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**Research Article**

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# Mechanisms of Disruption of the Articular Cartilage Surface in Inflammation

## Neutrophil Elastase Increases Availability of Collagen Type II Epitopes for Binding with Antibody on the Surface of Articular Cartilage

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### Abstract

We recently observed that specific antibodies to type II collagen do not bind in appreciable amounts to the intact surface of articular cartilage, whereas antibodies to the minor collagen types V, VI, and IX do. These results suggest that the outermost cartilage surface layer prevented interaction of the antibodies with the major collagen type in articular cartilage. The present studies were designed to investigate the pathogenic mechanisms involved in the disruption of the cartilage surface layer in inflammatory arthritis.

Articular cartilage obtained from rabbits undergoing acute antigen-induced arthritis of 72 h duration showed a significant increase in binding of anti-type II antibody to cartilage surfaces compared with normal control cartilage ( $P < 0.01$ ). Augmentation of anti-type II binding was also observed upon in vitro incubation of bovine articular slices or intact rabbit patellar cartilage for 1 h with human polymorphonuclear neutrophils (PMN), PMN lysates, or purified human PMN elastase. This increase was not inhibited by sodium azide, nor was it enhanced by incubation of cartilage with the strong oxidant hypochlorous acid. Chondrocyte-mediated matrix proteoglycan degradation in cartilage explants cultured in the presence of cytokines failed to increase antibody binding appreciably. The augmentation in antibody binding seen with PMN lysates was inhibited by the nonspecific serine-esterase inhibitor PMSF, but not by the divalent metal chelator EDTA. The elastase-specific inhibitor AAPVCMK also inhibited most of the PMN-induced increase in antibody binding, whereas the cathepsin G-specific inhibitor GLPCMK was much less effective. Incubation of intact cartilage with purified human PMN elastase indicated that this serine esterase could account for the increase in anti-type II collagen antibody binding to intact cartilage surfaces. These studies suggest that in an inflammatory response, PMN-derived elastase degrades the outer layer of articular cartilage, exposing epitopes on type II collagen. They also help clarify the pathogenic mechanisms involved in early articular cartilage damage in inflammatory joint diseases. (*J. Clin. Invest.* 1991; 87:1531-1536.) **Key words:** articular cartilage surface • cartilage damage • inflammation • PMN elastase • anti-collagen antibodies

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### Introduction

The outermost layer of articular cartilage confers on this tissue some of its peculiar biomechanical properties, probably including its unusual permeability characteristics to macromolecules (1, 2). Little is known, however, about the physicochemical properties of this important layer. MacConaill (3) described a distinct amorphous surface layer detected by phase microscopy. This observation was interpreted by others to be a film of synovial fluid macromolecules (4) or highly oriented superficial collagen fibers (2). Stanescu et al. (5) described a thin, irregular, granular, electron-dense layer which contained anionic charges. This layer resisted extraction with high-ionic strength reagents, hyaluronidase, chondroitinase ABC, and keratanase, but was partially disrupted by collagenase and was removed by trypsin (6). Moreover, the same group of investigators reported that this superficial layer contained type I collagen in addition to type II, the main collagen type in cartilage (7). Thus, it would appear that the most superficial layer of cartilage may include one or more unique proteins with a peculiar spatial organization. Because of this layer's anatomic location and susceptibility to enzymatic degradation, it would be expected to sustain damage early in the course of any pathologic process in which inflammatory cells appear in the joint cavity. Such damage might then lead to significant changes in the physical properties and nutritional characteristics of articular cartilage, contributing to the development of further sustained injury to this tissue.

In the present studies, we took advantage of our recent observation indicating that specific antibodies to type II collagen, the main collagen type in cartilage, do not bind significantly to the surface of intact articular cartilage, whereas antibodies to the minor collagen types V, VI, and IX do (8). From these observations it was concluded that the outermost layer of cartilage is organized in such a fashion that it obscures the collagen type II epitopes, making them inaccessible to interact with antibodies in the articular cavity. This observation suggests that measurement of collagen antibody binding could be used to detect early and subtle damage to the proteinaceous outer layer, and to study the pathogenic mechanisms involved in the disruption of this layer.

