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Human cartilage is degraded by rheumatoid arthritis synovial fluid but not by recombinant cytokines *in vitro*

A. P. HOLLANDER*, R. M. ATKINS†, D. M. EASTWOOD†, P. A. DIEPPE‡ & C. J. ELSON*
*Departments of *Pathology, †Orthopaedic Surgery and ‡Rheumatology, University of Bristol, Bristol, England*

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SUMMARY

Rheumatoid arthritis (RA) synovial fluid (SF) stimulated significant loss of glycosaminoglycans (GAG) from normal and pathological human cartilage biopsies over 2 days as compared with normal human serum. By contrast, 15 RA SFs failed to degrade killed normal cartilage, and degraded killed RA cartilage less effectively than living RA cartilage. Four RA SFs were treated with neutralizing anti-cytokine antisera prior to incubation with normal cartilage. The degrading effects of two of the fluids were reversed by anti-interleukin-1α (IL-1α) while degradation by the third and fourth fluids were reversed by anti-interleukin-1β (IL-1β) and anti-tumour necrosis factor-alpha (TNF-α), respectively. However, recombinant human IL-1α, IL-1β, TNFα or a combination of all three cytokines had no degrading effect in this 2-day culture system. It is concluded that RA SF degrades cartilage by a mechanism involving a synergistic interaction between cytokines and some other component of SF.

Keywords human cartilage degradation synovial fluid rheumatoid arthritis interleukin-1 tumour necrosis factor-alpha

INTRODUCTION

The main stimuli of cartilage degradation in rheumatoid arthritis (RA) are thought to be interleukin-1 (IL-1) and tumour necrosis factor (TNF). However, this view is based essentially on just two pieces of evidence. Firstly, IL-1 and TNF can degrade bovine, porcine, rat and rabbit cartilage *in vitro* (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980; Saklatvala, 1981; Saklatvala, Curry & Sarsfield, 1983; Saklatvala *et al.*, 1984; Saklatvala, Sarsfield & Townsend, 1985; Saklatvala, 1986; Steinberg, Hubbard & Sledge, 1987; Clay, Seed & Clement-Jewery, 1989) or *in vivo* (Pettipher, Higgs & Henderson, 1986; Dingle *et al.*, 1987; Henderson & Pettipher, 1988, 1989; van de Loo & van den Berg, 1990) by stimulating the chondrocytes to secrete cartilage-degrading metalloproteases (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980; Saklatvala, 1981; Saklatvala *et al.*, 1983). Secondly, IL-1 and TNF are spontaneously produced by RA synovial tissue cells (Wood, Ihrie & Hamerman, 1985; Buchan *et al.*, 1988; Yocum *et al.*, 1989) and both cytokines have been detected in RA synovial fluid (SF) by several assay methods (Fontana *et al.*, 1982; Wood *et al.*, 1983; Bendtzen *et al.*, 1985; DiGiovine *et al.*, 1986; Miossec, Dinarello & Ziff, 1986; Hopkins & Meager, 1988; Hopkins, Humphreys & Jason, 1988; Symons *et al.*, 1989).

Correspondence: Dr C. J. Elson, Department of Pathology, Medical School, University of Bristol, University Walk, Bristol BS8 1TD, UK.

If IL-1 and TNF are indeed the major mediators of cartilage degradation in RA, then it should be possible to satisfy three predictions: (i) IL-1 and TNF levels should only be raised in SF during periods of cartilage degradation; (ii) inhibitors of IL-1 and TNF should inhibit cartilage degradation in RA, or in models of RA; and (iii) recombinant human (rh) IL-1 and TNF should degrade human (as opposed to animal) cartilage *in vitro*. The first of these predictions does not appear to be satisfied, since the concentrations of a cartilage matrix component, glycosaminoglycans (GAG), in RA SFs do not correlate with the concentrations of IL-1 (Bensouyad *et al.*, 1990) or TNF (Hollander, 1990). Furthermore, very high TNF levels have been detected in two normal SFs (Westacott *et al.*, 1990). The second and third predictions have also not yet been satisfied, since cytokine inhibitors have not been tested in RA and there are few publications on human cartilage degradation by cytokines. The aim of this study was to test predictions (ii) and (iii) by developing an *in vitro* model of RA cartilage degradation.

