Immunohistochemical Detection and Immunochemical Analysis of Type II Collagen Degradation in Human Normal, Rheumatoid, and Osteoarthritic Articular Cartilages and in Explants of Bovine Articular Cartilage Cultured with Interleukin 1

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Abstract

Articular cartilage destruction and loss of function in arthritic diseases involves proteolytic degradation of the connective tissue matrix. We have investigated the degradation of cartilage collagen by developing immunochemical methods that permit the identification and analysis of type II collagen degradation in situ. Previously, a technique to specifically identify type II collagen degradation in situ in articular cartilage did not exist. These methods utilize a polyclonal antiserum (R181) that specifically reacts with unwound α-chains and CNBr-derived peptides, α1(II)CB11 and α1(II)CB8, of human and bovine type II collagens. The experimental approach is based on the fact that when fibrillar collagens are cleaved the helical collagen molecule unwinds, exposing hidden epitopes. Here we demonstrate the use of R181 in studying type II collagen degradation in bovine articular cartilage that has been cultured with or without IL-1 and in human normal, rheumatoid, and osteoarthritic articular cartilages. Compared to cartilages either freshly isolated or cultured without IL-1, bovine cartilage cultured with IL-1 for 3–5 d showed an increase in both pericellular and intercellular immunohistochemical staining. Extracts of these cartilages contained type II collagen α chains that were increased in amount after culture with IL-1 for 11 d. In addition, culture with IL-1 resulted in the appearance of α chain fragments of lower molecular weight. All human arthritic tissues examined showed areas of pronounced pericellular and territorial staining for collagen degradation as compared with nondiseased tissues, indicating that chondrocytes are responsible in part for this degradation as compared with nondiseased tissues. In most cases rheumatoid cartilage was stained most intensely at the articular surface and in the deep and mid-zones, whereas osteoarthritic cartilage usually stained more in the superficial and mid-zones, but less intensely. Distinct patterns of sites of collagen degradation reflect differences in collagen destruction in these diseases, suggesting possible different sources of chondrocyte activation. These experiments demonstrate the application of immunological methods to detect collagen degradation and demonstrate an increase of collagen degradation in human arthritides and in IL-1-treated viable bovine cartilage.

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Introduction

Most arthritic diseases result in the destruction of articular cartilage with a loss of function. The destruction is due, in part, to the degradation of the extracellular matrix, which is composed primarily of fibrillar type II collagen and aggregating proteoglycans. In articular cartilage, type II collagen fibrils are responsible for the tensile strength whereas the proteoglycans provide the compressive stiffness necessary for normal articulation and function (1). The precise mechanisms by which these connective tissue components are degraded are not fully understood. In mammals, one mechanism involves collagenase, an enzyme capable of a site-specific cleavage of helical (native) collagen (2). Incapable of maintaining a helical structure at physiological temperatures, collagenase-cleaved collagens unwind and become susceptible to further degradation by other proteinases in the extracellular space. In this regard, collagenase can be considered the rate limiting enzyme involved in collagen degradation. A variety of studies have provided indirect evidence that proteolytic enzymes are implicated in the destruction of articular cartilage, although there has been no direct evidence other than morphological (3, 4) that fibrillar collagen is being degraded at the tissue level, such as in cartilage. Limited proteolytic degradation of collagen in cartilage has been demonstrated to result in a loss of the tensile strength of cartilage with negligible release of collagen from the cartilage matrix, measured biochemically (5).

Since the maintenance of tissue integrity is essential to normal function, factors that influence the synthesis, secretion, and activity of proteinases, such as collagenase, are important to examine. The cytokine, IL-1, has been shown to stimulate collagenase synthesis and secretion from a variety of cells including synovial cells, fibroblasts, and chondrocytes (6–8). Even though IL-1 can be produced by different connective tissue cells and cells of the inflammatory response, its relationship to the pathogenesis of connective tissue diseases, such as rheumatoid and osteoarthritis, remains speculative. In vitro, stromelysin or neutral metalloproteinase has been shown to activate collagenase (9, 10): it is also coordinately released with collagenase after stimulation with agents such as IL-1 or urate crystals (10).

One major limitation on our ability to detect and study collagen degradation in health and disease has been the lack of a technique for identifying collagen breakdown in situ. We describe here both immunohistochemical and immunochemical methods that permit one to detect type II collagen degradation. These methods have been used to analyze collagen degradation in human and bovine articular cartilages. They employ an antibody (R181) that specifically reacts with fragmented and denatured human and bovine type II collagen α-chains and does not react with native helical collagens. Also, R181 does not react with bovine type V, VI, IX, and XI collagens, all of which are collagens found in cartilage matrix, nor with any

other molecules in bovine and human cartilage extracts. The studies are based on the premise that when fibrillar collagens are cleaved by collagenase the subsequent unwinding and fragmentation results in the exposure of hidden epitopes that are not exposed in the native molecule. Earlier work (11–14) identified antibody reactive epitopes on unwound collagen α chains. These determinants are usually sequestered in the triple helix and are not detectable until this unwinds. Experimentally, an antibody that only recognizes epitopes on unwound or fragmented type II collagen, provides a tool to study cartilage collagen degradation as a normal function of cartilage remodeling and in pathology. We report here experiments in which type II collagen degradation was demonstrated in situ and analyzed in explants of bovine articular cartilage, cultured with IL-1, and in human articular cartilages from normal, rheumatoid, and osteoarthritic patients.

Methods

Tissue. Bovine condylar articular cartilage was obtained from animals (12–18 mo of age) from a local slaughterhouse shortly after sacrifice. For tissue culture studies, condylar cartilage was aseptically removed from the metacarpal-phalangeal joint. Bovine nasal cartilage and bovine skin were obtained from similar animals for the isolation and purification of types II and I collagen, respectively.

Human articular cartilages were obtained from the femoral condyles of adult knee joints with the following exceptions: radial head cartilage from patient K.E. (33-yr-old female, rheumatoid arthritic, rheumatoid factor negative); tibial plateau cartilage from patient C.P. (73-yr-old male, osteoarthritic). All arthritic cartilages were obtained at surgery from rheumatoid or osteoarthritic patients undergoing total joint arthroplasty or at the time of autopsy within 10 h postmortem. Four osteoarthritic cartilages were examined from patients P.S., 69-yr-old male; V.M., 74-yr-old female; C.P., 73-yr-old male; C.R., 63-yr-old female. All patients were diagnosed as having classical noninflammatory osteoarthritis. Histologically each cartilage had identifiable surface fibrillation. Rheumatoid cartilages were obtained from patients L.M. (65-yr-old female) and D.K. (63-yr-old female) and both were rheumatoid factor positive. Cartilage from two rheumatoid factor negative patients A.D. (59-yr-old male) and K.E. (33-yr-old female), were also examined.