In the present studies, we show that incubation of articular cartilage with polymorphonuclear leukocytes (PMN)<sup>1</sup> or PMN lysates increases binding of antibodies to type II collagen to the cartilage surface. This increase in antibody binding could not be reproduced by exposing cartilage to oxidative injury, or by matrix proteoglycan depletion mediated by activated chondro-

1. *Abbreviations used in this paper:* AAPVCMK, methoxysuccinyl-alanine-alanine-proline-valine-chloromethylketone; GLPCMK, benzoyloxy-carbonyl-glycine-leucine-phenylalanine-chloromethylketone; PMN, polymorphonuclear neutrophils.

cytes. Studies with protease inhibitors suggested that neutrophil elastase was the enzyme mainly responsible for the increase in exposure of type II collagen epitopes at neutral pH. Moreover, increased binding of type II collagen antibodies was achieved by brief incubation of articular cartilage with purified human PMN elastase.

## Methods

**Materials.** Two sources of articular cartilage were used for these studies. Bovine articular cartilage was obtained from the carpometacarpal joints of young steers 1–2 h after slaughter. Rabbit articular cartilage was obtained from the patellas and supracondylar areas of normal New Zealand White rabbits weighing 2–2.5 kg. In the experiments involving intact rabbit cartilage, the patellar bones were excised *in toto*. Special care was taken not to damage the articular surface with the surgical instruments. The supracondylar cartilage pieces were designated as sliced rabbit cartilage throughout this work. The tissues were washed in HBSS without phenol red before use.

Hypochlorous acid was purified by vacuum distillation of a 5% solution of sodium hypochlorite, pH 7.5 (9). Concentrations were determined by spectrophotometric analysis ( $E_{235} = 100 \text{ M}^{-1}\text{cm}^{-1}$ ) (10). Human leukocyte elastase was purchased from Elastin Products Co. (Pacific, MO). The specific elastase inhibitor methoxysuccinyl-alanine-alanine-proline-valine-chloromethylketone (AAPVCMK) and the cathepsin G inhibitor benzyloxy-carbonyl-glycine-leucine-phenylalanine-chloromethylketone (GLPCMK) (11) were obtained from Enzyme Systems (Livermore, CA). Recombinant human interleukin-1 (IL-1) was purchased from Cistron Technology (Pine Brooks, NJ).

**Antibodies.** Type II collagen was purified by the method of Trelstad (12) from bovine articular cartilage. Type V collagen purified from human placenta was a generous gift from Dr. Stephan Gay (University of Alabama, Birmingham). Rat anticollagen antisera were generated by intradermal immunization of specific pathogen-free Lewis rats (Charles River Laboratories, Wilmington, MA) with 100  $\mu\text{g}$  types II or V collagen emulsified in incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). The animals were bled 3–4 wk later, and the sera were separated and stored at  $-20^\circ\text{C}$  until used. Control sera were obtained from rats injected with adjuvant alone. Affinity-purified goat anti-rat Ig was obtained from Tago Inc. (Burlingame, CA).

**Purification of blood leukocytes.** PMN were isolated from heparinized blood of normal human donors by differential centrifugation on Ficoll-Isopaque discontinuous gradients (13). The PMN were washed twice with HBSS without phenol red, and the contaminating erythrocytes were lysed with 0.16 M ammonium chloride solution (14). After three more washes, the cell concentration was adjusted as specified under Results. The cell suspensions contained  $> 92\%$  PMN, which were at least 97% viable as determined by Trypan blue dye exclusion. PMN lysates were obtained from cell suspensions containing  $5 \times 10^6$  cells frozen and thawed three times. The lysates were centrifuged at 2,000  $g$  for 15 min and the supernatants stored at  $-70^\circ\text{C}$ .