In the rheumatoid joint, degradation of cartilage matrix can occur at a distance from the cartilage/synovium junction as well as close to it (Mitchell & Shepard, 1978). This observation suggests that at least some of the degradative changes are mediated by component(s) of the SF in which the cartilage is bathed. It follows that if cartilage is incubated with RA SF *in vitro* then it should lose GAG from its matrix. Accordingly, measurements of GAG loss from normal human cartilage were

made and the contribution of cytokines to cartilage degradation in RA was explored by freeze-killing the cartilage (to test for chondrocyte dependency) and by treating the SF with neutralizing anti-cytokine antisera. Moreover, the effects of rhIL-1 and TNF on GAG concentrations in normal human cartilage were examined using the same *in vitro* system.

MATERIALS AND METHODS

Synovial fluids and serum

Samples of knee SF were obtained from RA patients attending the Rheumatology Clinic at Bristol Royal Infirmary. All patients fulfilled the American Rheumatism Association criteria for classical or definite RA (Ropes *et al.*, 1958) and were receiving a variety of non-steroidal anti-inflammatory drugs and slow-acting anti-rheumatic drugs. The SFs were collected in sodium citrate (3% w/v) and were centrifuged at 800 ***g*** for 10 min to remove cells and debris. After treatment with hyaluronidase (30 U/ml; Sigma, Poole, UK) for 30 min at 37°C they were stored at −70°C until use. Normal human serum (NHS) was obtained from healthy volunteers and stored at −70°C until use.

Cartilage

Slices of normal human articular cartilage were taken from the femoral heads of patients who were undergoing hemi-arthroplasty following osteoporotic subcapital fracture of the femoral neck. Samples were also obtained from the upper tibia of two patients following severe tibial plateau fracture. In these cases, small samples of cartilage which could not be reconstructed into the joint surface due to fracture comminution, were removed at the time of operative fixation. In all these cases there was no clinical, macroscopic or radiographic evidence of degenerative change in the joint prior to fracture. Slices of arthritic cartilage were obtained from RA patients undergoing total hip or knee replacement. Biopsies of 3×3 mm were obtained from the cartilage slices using a biopsy punch. Ten biopsies were taken at random from each joint and used to determine the intrinsic GAG content of the cartilage. The remaining biopsies were used in cartilage-culture experiments.

Tissue culture

For culture experiments, each biopsy was cut in half and the two pieces were placed in separate wells of a 24-well plate. One piece (control biopsy) was cultured in 2 ml of complete medium (CM) consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco Europe), 25 mM HEPES (Flow Labs) and 5% normal human AB serum (Blood Transfusion Centre, Bristol, UK) which was heat-inactivated (56°C for 30 min) before use. The other piece (treated biopsy) was cultured in 2 ml of CM containing one of the following stimuli: 15% SF; 15% NHS; rhIL-1α (a gift from Hoffman La-Roche, NJ; specific activity, 3×10^8 U/mg): rhIL-1β (a gift from Roussel, UCLAF; Specific activity, 1×10^8 U/mg) or rhTNF-α (a gift from Glaxo; Specific activity, 6×10^7 U/mg). Each SF, NHS or cytokine was tested on five biopsies alongside five paired control biopsies. In some experiments SFs were treated with neutralizing anti-cytokine antisera before addition to the culture wells. Sheep anti-IL-1α (Poole *et al.*, 1989), anti-IL-1β (Poole *et al.*, 1989) and pre-immune serum were generous gifts from Dr S. Poole; goat anti-TNF-α (neutralizing titre, $1/10^4$) was kindly provided by Dr T. Meager (both at the National Institute for Biological Standards and Control, Potters Bar, UK). They were all used at a final dilution of 1/1000. The cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2/air. At the end of the culture period the plates were stored at −20°C until the GAG concentration in each biopsy could be determined.

Freeze-killing of cartilage

In some experiments SF were tested for their ability to degrade cartilage in which the chondrocytes had been killed by repeated freeze–thawing. To this end, biopsies of cartilage in Eppendorf tubes containing 500 μl of CM were floated in a bath of liquid nitrogen for 10 min and then in boiling water for 10 min. This procedure was repeated twice. The biopsies were then cultured with or without SF in the same way as described above for living cartilage.