Nonarthritic (normal) cartilages were obtained from four individuals within 10 h postmortem at the time of autopsy, each having no known history nor signs of arthritic/joint abnormalities. The following age-matched cartilages were examined: 73-yr-old male, 61-yr-old male, 56-yr-old male, and 17-yr-old female. All tissues were handled identically and processed for immunohistochemistry, as described below.

Collagen preparation. Type II collagen was extracted and purified from adult bovine nasal septum or adult human femoral condylar cartilage by differential salt precipitation as described by Miller (15). Briefly, the cartilage was minced with a scalpel and twice extracted with 4 M guanidine-HCl in 0.15 mM potassium acetate, pH 6.3 to remove the proteoglycan. The resulting residue was rinsed in water and dissolved in 0.5 M acetic acid containing 1 mg/ml pepsin (Cooper Biomedical, Malvern, PA) for 24 h at 4°C, with stirring. After centrifugation at 15,000 g_{av} for 20 min the supernatant was adjusted to 0.9 M NaCl by slow addition of crystalline salt. The resultant precipitate was recovered by centrifugation and redissolved in 0.05 M Tris-HCl, pH 7.5 containing 1 M NaCl and dialyzed extensively against 0.02 M Na_2HPO_4. The precipitate was redissolved in 0.5 M acetic acid and NaCl was added to a final concentration of 2.22 M NaCl. The precipitated type II collagen was collected by centrifugation and redissolved in 0.5 M acetic acid and exhaustively dialyzed against the same to remove residual NaCl. The collagen was then lyophilized, checked for purity by SDS-PAGE, as described below, and stored at −20°C.

Bovine type I collagen was isolated from bovine skin by differential salt precipitation as previously described (16). Bovine collagen types V, VI, IX, and XI were kindly provided by Dr. David R. Eyre (Department of Orthopedics, University of Washington, Seattle, WA). Each was determined to be pure by SDS-PAGE analysis and used for ELISA analysis. Human type II collagen was a generous gift from Dr. Michel van der Rest (Genetics Unit, Shriners Hospital for Crippled Children, Montreal, Quebec).

Density gradient centrifugation. Growth plate or adult, bovine or normal, human cartilage was dissected into small pieces of 1 mm^3 and extracted with 4 M guanidine-HCl, 0.05 M EDTA, 0.15 M sodium acetate, pH 7.0, containing proteinase inhibitors as described for use in the immunohistochemical protocol. The extraction and subsequent fractionation were performed as described elsewhere (17). The cesium chloride density gradient centrifugation was performed under associative conditions as described, resulting in fractions designated A1 through A6 (nomenclature as described by Heinegård) (18).

Cyanogen bromide cleavage of collagen. Collagens were cleaved at methionine residues with cyanogen bromide (CNBr) using a method based on others (19, 20). Collagens were dissolved in 70% formic acid at a concentration of 5 mg/ml to which was added 12 mg/ml of CNBr. The tubes were flushed with nitrogen, sealed, and kept at 26°C. To ensure the most complete cleavage possible, the reaction was continued for 18–20 h and was terminated by a 10-fold dilution with distilled water. After lyophilization the collagen peptides were stored at −20°C. Purity and characterization were determined by SDS-PAGE.

Antibody production. Female New Zealand white rabbits weighing 2.5 kg (Ferme des Chenes Bleues Inc., Montreal, Quebec) were maintained on rabbit chow (Ralston Purina Inc., Montreal, Quebec). Before immunization, serum was obtained from a preimmune blood sample of 30 ml taken under anesthesia using a combination of ketamine-HCl, (25 mg/kg; Rogar/STP, Inc., Montreal, Quebec) with xylazine (3 mg/kg Hauer-Lockhard; Bayvet Div., Miles Laboratories, Ltd., Rexdale, Ontario) by cardiac puncture.

For the antiserum, R181, the primary immunization, 5 mg bovine type II collagen CNBr-derived peptides in 0.5 ml 0.9% NaCl, 0.01% acetic acid was emulsified with 0.5 ml complete Freund's adjuvant (Difco, Detroit, MI). Half total volume was injected intramuscularly into each hind leg. Booster injections of 1 mg bovine type II CNBr-peptides emulsified with incomplete Freund's adjuvant (Difco) as before were injected either intramuscularly or subcutaneously or both, every 2–3 wk. Each booster was followed 2 wk later by a test bleed and antibody titer determination by ELISA.

After two booster injections the antiserum was of sufficient titer and 50 ml of blood were obtained by cardiac puncture. For immunohistochemical studies a $F(ab')_2$ preparation was prepared by pepsin digestion, as previously described (21). The Fc portion and undigested IgG were removed by absorption to AH-Sepharose-protein A (Pharmacia, Uppsala, Sweden) (22). $F(ab')_2$ was also prepared from normal rabbit serum and was used in immunohistochemical work as a control for the antiserum (R181).

Antisera R167 and R254 were prepared in this laboratory and used in these studies as a positive control for cartilage matrix components. For antiserum R167, the immunogen was fractions A4, 5, 6 of an associative cesium chloride density gradient centrifugation of bovine fetal growth plate cartilage (17). To prepare antiserum R254, fractions A5 and A6 of a similar centrifugation gradient of bovine fetal growth plate were used to immunize the rabbit. Both antisera were prepared by a similar immunization protocol as described for R181, and were shown by ELISA to react with multiple proteins of whole-bovine cartilage extracts (Poole, A. R., unpublished observations).

The mouse monoclonal antibody AN9P1 reacts with keratan sulfate when it is bound to cartilage proteoglycan core protein and has been described previously (23).

Enzyme-linked immunosorbent assay. Purified collagens were dissolved in 0.1 M acetic acid at 2 mg/ml and diluted in 0.1 M carbonate buffer, pH 9.0 to 50 μg/ml. 50 μl was added to each well of a 96-well flat bottom tissue culture microtiter plate (No. 76-032-05; Flow Labo-

ratories, McLean, VA). Where indicated, collagens were denatured after dilution by heating for 15 min at 60°C. Collagens were bound overnight at 4°C to ensure the integrity of native collagens. The assay was continued as previously described (24) with the following modifications. The labeled second step antibody was a porcine anti-rabbit $F(ab')_2$ (21) labeled with alkaline phosphatase (25) that was prepared in this laboratory. The substrate used was disodium p-nitrophenylphosphate (Sigma 104; Sigma Chemical Co., St. Louis, MO) at 0.5 mg/ml in diethanolamine buffer (9.6% diethanolamine, 0.2 mM $MgCl_2$, pH 9.8). Routinely, the plates were incubated with the substrate at 37°C for 1–2 h before reading the optical density at 405 nm. Immune serum R181 was compared in all tests to the preimmune serum of R181.