**Acute antigen-induced arthritis.** New Zealand White rabbits were immunized with 10 mg crystalline ovalbumin emulsified with 1 ml complete Freund's adjuvant (Sigma Chemical Co.) given intramuscularly and intradermally in several sites in the nape of the neck. 2–3 wk later, the animals were injected in the knee joints with 5 mg ovalbumin in 1 ml sterile pyrogen-free saline solution. The rabbits were sacrificed 72 h later, and the patellas and supracondylar cartilage were excised for incubation with the collagen antisera.

**Experimental protocol.** Bovine cartilage slices were cut with a scalpel into  $4 \times 4$  mm squares weighing 20–30 mg each. Care was taken not to damage the articular surfaces. Quadruplicate tissue explants were washed with large volumes of HBSS for 30 min before incubation with the appropriate reagent at  $4^\circ\text{C}$  in 1-ml-capacity screwcap sterile plastic tubes (Sarstedt Co., Princeton, NJ), or with a suspension of PMN activated with 100 ng/ml phorbol myristate acetate, as indicated under Results, for 1 h at  $37^\circ\text{C}$  with continuous slow tumbling (5 rpm). They

were then transferred to  $12 \times 75$ -mm polystyrene tubes (Falcon; Becton-Dickinson Co., Lincoln Park, NJ), and washed three times for 20 min each with PBS solution, pH 7.2, containing 10% newborn calf serum (Gibco, Grand Island, NY) (PBS-NCBS) at  $4^\circ\text{C}$  with continuous tumbling. The tissues were then transferred back to fresh 1-ml tubes for incubation with 0.5 ml of the rat sera at 1:100 dilutions in PBS-NCBS for 1 h at  $4^\circ\text{C}$ . The tissues were washed again as described above and then incubated with 0.5 ml of a 2–4  $\mu\text{g}/\text{ml}$  solution of affinity-purified  $^{125}\text{I}$ -goat anti-rat Ig, sp act  $5 \times 10^5$ – $10^6$  cpm/ $\mu\text{g}$ , containing 0.1 M NaI, for 1 h. After three additional washes, the bound radioactivity was measured in a Packard Tricarb gamma scintillation spectrometer (Packard Instruments Co., Downers Grove, IL). Tissue wet weights were then obtained so that the results could be expressed as picograms anti-Ig bound per milligram tissue. The rat anticollagen sera used in the present experiments were selected by radioimmunoassay on the basis of comparable titers to their respective collagens and their lack of cross-reactivity with the alternative collagen type (8).

The rabbit patellas were treated in a similar fashion except that incubation with antisera was carried out in a volume of 4 ml. The cartilage was excised from the patellas after the last wash. In some experiments, anticollagen antibody binding to the intact articular cartilage was compared with patellar cartilage sliced before incubation with the antisera.

**Cartilage organ culture.** Quadruplicate bovine articular cartilage explants were cultured for 8 d with culture medium alone, with 100 U/ml interleukin 1 $\beta$ , or with a 10% dilution of a supernatant obtained from phytohemagglutinin-activated human mononuclear cells (15). After the 8-d incubation period, the tissue explants were treated as described above. Controls consisted of cartilage explants kept at  $4^\circ\text{C}$  for the same period of time. Proteoglycan released by the explants was measured by the colorimetric method of Farndale et al. (16).

**Statistics.** Analysis of significance was performed using unpaired two-tailed Student's *t* test.

## Results

Initially, experiments were carried out to investigate the effects of acute arthritis on the availability of type II collagen epitopes on the intact surface of articular cartilage. Acute antigen-induced arthritis was induced in 18 rabbits in three separate experiments. The results shown in Table I compare binding of antisera to collagen types II and V, and control rat sera to intact patellar and sliced supracondylar cartilage obtained from normal and inflamed joints. As previously observed (8), binding of anti-type II collagen antibodies to the intact patellar cartilage surface was not significantly different from that of control rat serum, whereas the binding of anti-type V collagen antibodies was substantially greater. Moreover, sliced normal cartilage

Table I. Effect of Acute Inflammation on Anticollagen Antibody Binding to Rabbit Articular Cartilage Surfaces

