration chromatography and enzymic digestion. Crude medium from 8-day control cultures (Fig. 8*a*) revealed a single high-molecular-weight peak essentially confined to the void volume. In comparison, medium pooled from 8-day cultures exposed to rheumatoid synovium showed a more polydisperse pattern (Fig. 8*b*), but a major component was still excluded from Sepharose 4B. Both media re-chromatographed in dissociative conditions (4M-guanidinium chloride) showed only minor changes, indicating that these large complexes were not simply aggregated products. Prior digestion with the specific *Streptomyces* hyaluronidase did not alter either initial elution pattern, indicating that hyaluronate was not an integral part of the complex. The significant protein content of this material was demonstrated by its

Table 1. *Effect of freeze–thawing versus heat-killing on spontaneous proteoglycan release from cartilage*
Freshly prepared cartilage discs were established in organ culture (*a*) directly (live), (*b*) after preliminary killing by two cycles of rapid freezing on a bed of solid CO_2 followed by thawing to 37°C, or (*c*) by immersion in a 100°C water bath for 4min. Culture media were changed at 2-day intervals. Values are means ± S.E.M. ($n = 7$).

	Rate of proteoglycan release into culture medium (μg/2 days per culture)			
Cartilage Day ...	0–2	2–4	4–6	6–8
(*a*) Live	434±33	333±25	275±29	165±6
(*b*) Frozen–thawed	630±86	352±26	265±62	196±59
(*c*) Heat-killed	128±15	122±15	135±26	76±4

Table 2. *Cartilage breakdown in vitro after cryopreservation*
Cartilage discs were restored from liquid N_2 as described in the Materials and Methods section. One half were 'heat-killed' by immersion in a 100°C water bath for 4min. Individual discs were established as organ cultures at zero time, and media were changed at 1 and 2 days. At the second medium change, minced human rheumatoid synovium was added to one half of the cultures in each group and media were changed at 2-day intervals thereafter. The total proteoglycan content of each culture was calculated from the sum of proteoglycan released into medium plus residual in cartilage after 10 days. Initial proteoglycan content determined in five discs at zero time was 6300±300 μg/disc. Values are means ± S.E.M. ($n = 5$).

		Cumulative proteoglycan (μg/culture)					
		Medium					Medium+ cartilage
Days after restoration ...		2	4	6	8	10	10
Cartilage	Synovium						
Thawed	Absent	174±13	443±45	768±82	1018±109	1281±146	6639±1300
	Present	—	557±57	989±52	1270±57	1506±62	6396±900
Thawed and heat-killed	Absent	98±8	201±10	333±2	452±4	578±1	6292±190
	Present	—	471±87	960±216	1352±258	1644±285	5838±560

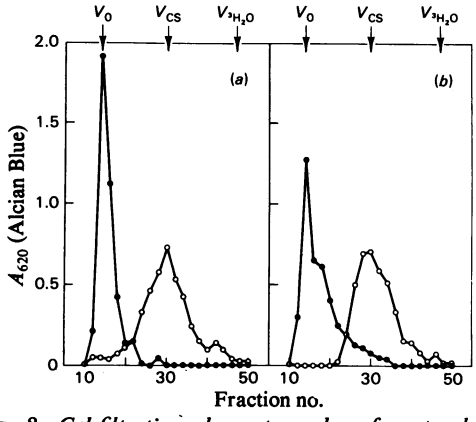

Fig. 8. *Gel-filtration chromatography of proteoglycans accumulated in 8-day culture media*

Media were prepared as detailed in the Materials and Methods section from (*a*) control cultures of cartilage discs and (*b*) complete cultures of cartilage plus human rheumatoid synovium. Portions of each before (●) and after (○) papain digestion were eluted from Sepharose 4B with 0.15M-NaCl/0.02M-sodium acetate buffer (pH5.8)/0.02% NaN_3. Alternate 1ml fractions were assayed by the Alcian Blue method. V_0, V_{CS} and $_{^3H_2O}$ V are the elution peak positions of Blue Dextran 2000, chondroitin sulphate and [^{3}H] water respectively. Profiles have been normalized to the same area under each curve.

decrease in size after digestion with papain, which shifted the elution profile to the position of single glycosaminoglycan chains (Figs. 8*a* and 8*b*). The proteoglycan nature of these polyanionic macromolecules was confirmed by their virtual elimination after bovine-testicular-hyaluronidase digestion. The resistant fraction averaged approx. 11% and chromatographed as a broad band (not shown) intermediate in size between V_0 and V_{CS}, with a peak at fraction no. 24. Together these data indicate that proteoglycan–chondroitin sulphate was the major product released in all culture media, and that the proteoglycan released in the presence of rheumatoid synovium was partially degraded.

Discussion

Bovine nasal-cartilage discs could be prepared in numbers sufficient for multiple replication. Weights were internally consistent and varied little between experiments. The cutting height produced 1mm discs that became measurably thicker during the course of culture, presumably due in part to water gain (Sokoloff, 1969); nonetheless, the overall loss of cartilage mass and accompanying erosion frequently evident in complete cultures pointed to significant breakdown. This was confirmed by the stoicheiometry of proteoglycan content. The net gain of proteoglycan during 8 days in culture was significantly less in the presence of synovium, indicating an overall 45% reduction compared with control cartilages. In addition, a major component (58%) of total proteoglycan was found in the culture medium. From a consideration of quantitative and specific-radioactivity data it was estimated that cartilage breakdown accounted for a minimum of 65–73% of the released proteoglycan.