Electrophoresis and immunoblot analyses. SDS-PAGE of collagens was performed according to Laemmli (26) in 1 mm thick 10% slab gels (16 × 18 cm format) with two exceptions where noted: (*a*) a 7.5–15% gradient gel was used to characterize CNBr-derived peptides of type II collagen, and (*b*) the mini-protean apparatus (7.5% gel, 1 mm thick, 7 cm × 8 cm; Bio-Rad Laboratories, Richmond, CA) was used for α1(II) chain analysis. Collagens in sample buffer containing 0.35 M 2-mercaptoethanol were heated to 60°C for 10 min and centrifuged for 2 min in a fixed speed Eppendorf centrifuge 5313 (Fisher Canada, Montreal) before loading to remove any undissolved material.

Molecular weight standards used were the following ^{14}C-methylated proteins that have approximate molecular weights (kD) as indicated: myosin, 200; phosphorylase b, 93; BSA, 69; lysozyme, 14 (Bio-Rad Laboratories). Gels were stained with Coomassie blue R-250 in 40% vol/vol methanol and 10% vol/vol acetic acid in water or transferred to nitrocellulose. Electrophoretic transfer to nitrocellulose was performed according to Towbin et al. (27) except that the transfer was carried out in methanol-free electrode buffer of 25 mM Tris, 192 mM glycine, pH 8.3. This resulted in a more complete transfer of collagens, since some collagen peptides are not transferred efficiently with methanol present (unpublished observations). The membrane was blocked for 16 h at 4°C with 3% BSA in 10 mM sodium potassium phosphate, pH 7.2, containing 0.145 M NaCl and 0.05% NaN_3 (PBS). After rinsing with PBS for 15 min at room temperature the membrane was incubated for 1.5 h at room temperature with the antiserum (R181) or a mixture of antisera R254 and R167, or preimmune serum, each at 10% concentration after dilution in PBS containing 1% BSA. After exhaustive washing with PBS containing 0.01% Tween 20, the immunoblot was incubated for 1.5 h at room temperature with ^{125}I protein A (Amersham Canada, Oakville, Ontario) (10^5 cpm/ml) in PBS containing 1% BSA. The membrane was then washed thoroughly in PBS, air dried and the radioactivity was visualized by radioautography using X-Omat AR x-ray film (Kodak, Rochester, NY). Routinely, the exposure time was overnight at −70°C with the use of a Cronex lightning plus intensifying screen (Dupont, Wilmington, DE).

Protein sequence analysis. CNBr-derived peptides of bovine type II collagen were electrophoresed in 1 mm thick 10% slab gels as described above. Separated proteins were electrophoretically transferred onto a polyvinylidine difluoride (PVDF)[1] membrane as described by Matsudaira for sequencing small quantities of proteins (28). Briefly, the PVDF membrane is quickly stained with Coomassie Blue R250 after transfer and destained sufficiently to identify the protein bands. For the purposes of peptide identification, three major bands were cut out and their amino acid sequences determined by automated Edman degradation using a microsequencer (model 470A; Applied Biosystems, Foster City, CA). The derivatized amino acids were identified by on line high performance liquid chromatography using an analyzer, (model 120A; Applied Biosystems).

Bovine articular cartilage explant cultures. For culture of cartilage explants, serum-free Dulbecco's modified Eagle's medium (DME-B) (Flow Laboratories) was used with the following additions: BSA (crystallized), 0.1 mg/ml; gentamicin sulfate, 100 μg/ml (both from Sigma); sodium pyruvate, 1 mM; nonessential amino acids, 0.1 mM (both from Gibco Laboratories, Grand Island, NY); 2-mercaptoethanol, 5×10^{-5} M. Bovine metacarpal cartilage was cut into small uniform pieces of ~ 3 mm × 7 mm × 1 mm. Tissue was rinsed, first with BSA-free DME containing gentamicin at 200 μg/ml and fungizone at 5 μg/ml (Flow Laboratories), and then with regular BSA-free DME. Explants were randomly distributed into 12-well, 2.4 cm diameter, Linbro tissue culture plates (No. 76.053.05; Flow Laboratories) with each well containing 2 ml DMEM-B. Human recombinant IL-1β (5 half-maximal units/milliliter) (Cistron, Pine Brook, NJ) was added where indicated. Explants were maintained for up to 14 d at 37°C in a humidified incubator containing 5% CO_2 in air. Medium was changed every 2 d, with fresh addition of rIL-1β to the stimulated cultures. Tissues were removed at intervals, rinsed with PBS, and prepared for immunohistochemistry as described below. Culture media were stored at −20°C until assayed for collagenase activity.

In some experiments, an aliquot of the culture medium was examined for the presence of collagen. Media were concentrated using a Savant Speed Vac (Emerston Instruments, Scarborough, Ontario). The dried material was rehydrated in one tenth the original volume in SDS-PAGE sample buffer, electrophoresed (25 μl per track) and immunoblotted as described for antibody characterization. Antiserum R181 was used to identify the presence of collagen on the immunoblot.

Cartilage extraction. Freshly isolated bovine articular cartilage, and cartilage cultured with or without IL-1, were subjected to extraction with 4 M guanidine HCl in 0.15 mM potassium acetate, pH 6.3 including the proteinase inhibitors as described for use in the immunohistochemical protocol. Equivalent quantities (~ 25 mg) of cultured and noncultured cartilage were cut into small pieces of < 2 mm^3 and extracted in 0.7 ml of the extraction solution, with rigorous shaking for 48 h at 4°C. The residual cartilage was removed by centrifugation for 15 min in 100 *g* and washed with 200 μl of 0.5 M acetic acid. The extracts were prepared for electrophoresis and immunoblotting by removing the guanidine HCl using microdialysis (Bethesda Research Laboratories, Bethesda, MD) against 0.5 M acetic acid. Each dialysate was collected and concentrated by freeze-drying using a Savant speed vac and rehydrated with 150 μl of electrophoresis sample buffer.

Collagenase assay. To measure collagenase activity, acid soluble guinea pig skin collagen was acetylated with [^{14}C]acetic anhydride (10–30 mCi/mmol) generously provided by Dr. Elaine Golds (Joint Diseases Laboratory, Shriners Hospital for Crippled Children, Montreal, Quebec) and used in the standard fibril assay as described by Cawston and Barrett (29). The assay was routinely performed for 18 h at 35°C in the presence of 4-aminophenylmercuric acetate 1 mM (APMA) to activate the latent enzyme. Test media were thawed before assay and centrifuged for 5 min using a fixed speed Eppendorf centrifuge 5313 (Fisher Canada) to remove any cells or debris.