Antisera	Cartilage			
	Intact		Sliced	
	Normal	Inflamed	Normal	Inflamed
	<i>pg anti-Ig/mg tissue <math>\pm</math> SEM*</i>			
Collagen type II	6.9 $\pm$ 1.1 <sup>†</sup>	17.9 $\pm$ 3.0 <sup>†</sup>	65.2 $\pm$ 4.0	72.3 $\pm$ 6.5
Collagen type V	16.3 $\pm$ 1.6	17.7 $\pm$ 1.7	49.2 $\pm$ 6.7	101.1 $\pm$ 18.6
Control	9.4 $\pm$ 1.8	6.3 $\pm$ 1.1	6.0 $\pm$ 0.9	9.1 $\pm$ 0.8

\* Results represent means $\pm$ SEM of six joints per group. <sup>†</sup>  $P < 0.01$  normal vs. inflamed cartilage.

bound more anti-type II collagen than anti-type V. If the results are corrected for the amount of antibodies bound to intact normal cartilage, then it can be calculated that roughly twice as much anti-type II as anti-type V was bound, presumably to the cut surfaces of the cartilage slices. The inflammatory insult provided by the acute Arthus arthritis resulted in a significant increase in binding of anti-type II collagen antibodies on the cartilage surface (6.9 vs. 17.9 pg anti-Ig/mg,  $P < 0.01$ ). In contrast to the increase of anti-type II antibody binding to the surface of cartilage obtained from inflamed joints, no significant increase in binding was seen with the sliced tissues from the same joints (72.3 vs. 65.2 pg anti-Ig/mg), suggesting that there was no change in type II collagen epitopes exposed on the cut surfaces of cartilage obtained from inflamed joints. This result also contrasted with that obtained with anti-type V collagen sera. In this case, inflammation caused no increase in antibody binding to the intact cartilage surface, but the binding to the cut surfaces doubled, when compared to the slices obtained from normal joints. These results suggest that resident chondrocytes in the deeper layers of cartilage may have responded to the inflammatory insult by increasing the synthesis of type V collagen.

Because PMN are the principal effector cells of the acute inflammatory response that resulted in increased anti-type II collagen antibody binding, *in vitro* experiments were carried out to determine if isolated PMN could account for the effect seen in the *in vivo* inflammatory response. For this purpose, quadruplicate bovine cartilage slices were incubated for 1 h with increasing numbers of activated human PMN. The results in Fig. 1 show significant increases in antibody binding with as little as  $10^5$  PMN/ml. Maximal increases occurred between  $5 \times 10^5$ – $2.5 \times 10^6$  PMN/ml. The effect of PMN incubation on the increase in collagen type II epitope availability could not be inhibited by sodium azide (results not shown). Similar results were obtained using intact or sliced rabbit cartilage (Table II). In this experiment, binding of antibody to the intact cartilage surface increased fourfold.

One of the pathogenic mechanisms implicated in the process of irreversible degradation of articular cartilage in rheumatoid arthritis and osteoarthritis involves degradation of matrix macromolecules by cytokine-activated resident chondrocytes (15). To investigate if chondrocyte-mediated cartilage degradation results in increased binding of anti-type II collagen antibody to cartilage surfaces, we incubated bovine cartilage ex-

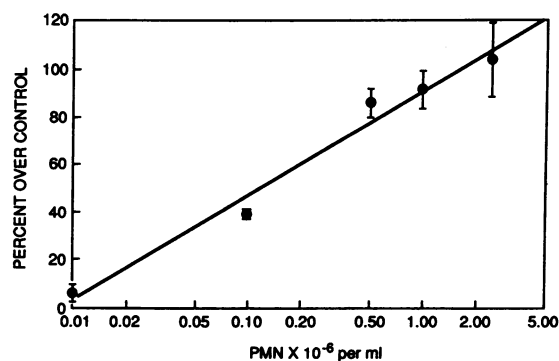


Figure 1. Effect of incubation of bovine articular cartilage with polymorphonuclear leukocytes on binding of anti-type II collagen antibodies.