The Alcian Blue-complexing method provided a simple assay of proteoglycan content and readily permitted the detection of group differences. Complete separation was effected between sulphated glycosaminoglycans and the significant hyaluronate content of culture medium, provided the appropriate critical electrolyte ($MgCl_2$) concentration (Scott, 1960) was used, as recommended by Whiteman (1973). The spontaneous release of cartilage proteoglycan in our experiments represented approx. 35–60% of the amount released in synovium-supplemented cultures. This was comparable with data reported by Dingle *et al.* (1975), who used retinol-treated pig articular cartilage. Such relatively high cartilage 'blank' values may be the result of the slicing procedure, with consequent exposure of denuded matrix at the surfaces, permitting ready diffusion between tissue and medium. The gradual decline in basal proteoglycan release (see Figs. 3 and 7*a*) may have been due to a decline in autolysis or in synthesis and metabolic 'shunting'. These possibilities could not be distinguished from the available data. However, observations in radioactively labelled cultures are consistent with preferential early release of recently synthesized proteoglycan, perhaps from surface or pericellular pools (J. Steinberg, unpublished work).

The addition of rheumatoid or antigen-induced arthritis synovium stimulated proteoglycan release approx. 2-fold, but usually only after an initial lag phase. Despite the apparent lack of early quantitative differences, significant qualitative changes in the proteoglycan products have been noted soon after treatment with synovium (Steinberg *et al.*, 1978). Thus difficulties with enzyme penetration and substrate accessibility are unlikely explanations for the lag. Differential enzyme activities yielding early products that may not be detected by the dye-complexing method have been proposed (Steinberg *et al.*, 1978).

The appropriateness of bovine nasal cartilage as a model for articular-cartilage degradation may be questioned. Although the relative proportions of chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate may differ from human articular cartilage (Mankin, 1973), proteoglycan structure is essentially similar (Keiser & Sandson, 1974). The major polyanionic material accumulating in our cultures has been identified as proteoglycan–chondroitin sulphate. In the presence of rheumatoid

synovium this was partially degraded, the chromatographic pattern suggesting limited proteolytic cleavage. The minor constituent intermediate in size between intact proteoglycan and glycosaminoglycan chains, and revealed by testicular hyaluronidase treatment, was tentatively identified as proteoglycan–keratan sulphate. Since other hyaluronidase-resistant proteoglycans (e.g. heparin, heparan sulphate, dermatan sulphate) are essentially absent in cartilage (Sajdera & Hascall, 1969), macromolecules enzymically stripped of their chondroitin sulphate chains would be expected to contain only keratan sulphate. Our value of 11% resistant material agrees with the reported keratan sulphate content of nasal cartilage (Sajdera & Hascall, 1969).

Which cells are specifically responsible for the degradation is difficult to assess in the rheumatoid joint because of the proliferation of diverse types, including synovial cells, inflammatory and other connective-tissue cells. Blood cells and vascular elements are prominent in rheumatoid pannus. Leucocyte enzymes including neutral proteinases have been shown to penetrate and produce depolymerization of cartilage proteoglycan (Janoff *et al.*, 1976; Keiser *et al.*, 1976), and have been proposed as playing a key role in the pathogenesis of human arthritis. A related observation has been made by Dingle *et al.* (1975) that, in the presence of complement-sufficient antiserum, cartilage explants that included subjacent invasion zone (derived from the marrow cavity) underwent significant degradation in organ culture, whereas pure articular cartilage was virtually unaffected.

Synovial elements apart from inflammatory cells, but including capsular tissue, have been shown to degrade pure cartilage matrix (Fell & Barratt, 1973; Barratt, 1973). More recently, Fell & Jubb (1977) have demonstrated that isolated synovial villi are also very potent in this regard, both directly and by a mechanism mediated through chondrocytes. The role of the chondrocyte in matrix turnover, and its potential contribution to cartilage breakdown in various pathologic states, has been attributed to a number of well-characterized enzyme activities found in cartilage (reviewed in Barrett, 1975). These include acid cathepsins (Ali & Evans, 1973; Sapolsky *et al.*, 1976), neutral metalloproteinases (Sapolsky *et al.*, 1974, 1976) and collagenase (Ehrlich *et al.*, 1977). Dingle *et al.* (1975) demonstrated in isolated cartilage the significant stimulation by vitamin A of proteoglycan breakdown, and to a lesser extent, collagen breakdown. The interaction of synovium plus cartilage, occurring even at a distance within the same culture vessel (Fell & Jubb, 1977), shows an absolute requirement for live chondrocytes. This is compatible with the hypothesis that diffusible products released from synovium may be responsible for chondrocyte-mediated resorption.

That cartilage did not serve merely as an inert substrate was demonstrated in our experiments, and is in broad agreement with the observations of Fell & Jubb (1977). The requirement for both live synovium and live cartilage to obtain maximal resorption, and the limited capacity of living synovium to resorb dead cartilage, are strong evidence for a chondrocyte-mediated component. Moreover, the peak rate of proteoglycan release in the completely live co-culture was greater than the sum of its components, suggesting a positive interaction between synovium and cartilage, amplifying the degradative response.

Although antigen-induced arthritis in rabbits bears many similarities to human rheumatoid arthritis (Twenty-Second Rheumatism Review, 1976) and the two disorders show strikingly similar patterns of cartilage breakdown in our system *in vitro*, it is not to be inferred that the degradative response is specific to these two models. Rather, there is mounting evidence that cartilage destruction is dependent on the clinical activity of the synovitis from whatever cause (Waxman & Sledge, 1973; LeMarshall *et al.*, 1977; Muirden, 1972) and may be mediated through a 'final common pathway' (Hollander *et al.*, 1965). This view has been supported by observations in other experiments using human synovia: very similar patterns of breakdown were noted with active synovia from patients with osteoarthritis, pseudo-gout and pigmented villonodular synovitis (J. Steinberg, unpublished work).

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