GAG assay

Estimation of sulphated GAG in cartilage was carried out using the colourimetric method of Farndale, Sayers & Barret (1982) and Farndale, Buttle & Barrett (1986) with some modifications. Each cartilage biopsy was blotted dry, weighed and then placed in an Eppendorf tube containing 0·5 ml of papain (16·3 U/ml; Sigma) in digestion buffer (DB) consisting of 20 mM disodium-hydrogenorthophosphate dihydrate, 1 mM EDTA (both from BDH) and 2 mM and 2 mM dithiothreitol (Sigma). The capped Eppendorf tubes were incubated at 65°C for 2 h. They were then vortex-mixed and 50 μl of each cartilage digest were diluted 1/8 with DB. Duplicate 10 μl aliquots of the diluted cartilage digest and of standard whale chondroitin sulphate (Sigma) were added to the wells of a round-bottomed microtitre plate. DMB was prepared by dissolving 16 mg of the dye (Serva, FRG), 3·04 g glycine (BDH) and 2·37 g NaCl (BDH) in deionized water (final volume 1 *l*). The pH was adjusted to 3·0 with concentrated hydrochloric acid. DMB (200 μl) was added to each well and the absorbance at 525 nm (reference wavelength, 690 nm) was read immediately using a Multiskan MCC/340 MKII plate-reader (LabSystems). The change in absorbance was calculated from the mean absorbance of six blank wells in which only buffer and dye were added. The plate reader was interfaced with an Amstrad computer to facilitate calculation of the concentration of GAG in the samples from the standard curve.

IL-1 bioassay

The efficacy of the rhIL-1 and anti-IL-1 antisera were tested in a conventional lymphocyte-activating factor (LAF) assay. Thymocytes from three CBA/HT mice were cultured in triplicate in 96-well round-bottomed plates (Nunc). Each well contained 5×10^5 thymocytes in 200 μl of α-MEM medium (Gibco) containing 100 U/ml penicillin (Glaxo), 100 μg/ml streptomycin (Evans), 5×10^{-5} M 2-mercaptoethanol (Sigma), 10 mM glutamine (Gibco), 0·5% normal mouse serum, 1·25 μg/ml phytohaemagglutinin (PHA; Wellcome), and 100 μg/ml cytokine with or without neutralizing anti-serum. Control wells contained thymocytes alone or thymocytes and PHA. The cells were incubated for 48 h at 37°C in an atmosphere of 5% CO_2/air and pulsed with 1 μCi/well of ^{3}H-thymidine (Amersham) for the last 6 h of culture. The cells were harvested with an automatic cell-harvester and thymidine uptake was measured using a liquid

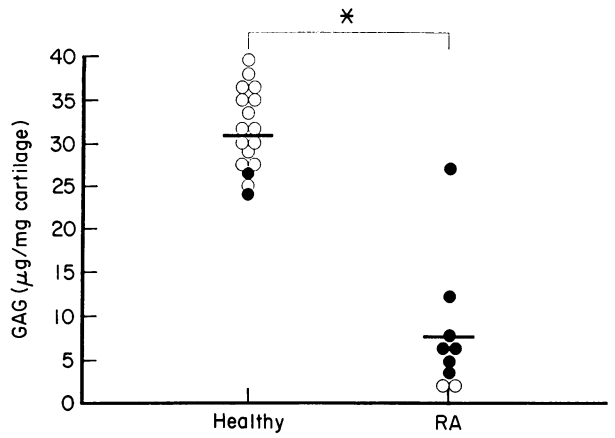

Fig. 1. Intrinsic glycosaminoglycan (GAG) concentration in human cartilage from 17 healthy subjects and nine patients with rheumatoid arthritis (RA). Each point is the mean concentration in 10 cartilage biopsies taken at random from the femoral head (○) or knee (●) of one patient. Bars indicate the mean concentration (hips and knees) for each patient group. $*P<0.01$.