Table II. Binding of Anti-Type II Collagen Antibodies to Intact and Sliced Rabbit Articular Cartilage: Effect of *In Vitro* Incubation with Human Polymorphonuclear Neutrophils

Cartilage	Incubation with*	
	Control	PMN
	<i>pg anti-Ig/mg tissue</i>	
Intact	9.8	41.2
Sliced	113.3	269.0

\* Intact rabbit patellas or sliced cartilage pieces were incubated with HBSS or with PMN  $10^6$ /ml for 1 h at 37°C.

plants for 8 d with interleukin-1 $\beta$  or a supernatant derived from activated human mononuclear cells. Although the explants incubated with the culture supernatant showed a modest increase in antibody binding, compared with the control explants incubated with culture medium alone either at 4 or 37°C, the differences were not statistically significant (Table III). Both PHA supernatants and IL-1 $\beta$  induced a marked increase in matrix proteoglycan release. Additional evidence for chondrocyte activation was provided by the appearance of caseinase activity in the culture supernatants of the cartilage explants incubated with cytokines (results not shown). These data suggest that the proteases secreted by activated chondrocytes, which degrade matrix proteoglycan and collagen (15, 17), were not able to reach and/or digest the surface layer of cartilage.

The next series of experiments was carried out to identify the PMN-derived factors involved in the observed increase in anticollagen antibody binding to cartilage surfaces. To test whether hyaluronidase or reactive oxygen species might be involved, bovine and rabbit cartilage were incubated with PMN lysates, testicular hyaluronidase, and hypochlorous acid (Table IV). Incubation of cartilage with PMN lysates induced effects similar to those observed with live PMN. The antibody binding increase was particularly impressive with intact rabbit patellar cartilage, in which an almost sixfold increase over control was observed. Incubation with testicular hyaluronidase induced only a marginal increase in antibody binding, and treatment of cartilage with the strong oxidant hypochlorous acid had no

Table III. Anti-Type II Collagen Antibody Binding to Bovine Cartilage Explants: Effect of Chondrocyte Activation

Incubation with*	Antibody bound	Proteoglycan release
	<i>pg anti/mg tissue <math>\pm</math> SEM</i>	<i><math>\mu</math>g/mg tissue <math>\pm</math> SEM</i>
Control 4°C	103.2 $\pm$ 16.4	—
Control 37°C	126.9 $\pm$ 3.6 <sup>§</sup>	5.4 $\pm$ 0.4
PHA supernatant <sup>‡</sup>	150.9 $\pm$ 22.7 <sup>§</sup>	13.2 $\pm$ 0.8 <sup>  </sup>
Interleukin-1	135.4 $\pm$ 11.0 <sup>§</sup>	10.0 $\pm$ 1.1 <sup>†</sup>

\* Quadruplicate cartilage explants were incubated for 8 d with culture medium, 10% PHA supernatant, or 100 U/ml recombinant human interleukin 1 $\beta$ . <sup>‡</sup> From phytohemagglutinin-activated blood mononuclear cells. <sup>§</sup> Nonstatistically significant with respect to control. <sup>||</sup>  $P < 0.001$  with respect to control. <sup>†</sup>  $P < 0.01$  with respect to control.

Table IV. Effect of Polymorphonuclear Neutrophil Lysate, Testicular Hyaluronidase, or Hypochlorous Acid on Binding of Anti-Type II Collagen Antibodies to Articular Cartilage

Incubation with	Cartilage	
	Rabbit	Bovine
	% increase over control*	
PMN lysate <sup>‡</sup>	563.9±163.5	88.2±32.7
Testicular hyaluronidase, 100 µg/ml	42.5±4.0	37.7±9.9
Hypochlorous acid, 1 mM	ND	-28.0±10.9 <sup>§</sup>

\* Controls incubated with HBSS. <sup>‡</sup> Obtained from 10<sup>6</sup> PMN/ml.