Immunohistochemistry. Cartilage explants were mounted in OCT embedding media (Miles Laboratories, Naperville, IL), frozen and 6-μm thick sections were cut perpendicular to the articular surface at −20°C using a Tissue-Tek II cryostat (Miles Laboratories). Sections were picked up on glass microscope slides, precoated with a solution of 0.5% (wt/vol) gelatin in H_2O containing 1 mM chromium potassium sulfate as described by Pappas (30) to enhance adherence of the tissue sections to the slide.

Sections were either immediately frozen and stored at −20°C or used immediately. Sections of bovine tissue were fixed for 5 min in 4% formaldehyde freshly prepared from paraformaldehyde in PBS, pH 7.4 for 5 min (21), washed in PBS for 15 min and then for 15 min in 0.1% normal donkey serum (Serotec/Daymar Laboratories, Toronto, Ontario) in PBS, to block unreactive aldehyde groups for 15 min. These and subsequent manipulations were at room temperature. To enhance permeability of the tissue, sections were treated with chondroitinase ABC (Miles Laboratories) at 0.0125 U/50 μl per section in Tris-acetate buffer, pH 7.6, for 90 min at 37°C in a humid environment to remove chondroitin sulfate (19). Proteinase inhibitors included during enzyme treatment were: pepstatin (1 μg/ml), PMSF (1 mM), iodoacetamide (1 mM), and EDTA (1 mM). To examine bovine articular cartilage for

1. *Abbreviations used in this paper:* APMA, 4-aminophenylmercuric acetate; PVDF, polyvinylidene difluoride.

the accessibility of the antigen for antibodies (R181), full thickness pieces of normal bovine articular cartilage were incubated in DME with 0.125% (wt/vol) trypsin type XIII (No. T8642; Sigma), 1 mM EDTA, for 3 h at 37°C. The enzyme activity was blocked by washing the tissue for 15 min with 1% normal donkey serum at room temperature. Frozen sections were prepared, fixed, and immunolocalization performed as described.

Sections of human cartilage were fixed for 8 min with 4% formaldehyde in PBS, pH 7.4, then washed and blocked in the same manner as described above for bovine tissue. Keratanase (Miles Laboratories) was added to the enzyme digestion solution for human tissue at 0.025 U/50 μl per section and the digestion was conducted for 18 h at 37°C in a humidified chamber. After enzymatic pretreatment, all tissues were washed twice for 10 min with PBS containing 0.1% BSA.

Sections were incubated for 90 min at room temperature in a humidified chamber with 50 μl/section of R181 $F(ab')_2$ or normal rabbit serum (NRS) $F(ab')_2$ diluted in PBS containing 1% BSA including 0.25% vol/vol NP-40 (BDH Chemicals) at a concentration of 0.1 mg/ml. Controls were prepared by absorbing R181 $F(ab')_2$ before use with 1 mg/ml of denatured type II collagen at 37° for 1 h followed by 18 h at 4°C and centrifugation before use to remove any precipitate. Sections were washed twice as above for 10 min after each antibody incubation. A biotin-streptavidin detection system (Amersham Corp., Arlington Heights, IL) was used consisting of a donkey $F(ab')_2$ anti-rabbit Ig labeled with biotin, and either fluorescein-labeled streptavidin or gold (5–15 nM)-labeled streptavidin as the indicator. The biotinylated donkey anti-rabbit $F(ab')_2$ was used at a dilution of 1/100 in PBS with 1% BSA at 50 μl/section for 1 h. After washing twice, the sections were treated with 50 μl of the fluorescein-labeled streptavidin at a dilution of 1/50 in PBS with 1% BSA for 20 min at room temperature. After washing in PBS, the sections that were stained with fluorescein-labeled streptavidin were counterstained with 0.001% (wt/vol) ethidium bromide in H_2O for 1 min (to counterstain nuclei which fluoresce red) and mounted in ~ 25 μl of a mixture of Tris-glycerol, pH 8.6 (31). Slides were examined for fluoresence immediately with incident illumination using a Zeiss photomicroscope III fitted with a 75-W xenon lamp.

In studies where gold-labeled streptavidin was used, the silver enhancement method of Danscher and Nörgaard (32), as modified by others (Lee, E., and A. R. Poole, manuscript in preparation), was utilized. Thus after sections were incubated with the biotinylated donkey anti-rabbit $F(ab')_2$ and washed as described above, the sections were incubated with gold (5–15 nm)-labeled streptavidin at a dilution of 1/20 in PBS containing 1% BSA for 30 min at room temperature. After washing twice, the slides were dried slowly and held 4°C until next day, or when convenient, before proceeding. To proceed, the slides were dipped in a 0.5% gelatin in water and allowed to air dry. The physical developer was prepared by combining a 50% solution of gum arabic in water (BDH Chemicals, Montreal, Quebec), 6.67 ml citric buffer (2.0 M), 10 ml of 50 mM hydroquinone, and 10 ml of 40 mM silver lactate in the dark. The slides were dipped in this solution for 1 h at 30°C in the dark, after which the slides were flushed with tap water at ~ 40°C for 30 min before exposure to light. The slides were then air-dried and the sections mounted in Permount (Fisher Canada). Using the light microscope the presence of a positive reaction could be visualized as a black to brownish, grain-like staining.

In experiments where the murine monoclonal antibody, AN9P1 was used the ascites fluid containing the antibody was diluted 1/32 in PBS containing 1% BSA and 0.25% vol/vol NP-40, as used for other antibodies. Normal ascites fluid (BRL) was used as a control diluted in the same manner. When AN9P1 was used a second step antibody was needed in order to utilize the biotinylated-donkey anti-rabbit antibody. A rabbit antiserum to mouse IgG, prepared in this laboratory, was used at a dilution of 1/10 in PBS as described above. Reaction was visualized by silver enhancement of the gold-labeled streptavidin as described above.