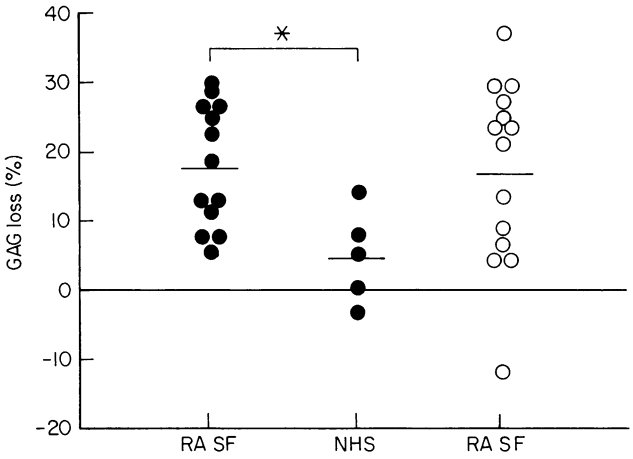

Fig. 2. Glycosaminoglycan (GAG) loss, induced by synovial fluid (SF), from normal femoral head cartilage. Biopsies from one patient (●) were incubated with and without rheumatoid arthritis (RA) SF ($n=13$) or normal human serum (NHS) ($n=5$). A second group of RA SFs ($n=14$) were tested on cartilage from another patient (○). Each point is the mean result for five pairs of biopsies (five treated and five control). Bars indicate the mean GAG loss for each group of fluids. $*P<0.01$.

scintillation counter. Results were expressed as the mean ± s.d. of triplicate cultures.

Analysis of data

The loss of GAG from cartilage induced by each SF, NHS or cytokine was estimated using the calculation:

$$\% \text{ GAG loss} = 10 - \left(\frac{\text{Mean (GAG) in five treated biopsies}}{\text{Mean (GAG) in five control biopsies}} \times 100 \right)$$

The significance of differences between groups of data was tested using the two-tailed Student's *t*-test. $P<0.05$ was taken as significant.

RESULTS

Intrinsic GAG levels in human cartilage

In order to test whether the effect of SF on cartilage GAG levels *in vitro* was likely to be a good model of RA cartilage degradation, it was considered important to demonstrate GAG loss as a major feature of cartilage from RA patients. Accordingly, the intrinsic GAG concentration in RA cartilage was measured and compared with GAG levels in normal human cartilage. The mean concentration of GAG in 10 biopsies taken at random from each cartilage sample is shown for different groups of patients in Fig. 1. The mean GAG concentration (+s.d.) in normal hip and knee cartilage from 17 patients was 31·4 ± 4·6 μg/mg. The equivalent value for cartilage from nine RA patients was 8·1 ± 7·7 μg/mg. The two groups were significantly different from each other ($P<0.01$).

Unstimulated cartilage culture

In order to test whether either normal or arthritic cartilage spontaneously degrade in culture, the concentration of GAG was measured either immediately or after 2 days of culture in CM without any stimulus. No significant spontaneous GAG loss could be detected in normal or RA cartilage. Thus, the GAG concentrations in normal cartilage from three patients were, respectively, 35, 36 and 38 μg/mg immediately; and 35, 37 and 38 μg/mg after days of culture. Similarly, the GAG concentrations in RA cartilage from three patients were, respectively, 2, 4 and 6 μg/mg immediately; and 3, 3 and 7 μg/mg after 2 days of culture. Normal and RA cartilage samples from other patients were equally stable in unstimulated culture.

Table 1. Loss of glycosaminoglycans (GAG) induced by synovial fluid (SF) from normal cartilage and from cartilage of patients with rheumatoid arthritis (RA)

	Normal cartilage		RA cartilage	
SF	1	2	1	2
1	6	4	ND	17
2	30	38	44	37
3	19	ND	40	ND
4	26	ND	34	ND

Four RA SF were tested on cartilage from different patients. Each value is the mean % GAG loss for five pairs of biopsies (five treated and five control).

ND, not determined.