<sup>§</sup> Decrease in binding.

effect, in agreement with our previous negative results using sodium azide to inhibit the antibody binding increase mediated with live PMN. These results excluded reactive oxygen radicals and enzymes capable of degrading acidic polysaccharides from consideration as possible factors responsible for the exposure of type II collagen epitopes on the articular cartilage surface, and suggested that proteases active at neutral pH may be responsible for these observations.

This hypothesis was tested by treating the PMN lysate with a series of specific and nonspecific protease inhibitors in an attempt to block the increase in anticollagen antibody binding to cartilage treated with the lysate. As indicated in Table V, the specific elastase inhibitor AAPVMCK markedly inhibited the antibody binding increase mediated by the PMN lysate on both rabbit and bovine cartilage by 84.9 and 75.3%, respectively. Inhibition of metalloproteases with EDTA, or of cathepsin G with the specific inhibitor GLPCMK, failed to reduce binding appreciably, whereas the nonspecific esterase inhibitor PMSF showed an inhibitory effect comparable with that of AAPVMCK.

The role of elastase was confirmed in experiments involving treatment of bovine or rabbit cartilage with purified human PMN elastase. Incubation of intact and sliced cartilage with 1 µg/ml elastase for 1 h significantly increased binding of anticollagen antibodies to cartilage surfaces (Table VI). The increase was particularly impressive in the intact patellar cartilage incubated with elastase, in which a fivefold increase in antibody

Table V. Effect of Enzyme Inhibitors on Binding of Anti-Type II Collagen Antibodies to Articular Cartilage

Inhibitor	Proteolytic enzymes inhibited	Concentration	Cartilage	
			Rabbit	Bovine
			% inhibition±SEM*	
EDTA	Metalloproteases	0.05 M	ND	0
PMSF	Serine-esterases	10 mM	ND	83.2±6.2
AAPVMCK	Elastase	20 µM	84.9±5.8	75.3±2.2
GLPCMK	Cathepsin G	20 µM	31.6±5.4	ND

\* With respect to cartilage incubated with PMN lysate alone. Results represent means of two separate experiments.

Table VI. Effect of Purified Human Leukocyte Elastase on Binding of Anticollagen Antibodies to Bovine and Rabbit Cartilage

Antibody	Elastase	Bovine	Cartilage	
			Intact	Sliced
			pg anti-Ig/mg tissue±SEM*	
Type II collagen	-	108.2±13.8	7.4±1.5	63.7±4.5
	+	184.0±14.9	36.5±8.0	156.9±13.8
Type V collagen	-	81.2±6.7	138.2±22.8	310.3±45.5
	+	151.9±9.4	293.4±13.9	789.3±69.1
Control	-	2.3±0.3	5.8±1.5	8.8±0.7
	+	3.4±0.7	7.5±2.0	19.1±1.3

\* Cartilage slices or intact patellas incubated with 1 µg/ml elastase or HBSS for 1 h at 37°C. The results shown were obtained from three to five cartilage specimens.

binding was observed. This effect is even more marked if corrected for nonspecific binding by control rat sera.

## Discussion

The present studies were prompted by our recent observation indicating that specific antibodies to type II collagen, the main collagen type in articular cartilage, failed to bind in significant amounts to the intact outer surface of this tissue, whereas significantly greater binding to intact articular cartilage was seen with antibodies to the minor collagen types V, VI, and IX (8). This finding suggested that the outermost proteinaceous layer of cartilage might be organized in such a fashion so as to exclude type II collagen antigenic determinants from interaction with antibodies in the joint space. It is unlikely that this observation was due to lack of cross-reactivity of the rat antisera with rabbit type II collagen because the cut surfaces of sliced rabbit cartilage bound significant amounts of antibody (Table I). In fact, if correction is made for the antibody bound to intact cartilage surfaces, then anti-type II collagen bound roughly twice as much antibody as anti-type V. We reasoned that an increase in anti-type II collagen antibody binding to articular cartilage surfaces might be associated with early and subtle damage to the outer layer. The present studies were undertaken to identify the pathogenic mechanisms involved in the disruption of the outer layer of articular cartilage that results in increased binding of type II collagen antibodies to bovine and rabbit articular cartilage surfaces.