Results

Characterization of the antibody

The reactivity of antibodies in antiserum R181 with different cartilage collagens was investigated by ELISA analyses (Fig. 1). R181 reacted specifically with denatured (unwound) or

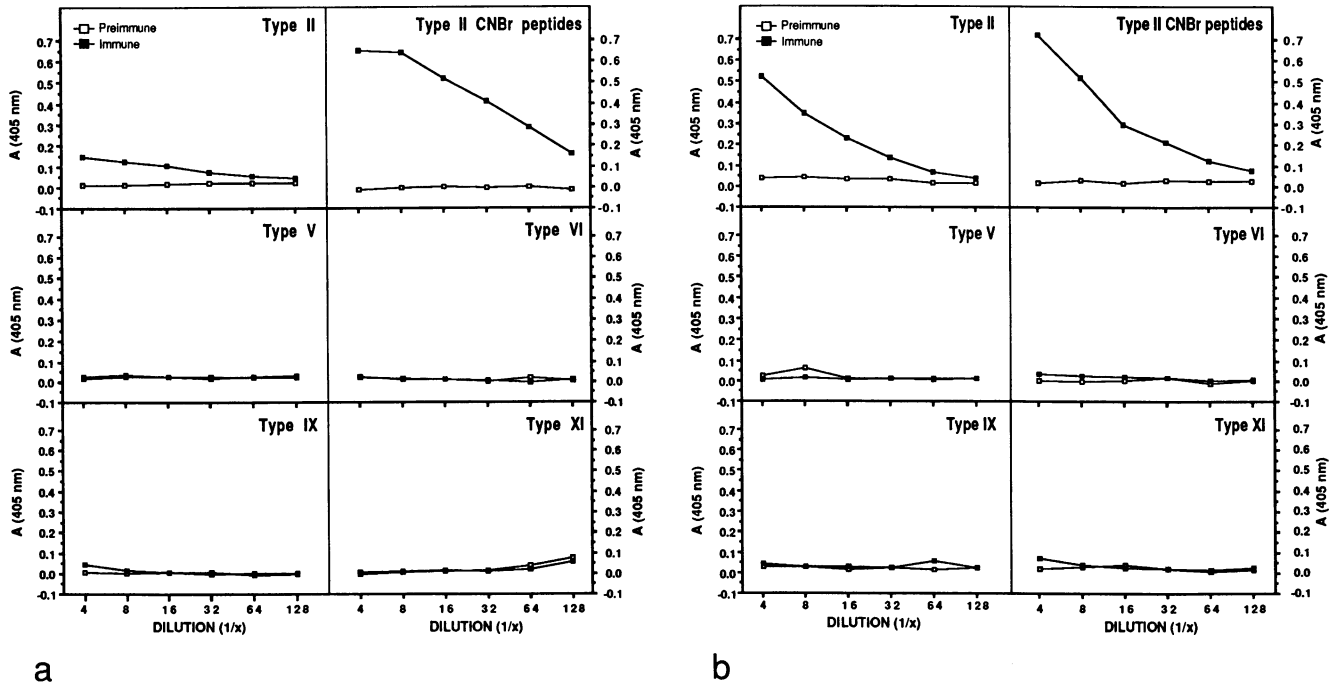

Figure 1. ELISA analyses of antiserum (R181) specificity with purified bovine cartilage collagens. Reaction profiles of R181 are shown (*a*) to bovine CNBr-derived peptides and native bovine collagen types II, V, VI, IX, XI and (*b*) to denatured collagen types II, V, VI, IX, XI.

CNBr-cleaved bovine type II collagen. Antibody reactions were assessed to CNBr-derived peptides of bovine type II collagen and native and denatured bovine collagen types II, V, VI, IX, XI (Fig. 1, *a* and *b*). The strongest reactions were detected to the immunogen (type II CNBr-peptides) and to denatured type II collagen. A small reaction with native type II collagen was observed that probably resulted from the presence of a trace amount of denatured collagen in the type II preparation as a result of purification and handling. This was demonstrated by analysis of type II collagen by SDS-PAGE followed by transfer to nitrocellulose and immunostaining with antibody R181. At even low collagen concentrations, as shown in Fig. 2, a small reaction with material of lower molecular weight was observed. This was not detectable by conventional Coomassie blue staining. There were no reactions with any other collagen tested. Preimmune R181 exhibited no reaction with any of the collagens tested (Fig. 1 *a* and *b*). The reactivity of antiserum R181 with human type II collagen was also determined by ELISA (Fig. 3). Native, denatured, and CNBr-derived peptides of human type II collagen were examined and compared in their reactivity to bovine type II collagen CNBr-derived peptides. Equivalent reactions were seen to human and bovine type II CNBr-derived collagen peptides and denatured type II collagen, whereas in this case there was no reaction with native human type II collagen over and above that shown by preimmune serum, which showed no reaction with any of the other collagens tested.

To characterize further the specificity of R181, purified type I and type II collagens, type II CNBr peptides and whole cartilage extract and fractions thereof separated by cesium chloride density gradient centrifugation, were electrophoresed in a 10% SDS-polyacrylamide gel under reducing conditions, and the separated proteins were then electrophoretically transferred onto nitrocellulose. The transblot was either incubated with preimmune, immune R181 serum or a mixture of R254 and R167, each at a dilution of 10%. As seen in Fig. 4 *a*, preimmune R181 showed no reactivity to any of the proteins examined, whereas immune serum R181 (Fig. 4 *b*) reacted with purified, denatured bovine α(II) chains, and peptides of bovine type II collagen. No reaction was detected with purified bovine type I collagen, nor with any proteins found in the bovine cartilage extract except for a weak band of reactivity detected in fraction 6 of the cesium chloride density gradient, corresponding in size to the α1(II) chains. To demonstrate the presence of other cartilage proteins on the transblot which were unreactive with R181, we incubated the same transblot

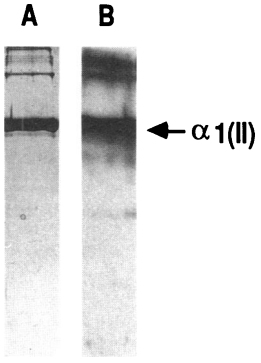

Figure 2. Demonstration of small quantities of immunoreactive fragments of type II collagen in purified bovine type II collagen. Purified bovine type II collagen (5 μg) was characterized in SDS-PAGE (7.5%) using a Bio-Rad mini-protean apparatus. Lane *A* represents the protein stained with Coomassie blue; lane *B* is an autoradiograph of an identical track after transfer onto nitrocellulose and incubation with R181, after detection with ^{125}I-protein A. The immunoreactive β and γ components of higher molecular weight can also be seen.