SF-induced GAG loss

The GAG content of cartilage biopsies was measured after incubation for 2 days with or without SF. As can be seen from Fig. 2, RA SF induced up to 31% loss of GAG from normal cartilage whereas NHS induced no more than 14% GAG loss. These differences were significant ($P<0.01$). Similar results were obtained when groups of RA SF were tested on normal cartilage from three further patients and the results of one such experiment are shown in Fig. 2. In subsequent experiments, RA

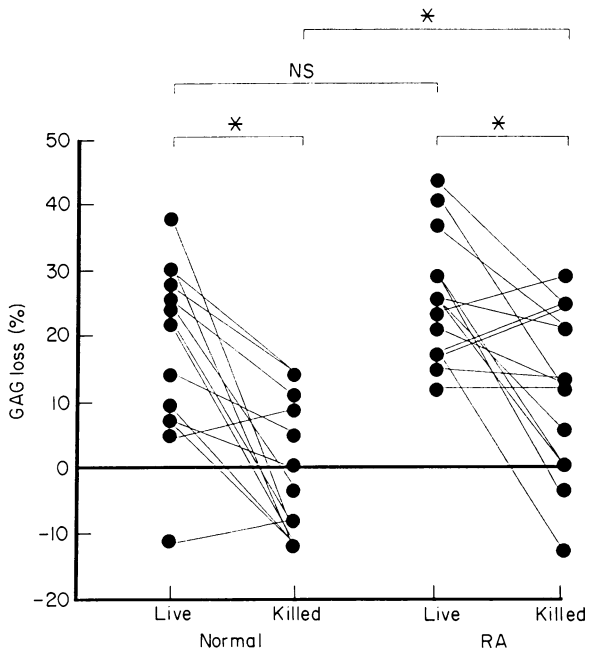

Fig. 3. Glycosaminoglycan (GAG) loss, induced by synovial fluid (SF), from living and killed cartilage. Biopsies from one normal femoral head and one rheumatoid arthritis (RA) knee were incubated with and without RA SF. Each line represents one SF. All 15 SFs were tested against living and killed cartilage from both patients. Each point is the mean result for five pairs of biopsies (five treated and five control). NS, not significant. $*P < 0{\cdot}01$.

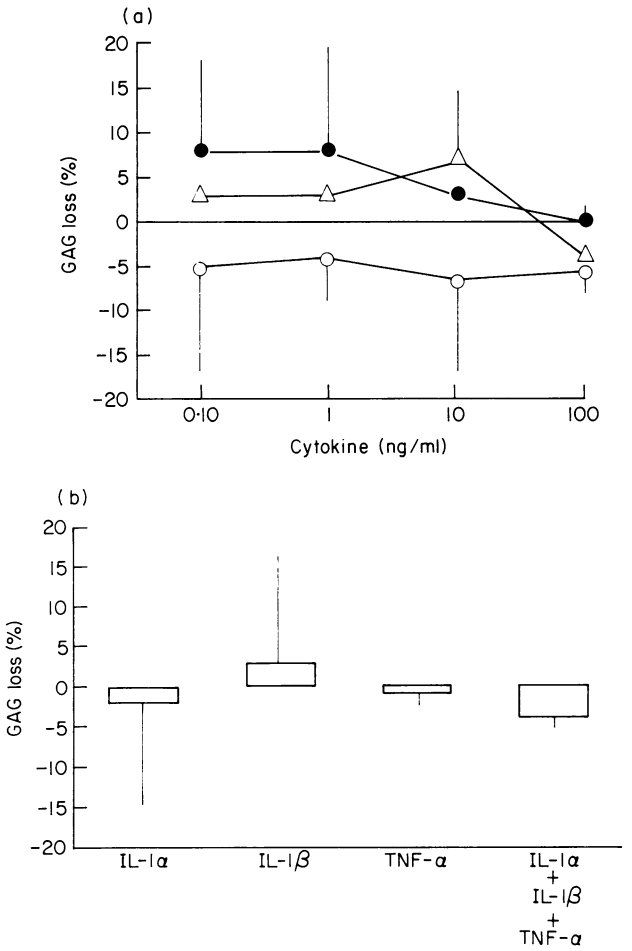

Fig. 5. Cytokine induced glycosaminoglycan (GAG) loss from normal femoral head cartilage. Biopsies were incubated with and without recombinant human interleukin-1α (IL-1α) (●); IL-1β (○); or tumour necrosis factor-alpha (TNF-α) (△). Each concentration of cytokine was tested on five pairs of biopsies and compared with five controls (medium only). (a) Mean ± s.d. for three experiments using individual cytokines; (b) Mean ± s.d. for three experiments using 10 ng/ml of each cytokine either alone or together.