The in vivo relevance of this phenomenon was investigated with articular cartilage obtained from rabbit knee joints undergoing acute antigen-induced arthritis of 72 h duration. In these experiments, we used the entire patella as a source of intact cartilage because cut-tissue surfaces would of necessity reveal collagen antigenic sites not normally available for interaction with antibody. When cartilage slices were used, it was assumed that at least part of the observed increase in antibody binding was due to exposure of collagen antigenic sites on the cut surfaces of the tissue. The in vivo experiments with antigen-induced arthritis were consistent with our earlier findings that normal articular cartilage surfaces did not bind appreciable amounts of anti-type II collagen antibodies, but did bind anti-type V collagen (8). In contrast, cartilage obtained from in-

flamed joints showed a significant increase in binding of anti-type II collagen antibodies when compared with control sera. Inflammation did not appear to increase the magnitude of binding of anti-type V collagen, suggesting that damage to the surface layer did not increase exposure of this collagen type. It is also possible that if there were an increase in accessibility of collagen type V epitopes, the proteases in the inflammatory fluid may have partially degraded them. The latter possibility is suggested by the consistent increase in binding of anti-type V collagen antibody to inflamed cartilage slices, which may have been due to an increase in type V collagen synthesis by the chondrocytes in the deeper cartilage layers in response to the inflammatory insult. Alternatively, the type V collagen epitopes on the cut surfaces may have been protected from protease digestion.

While investigating the pathogenic mechanisms involved in the disruption of the cartilage surface that resulted in increased anti-type II antibody binding, we observed consistent increases in antibody binding to cartilage with exposure of the tissues to as few as  $10^5$  PMN/ml (Fig. 1), or to lysates derived from cell suspensions containing  $2.5 \times 10^5$  PMN/ml (results not shown). This finding raised the question of whether one or more PMN-derived factors might be involved in damage to the proteinaceous outer layer.

The increase in antibody binding mediated by live PMN was not inhibited by sodium azide, nor was there an increase in antibody binding upon incubation of cartilage with the strong oxidant hypochlorous acid, which is normally generated by activated PMN (18). These results suggest that reactive oxygen radicals are not among the PMN-derived factors that render articular cartilage more susceptible to binding anticollagen antibodies. Similar negative results were obtained with testicular hyaluronidase. This finding is consistent with the report of Stanescu et al. (6), which indicated that hyaluronidase treatment of cartilage failed to remove the anionic charges present on the articular surface. This report (6) also indicated that trypsin treatment disrupted the proteinaceous layer, and it thus seemed plausible that PMN proteases active at neutral pH might be responsible for the damage of this layer in inflammation. Metalloproteases were ruled out by the inability of EDTA to suppress antibody binding (Table V), and by the failure of resident chondrocyte activation in the organ culture experiments (Table III) to increase antibody binding, in the face of clear evidence of matrix proteoglycan degradation and of increase in neutral proteinase secretion by the activated cells. Marked suppression of the increase in antibody binding by the nonspecific serine esterase inhibitor PMSF implicated one or both of the PMN serine esterases active at neutral pH: elastase and cathepsin G (19). The use of peptidyl chloromethylketone compounds specific for each protease (11) implicated PMN elastase as the enzyme mainly responsible for the observed phenomenon. This conclusion was strengthened by the experiments in which highly purified human PMN elastase induced increased binding of type II collagen antibodies to cartilage.

Elastase is thought to be the most abundant protease in PMN, amounting to 1% of the total cellular protein (20). Moreover, this enzyme has a broad substrate specificity (19), and is able to degrade the terminal, nonhelical peptides and cross-links of type II collagen (21) and cartilage proteoglycan (22). Pertinent to our observations are the findings of Janoff et al. (23). These authors observed that when articular cartilage was incubated with purified PMN elastase, a 10-min lag period en-

sued before detection of proteoglycan digestion products. It was concluded that penetration of the enzyme into the cartilage occurred during this lag period. In the light of the present work, this lag period may be accounted for by the time needed by elastase to disrupt the proteinaceous cartilage surface layer and diffuse into the tissue.