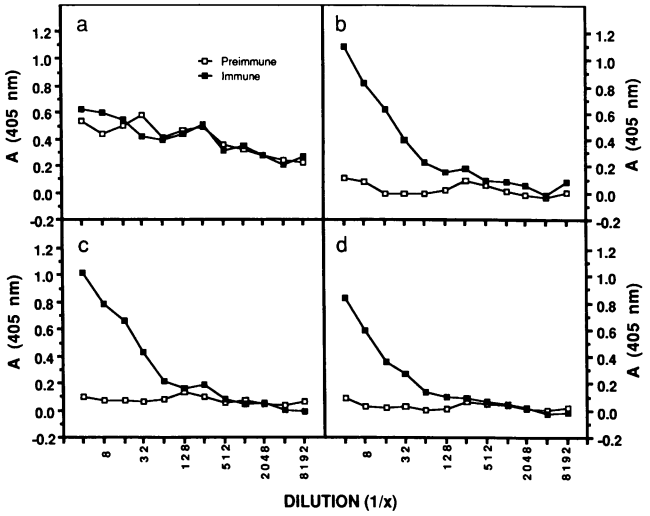

Figure 3. ELISA analyses of antiserum (R181) to demonstrate cross-reaction with human type II collagen. Binding of preimmune and immune R181 to human type II collagens (*a*) native, (*b*) denatured, and (*d*) CNBr-derived peptides are shown. For comparison, binding to the immunogen, bovine type II collagen CNBr-derived peptides (*c*) is shown.

with a mixture of two antisera designated R167 and R254. As can be seen in Fig. 4 *c*, these antisera react with a wide variety of proteins that are present on the nitrocellulose, none of which react with R181 except type II collagen α chains. CNBr-derived peptides of type II collagen, which have epitopes reactive with R181 were identified by their relative molecular sizes using SDS-PAGE analyses and by microsequencing of these electrophoretically separated peptides. In Fig. 4 *b* the major antibody reactive peptides were identified as α1(II)CB8 and α1(II)CB11.

This identification was made possible by comparing a Coomassie blue stained lane of electrophoresed CNBr-derived peptides of α1(II) to an immunoblot of the same stained with the antiserum R181 (Fig. 5). Peptides α1(II)CB8,10,11 were conclusively identified by sequencing the first 10–20 amino acids of the peptides obtained from an identical SDS-PAGE as shown in Fig. 5 and comparing the amino acid sequences with those which have been published (33) (Fig. 6). Two R181 reactive bands, (α1(II)CB8 and α1(II)CB11 were identified by amino acid sequencing and a major band, as seen by Coomassie blue staining, was identified by amino acid sequence as α1(II)CB10. This peptide was clearly unreactive with the antiserum R181 (Fig. 5). Based on previous studies of CNBr-derived peptides of type II collagen (34), the largest peptide is α1(II)CB10. Therefore any peptide species larger than α1(II)CB10 results from incomplete cleavage at methionine residues or the presence of nonreducible crosslinks.

These data demonstrate the specificity of R181 for denatured bovine and human type II collagen and two CNBr-derived peptides of type II collagen. The two major reactive peptides are located well within the helical region of the alpha chain in the NH_2-terminal half of the molecule. Fig. 7 represents a CNBr-derived peptide map of bovine type II collagen based on a review by Fietzek and Kühn (34), illustrating the relative positions of α1(II)CB8, α1(II)CB11, their relative size and the cleavage position of mammalian collagenase. This

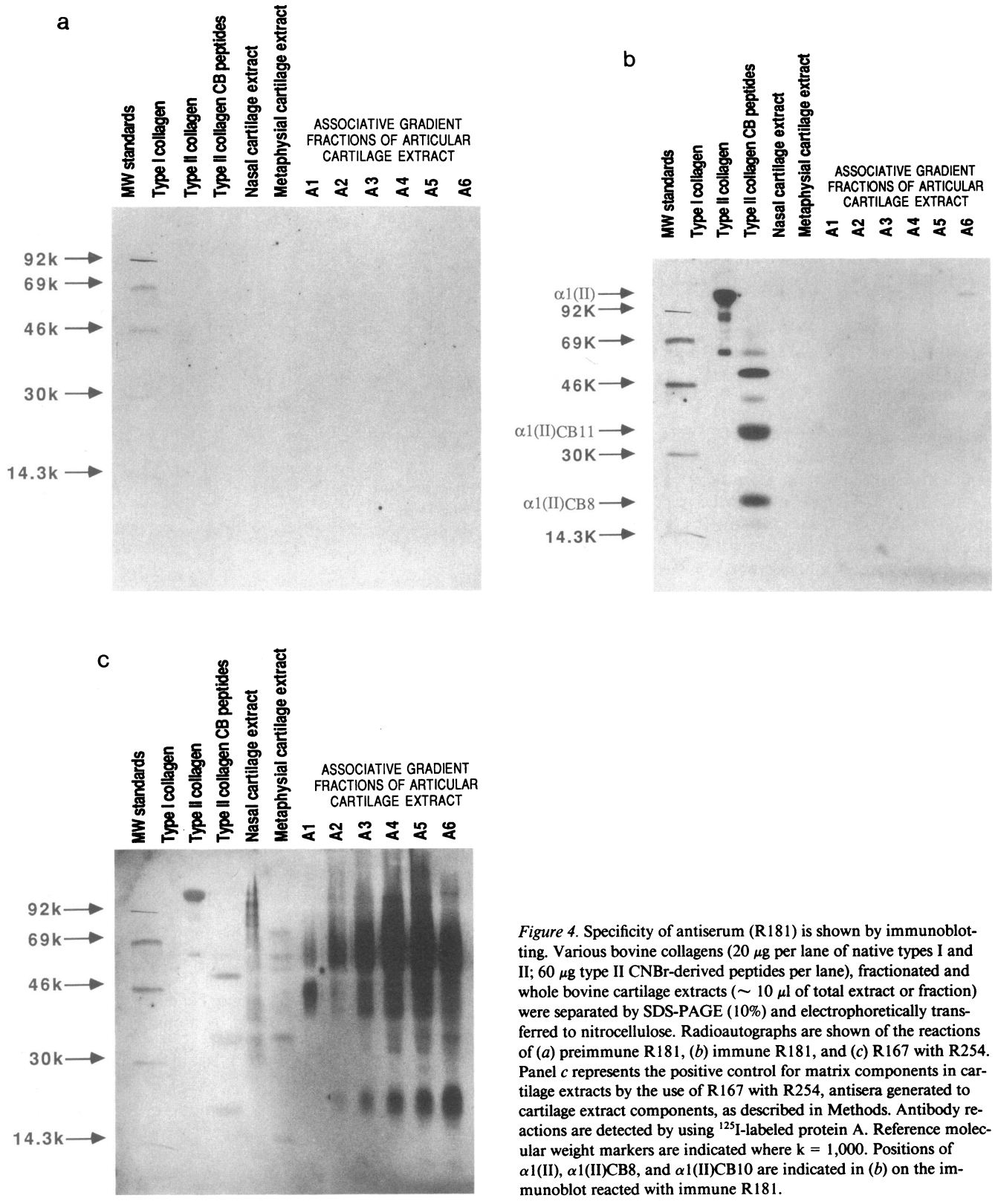

Figure 4. Specificity of antiserum (R181) is shown by immunoblotting. Various bovine collagens (20 μg per lane of native types I and II; 60 μg type II CNBr-derived peptides per lane), fractionated and whole bovine cartilage extracts (~ 10 μl of total extract or fraction) were separated by SDS-PAGE (10%) and electrophoretically transferred to nitrocellulose. Radioautographs are shown of the reactions of (*a*) preimmune R181, (*b*) immune R181, and (*c*) R167 with R254. Panel *c* represents the positive control for matrix components in cartilage extracts by the use of R167 with R254, antisera generated to cartilage extract components, as described in Methods. Antibody reactions are detected by using ^{125}I-labeled protein A. Reference molecular weight markers are indicated where k = 1,000. Positions of α1(II), α1(II)CB8, and α1(II)CB10 are indicated in (*b*) on the immunoblot reacted with immune R181.

demonstrates that the epitopes recognized by these polyclonal antibodies are in the region adjacent to the area of the α chain which contains the cleavage site of mammalian collagenase (35).