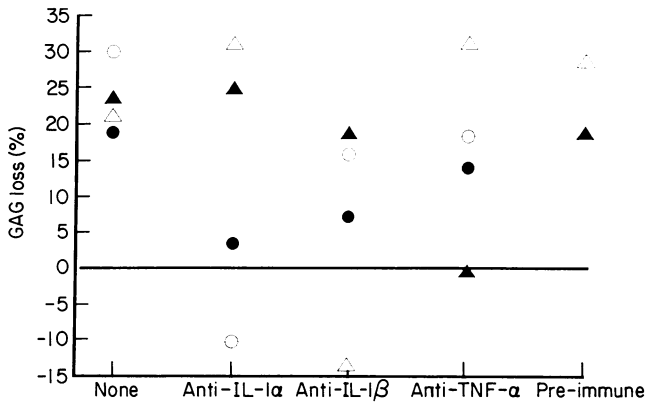

Fig. 4. Effect of anti-cytokine antisera glycosaminoglycan (GAG) loss, induced by synovial fluid (SF), from normal femoral head cartilage. Four rheumatoid arthritis (RA) SFs (SF1, ○; SF2, ●; SF3, △; and SF4, ▲) were cultured with cartilage either on their own or after treatment with a neutralizing antiserum (or, in two cases, with pre-immune serum). SF1 and SF2 were tested on cartilage from one patient and SF3 and SF4 on cartilage from a second patient. Each point is the mean result for five pairs of biopsies (five treated and five control). IL-1, interleukin-1; TNF, tumour necrosis factor.

SF was found to degrade cartilage from RA and ankylosing spondylitis (AS) patients. For 15 RA SF tested on one RA cartilage the mean GAG loss was 23% (range 11–43%), while for six RA SF tested on one AS cartilage the mean GAG loss was 21% (range 19–53%). Table 1 shows the effect of selected RA SF on different cartilage samples. It is evident that any one SF exerts a similar effect on cartilage from different patients.

To determine whether SF degrades cartilage directly or by stimulating chondrocytes, 15 RA SF were tested for their ability to degrade killed normal and RA cartilage. As shown in Fig. 3, the SF degraded dead cartilage significantly less well than living cartilage from normal subjects as well as from RA patients. However, whereas none of the SF induced any marked GAG loss from dead normal cartilage, six of them induced a GAG loss of greater than 20% from dead RA cartilage. Thus, while there was no significant difference in the ability of the SF to degrade living normal and living RA cartilage, the same SF degraded dead RA cartilage significantly more than dead normal cartilage (Fig. 3).

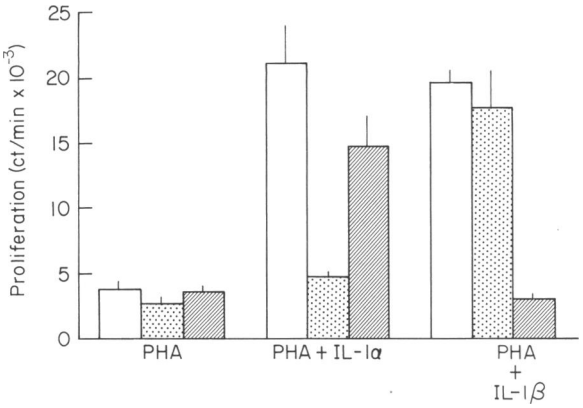

Fig. 6. Proliferation of mouse thymocytes in response to 1·2 μg/ml phytohaemagglutinin (PHA) and 100 pg/ml recombinant interleukin-1α (IL-1α) or IL-1β with or without neutralizing antisera. □, no antiserum; ▨, anti-IL-1α included in culture; ■, anti-IL-1β included in culture. Results are the mean ct/min ± s.d. of triplicate cultures in one experiment.