The possible pathogenic role of neutrophil elastase in the mediation of cartilage damage in rheumatoid arthritis is underscored by the studies of Menninger et al. (24) demonstrating the presence of this protease at the pannus-cartilage junction. This enzyme has also been detected in extracts of cartilage obtained from animals with experimental arthritis (25).

Recent studies on the characterization of serine proteases contained in the azurophil granules of PMN indicate that in addition to elastase, another enzyme with similar characteristics may also constitute a major component of these granules (26). It is of interest that this serine protease (proteinase 3 or myeloblastin) has recently been identified as a major autoantigen for the family of antineutrophil cytoplasmic antibodies associated with Wegener's granulomatosis (27). Whether this newly identified protease plays a significant role in the unmasking of collagen epitopes at the cartilage surface remains to be determined.

Anti-type II collagen antibodies are thought to play an important role in the induction of inflammatory arthritis in animal models. A transient synovitis can be observed in rodents after passive transfer of anti-type II native collagen antibodies (28–30). A more sustained arthritis develops when lymphocytes from animals sensitized to collagen type II are infused into naive recipients (31). The present findings suggest that these antibodies may not be able to bind type II collagen on the intact cartilage surface, so that the phlogogenic properties of such antibodies may be due to cross-reactivity with the minor collagen types available at the surface of articular cartilage. Alternatively, the antibodies may induce an as yet unrecognized inflammatory process localized in the synovium, leading to damage of the outer layer of cartilage, and to increase accessibility of collagen type II epitopes for antibody binding. It is also possible that small amounts of antibody are able to bind to intact cartilage surfaces, thus giving rise to an inflammatory reaction which in turn would increase the availability of type II collagen epitopes for binding to more antibody.

Passive transfer of sensitized lymphocytes might induce synovitis by secretion of proinflammatory cytokines, and thus prepare the cartilage surface for increased antibody binding. Anti-type II collagen antibodies have also been shown to exert a remarkable synergistic effect on the development of inflammatory arthritis in rats with Freund's adjuvant-induced disease when injected in amounts that do not induce clinically apparent synovitis when given alone (32). In the light of the present findings, it is reasonable to conclude that the enhanced effects of the passively infused antibodies are due to the unveiling of type II collagen epitopes by the adjuvant-induced arthritis, which results in increased binding of such antibodies to the articular surface. Our findings also support the notion that anticollagen antibodies may not play a primary pathogenic role in human disease. These antibodies have been detected in blood and synovial fluid in a variety of diseases where sustained injury to connective tissue is present (33–37). Their possible modulating effects on chronic inflammatory arthritides might depend on their ability to bind to previously damaged cartilage surfaces (38).

Little is known about the physicochemical properties of the outermost layer of articular cartilage. MacConaill (3) described an amorphous surface layer detected by phase microscopy, the lamina splendens. Other investigators have interpreted these findings as artifacts, either as synovial fluid macromolecules attached to the articular surface (4), or as an optical image created by the presence of highly oriented superficial collagen fibers (2). Stanescu et al. (5, 6) however, showed that the superficial, granular, anionic, electron-dense layer could resist extraction with high ionic strength reagents and polysaccharidases, but was removed by trypsin. The same investigator published evidence that this layer contained both type I and type II collagen (7). It should be pointed out that these studies were done before the characterization of the additional minor collagen types in cartilage (38), so that it is possible that the peptide chains derived from the cartilage surface demonstrated in that work may actually be part of the minor collagen types.

The present studies suggest that the use of immunologic probes such as anti-type II collagen antibodies, or antisera against unique proteins present on the surface of intact articular cartilage, may allow for the detection of early changes of the articular cartilage surface in inflammatory arthritis. Because of this layer's strategic anatomical location, its disruption in inflammatory states may lead to significant changes in the biomechanical properties and permeability characteristics of the underlying cartilage. Biochemical characterization of this structure will be an important step in the delineation of its function.

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