R181 reactivity with human and bovine type II collagen CNBr-derived peptides

To ascertain the reactivity of R181 with human type II collagen, α chains, and CNBr-derived peptides thereof and to com-

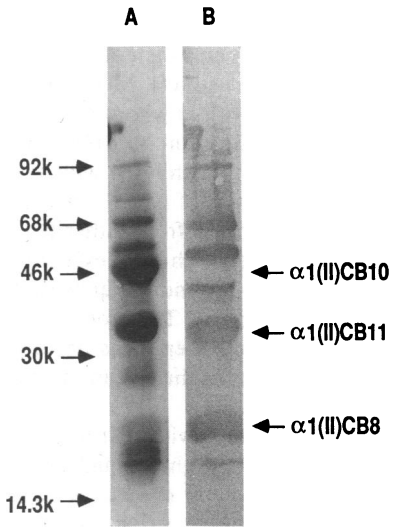

Figure 5. Identification of CNBr-derived peptides of bovine type II collagen. CNBr-derived peptides (60 μg/lane) of bovine type II collagen were separated by SDS-PAGE (7.5%–15% gradient). Lane *A* represents the peptides after staining with Coomassie blue. An identical track was transferred to nitrocellulose and incubated with R181 and ^{125}I-protein A (lane *B*). Peptides identified by microsequencing analysis (Fig. 6) are indicated. Radiolabeled molecular weight markers were used as reference points are indicated.

pare the reactivity with that of bovine type II collagen CNBr-derived peptides, immunoblots of bovine and human type II collagen α chains and CNBr-derived peptides were incubated with 10% R181 and 10% preimmune serum. Fig. 8 illustrates the radioautograph of the immunoblot showing the specific reaction of R181 with bovine and human type II collagen. The peptide reactions are identical and demonstrate the same specificity of R181 for human and bovine type II collagen peptides.

Investigation of the degradation of type II collagen in bovine articular cartilage explants

Collagenase activity in explant media. Media from bovine articular cartilage explant cultures were collected every second day and examined for collagenase activity. Fig. 9 is a representative experiment of five separate experiments showing accumulative collagenase activity measured in medium from explant cultures treated with or without IL-1. Collagenase was progressively released with time over 9 d. All the activity was latent and was detected only after activation with APMA. Little or no active collagenase was ever detected in the medium from any experiment.

Residue	α1(II)CBII	Residue	α1(II)CB8	Residue	α1(II)CB10
124	GLY	403	GLY	552	HYP
125	PRO	404	PHE	553	GLY
126	ARG	405	HYP	554	GLU
127	GLY	406	GLU	555	ARG
128	LEU	407	PRO	556	GLY
129	HYP	408	HYL	557	ALA
130	GLY	409	GLY	558	ALA
131	GLU	410	ALA	559	GLY
132	-	411	ASN	560	ILE
133	GLY	412	GLY	561	ALA
		413	GLU	562	GLY
		414	HYP	563	PRO
		415	GLY	564	HYL
		416	LYS	565	GLY
		417	ALA	566	ASP
				567	ARG
				568	GLY
				569	ASP
				570	VAL
				571	GLY

Figure 6. Amino acid sequence analysis of CNBr-derived peptides of bovine α1(II). Individual peptide bands blotted onto PVDF membranes were cut out and were sequenced from the NH_2-terminal by limited Edman degradation as described in Methods. Presented are the sequence analyses and their corresponding residue number as matched with data from a number of laboratories as compiled by D. Galloway (33).

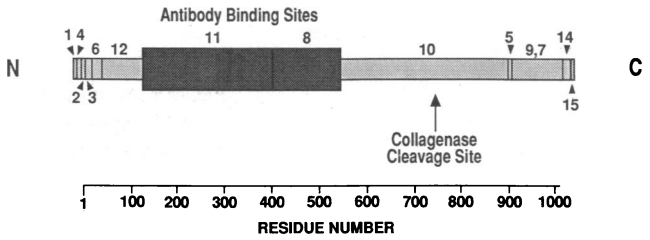

Figure 7. Diagramatic representation of position and relative sizes of CNBr-derived peptides of bovine type II collagen. R181-reactive peptides and the cleavage site for mammalian collagenase (32) are indicated.

Immunohistochemical studies of type II collagen degradation. Bovine articular cartilage was examined with immunofluorescence using antiserum R181 before culture and after various culture periods in the presence or absence of IL-1. In all experiments, tissue reacted with R181 was compared to tissue incubated with normal rabbit serum $F(ab')_2$ (NRS) under identical conditions to establish the specificity of antibody binding. The matrix of these cartilage sections treated

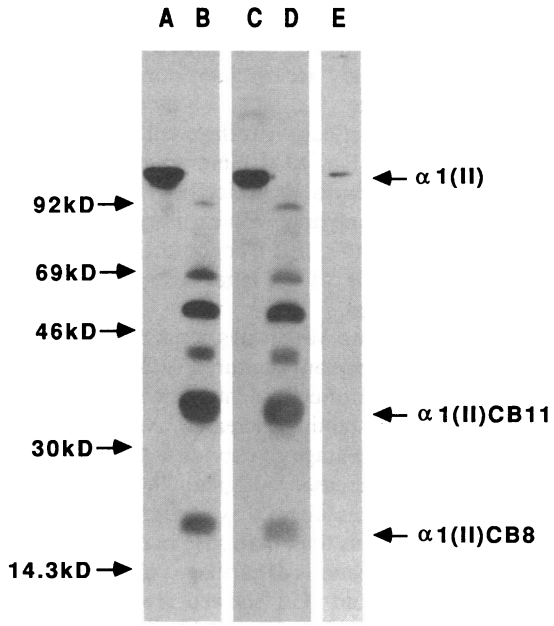

Figure 8. Reaction specificity of antiserum R181 with human type II collagen α chains, CNBr-derived peptides and an unfractionated extract of human articular cartilage. After SDS-PAGE (10%) and electrophoretic transfer onto nitrocellulose, immunoblots were prepared. Shown are radioautographs of immunoblots incubated with immune R181. In lane *A*, 15 μg purified bovine type II collagen; lane *B*, 60 μg bovine type II collagen CNBr-derived peptides; lane *C*, 15 μg human type II collagen; lane *D*, 60 μg human type II collagen CNBr-derived peptides; lane *E*, 10 μl unfractionated extract of human articular cartilage. Molecular weight markers are indicated in kilodaltons. Reaction of R181 has been detected by using ^{125}I-protein A. Positions of α1(II), α1(II)CB11, and α1(II)CB8 are indicated. No reactive bands were observed in an identical immunoblot incubated with preimmune R181 (not shown).