Role of cytokines in SF-induced GAG loss

Four of the RA SF which were shown to induce a marked GAG-loss from normal cartilage were subsequently tested on normal cartilage in the presence and absence of neutralizing antisera against different cytokines. The degrading effect of two of the fluids (SF1 and SF2) was abolished by anti-IL-1α antiserum and partially reduced by anti-IL-1β and anti-TNF-α (Fig. 4). The degrading effects of the third and fourth fluids (SF3 and SF4) were abolished by anti-IL-1β and anti-TNF-α, respectively, with no apparent reduction by any of the other antisera, including pre-immune serum. However, rhIL-1α, IL-1β and TNF-α at the dose range 0·1–100 ng/ml induced little or no loss of GAG from normal cartilage (Fig. 5a). There was also no degradation by 10 ng/ml of each of the cytokines in combination (Fig. 5b) or by the individual cytokines (at 1 and 10 ng/ml) over a 6-day culture period (data not shown). The rhIL-1α and IL-1β were both effective in stimulating proliferation of murine thymocytes and this effect could be selectively reversed using the relevant specific sheep antiserum (Fig. 6).

DISCUSSION

The results reported here show for the first time that cartilage degradation in RA is unlikely to be mediated by IL-1 and TNF on their own. The recombinant human cytokines, either alone or in combination with each other and at concentrations up to 10-fold in excess of their levels in RA SF (DiGiovine *et al.*, 1986; Hopkins *et al.*, 1988; Symons *et al.*, 1989; Yocum *et al.*, 1989), were unable to degrade normal human cartilage in this system, even after 6 days of culture. The rhIL-1α and IL-1β were clearly active since they were both potent inducers of mouse thymocyte proliferation. Furthermore, in our hands the same IL-1α and IL-1β preparations are able to degrade rat femoral head cartilage when used at 100 ng/ml (unpublished results). The IL-1β is also effective at priming neutrophils for myeloperoxide release, even when used at concentrations as low as 20 pg/ml (Dularay *et al.*, 1990), while TNF-α is effective at inducing bone resorption in mouse calveriae (M. Gowen, personal communication).

Our finding that recombinant human cytokines are unable to degrade human cartilage over 2 or 6 days of culture contrasts strikingly with the numerous reports of cytokine-mediated degradation of animal cartilage and also with the novel demonstration that RA SF induces marked degradation of human cartilage over a 2-day culture period. The RA SF did not degrade killed normal cartilage and degraded killed RA cartilage less effectively than living RA cartilage. These results suggest that SF degrades cartilage by a chondrocyte-dependent mechanism, thus implicating endogenous IL-1 and TNF in the SF as the mediators of GAG loss (Dingle *et al.*, 1979; Saklatvala & Dingle, 1980; Saklatvala, 1981; Saklatvala *et al.*, 1983). The possibility of a central role for these cytokines is supported by the finding that anti-cytokine antisera can reverse the SF-mediated degradation, although different antisera were effective with different SFs. These results are consistent with the view that each of the cytokines is capable of degrading human cartilage and that it is the relative concentrations of each in any one SF which accounts for the difference in antisera efficacy with different SFs. However, since recombinant human cytokines do not degrade human cartilage in this system, the endogenous cytokines in SF must be acting in concert with some other component of the SF. One possibility is that SF sensitizes chondrocytes to IL-1 and TNF. Alternatively, it may be that the cytokines in SF stimulate latent metalloprotease release from chondrocytes and these proteases are then activated by other SF enzymes such as plasmin, tissue (pancreatic) kallikrein, plasma kallikrein, cathepsin B, cathepsin G or elastase (Eekhout & Vaes, 1977; Werb *et al.*, 1977; Nagase *et al.*, 1982). Thus, recombinant human cytokines would stimulate the release of latent metalloproteases which could not exert any effect on cartilage matrix. Furthermore, our finding that killed RA cartilage, unlike killed normal cartilage, is degraded by some RA SFs might be due to the diseased tissue containing a high level of endogenous latent metalloproteases, so by-passing the need for chondrocyte activation by cytokines. In this respect it may be significant that high levels of metalloproteases have previously been reported in osteoarthritic cartilage (Dean *et al.*, 1989). We are currently investigating the roles of chondrocyte sensitization, metalloproteases and protease-activating enzymes in SF-induced cartilage degradation.

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