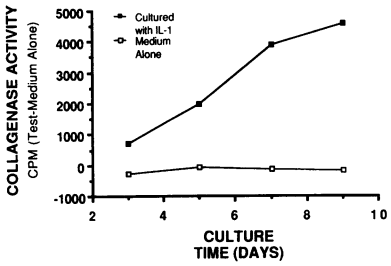

Figure 9. Accumulation of latent collagenase activity in supernatants from bovine articular cartilage explants cultured with and without IL-1.

with NRS F(ab')$_2$ did not stain. Only the cell nuclei were visible due to the counterstain, ethidium bromide (Fig. 10 *a*). When R181 F(ab')$_2$ was preabsorbed with denatured type II collagen before use, staining was considerably reduced demonstrating removal of antibodies to denatured type II collagen α chains.

Sections of bovine articular cartilage that were pretreated with trypsin to remove proteoglycan were stained with R181 in an identical manner with no increase in staining in both those sections from cartilage treated with or without trypsin. Staining with antibody AN9P1 to keratan sulfate of trypsin-treated cartilage was significantly reduced as compared to that observed in nontrypsin treated cartilage. Similar sections of cartilage were stained with toluidine blue and showed a near complete removal of glycosaminoglycans after trypsin pretreatment (data not shown). These observations provide further evidence that there is no masking of collagen epitopes by proteoglycans and that enhanced staining seen with R181 is not due to increased antibody accessibility, nor is it reactive with exposed helical collagen.

When cryosections of articular cartilage cultured for 5 d with IL-1 were examined (Fig. 10 *d*), intense green staining with R181 was observed both in pericellular regions and throughout the matrix as compared with cartilage cultured for the same time without IL-1 (Fig. 10 *c*), or control cartilage that was not cultured (Fig. 10 *b*). In the latter cases only limited pericellular staining was seen.

The most intense staining was always pericellular and was observed in cartilage from explants cultured with IL-1 for 3–5 d. Up until 5 d the number of cells identified with an area of intense broad pericellular staining increased. After day 5, in most experiments, the percentage of chondrocytes with positive surrounding areas decreased, although the pericellular staining that remained was always as earlier, covering a broader zone around the cells than than seen in controls without IL-1. In comparison, sections of cartilage cultured for the same period of time without IL-1 showed fewer cells with pericellular staining (data not shown). Freshly isolated articular cartilage was characterized by weaker and smaller zones of pericellular staining and intercellular matrix staining of low intensity (Fig. 10 *b*). In general, the articular surface was lightly stained in all cartilages. This staining showed some increase with culture time and/or the presence of IL-1. The cut edges in uncultured cartilage were not usually stained, whereas after culture the cut edges stained brightly with R181.

To further control the various steps in the staining protocol, sections were also incubated without the primary antibody F(ab')$_2$ and with the appropriate dilution of biotinylated donkey anti-rabbit or fluorescein-labeled streptavidin. Cartilage sections were not stained when treated with these reagents separately or together (data not shown).

Immunochemical analysis of extracts of bovine cartilage explants and of culture media for the presence of collagen type II degradation products. As a corollary to the in situ detection and demonstration of collagen degradation, we investigated whether or not collagen α chains or fragments thereof could be detected in culture media and cartilage extracts. Media were removed from explant cultures treated with or without IL-1 at days 5, 9, and 11 and examined by immunoblotting with R181. There was no evidence of reactivity with R181 with any protein, smaller than the intact alpha chain of type II collagen from the medium taken at days 5, 9, or 11 from either IL-1 treated tissue or control tissue not treated with IL-1, except in one experiment where a fragment of low molecular weight ($\sim$ 15 kD) was detected (data not shown). Therefore, fragments of collagen-bearing immunoreactive epitopes are not ordinarily present in sufficient quantities in the culture media to be detected by this methodology.

In contrast, extracts of cartilage cultured with IL-1 for 11 d contained an immunoreactive band of a slightly smaller size than an intact α chain that was not detectable in extracts from cultured cartilage without IL-1 after identical periods, or from normal, uncultured bovine cartilage (Fig. 11 *b*). The larger band could correspond to a cleaved or partially degraded α chain initiated by collagenase cleavage (33). This remains to be established. An additional smaller band migrating near the 30-kD globular standard was observed in extracts from cartilage cultured for 11 d with IL-1. This could represent a product of further proteinase action on the α chain, particularly since the R181-reactive epitopes of this fragment are located upstream from the mammalian collagenase cleavage site. These R181-reactive bands were not seen in the control immunoblot which was incubated with preimmune serum (Fig. 11 *a*). Intact type II collagen was extractable from cartilage treated with or without IL-1 at each time point, although more was extracted from cartilage of 11-d-old cultures incubated with IL-1.

Immunohistochemical identification of collagen degradation in human normal, osteoarthritic, and rheumatoid arthritic articular cartilages

Human normal cartilages from healthy joints, osteoarthritic and rheumatoid arthritic articular cartilages were examined for type II collagen degradation using antiserum R181. Patients were undergoing total knee arthroplasty. Normal cartilage was obtained at the time of autopsy within 10 h of death. Each arthritic patient was diagnosed as having advanced forms of osteoarthritis or rheumatoid arthritis and are discussed in Methods. In the case of osteoarthritic cartilages, histological examination of the cartilage confirmed the diagnosis: gross irregularities in the articular surface were seen with fibrillation. In the example shown (patient P.S.), the most striking staining was throughout the regions of the superficial fibrillation (Fig. 12 *b*) and in the interterritorial matrix in the superficial and mid-zones (Fig. 12 *d*). The NRS F(ab')$_2$ incubated tissue did not stain (Fig. 12 *a*). Evidence of pericellular degradation was also seen in the deep zone (Fig. 12 *f*), although this was restricted in this example, to the pericellular areas of the deep zone chondrocytes with little or no intercellular matrix staining elsewhere in this zone. These areas were not stained with the control NRS F(ab')$_2$. Three of four osteoarthritic cartilages examined showed this pattern of staining, while cartilage from patient CR was stained with R181 at the articular surface (not

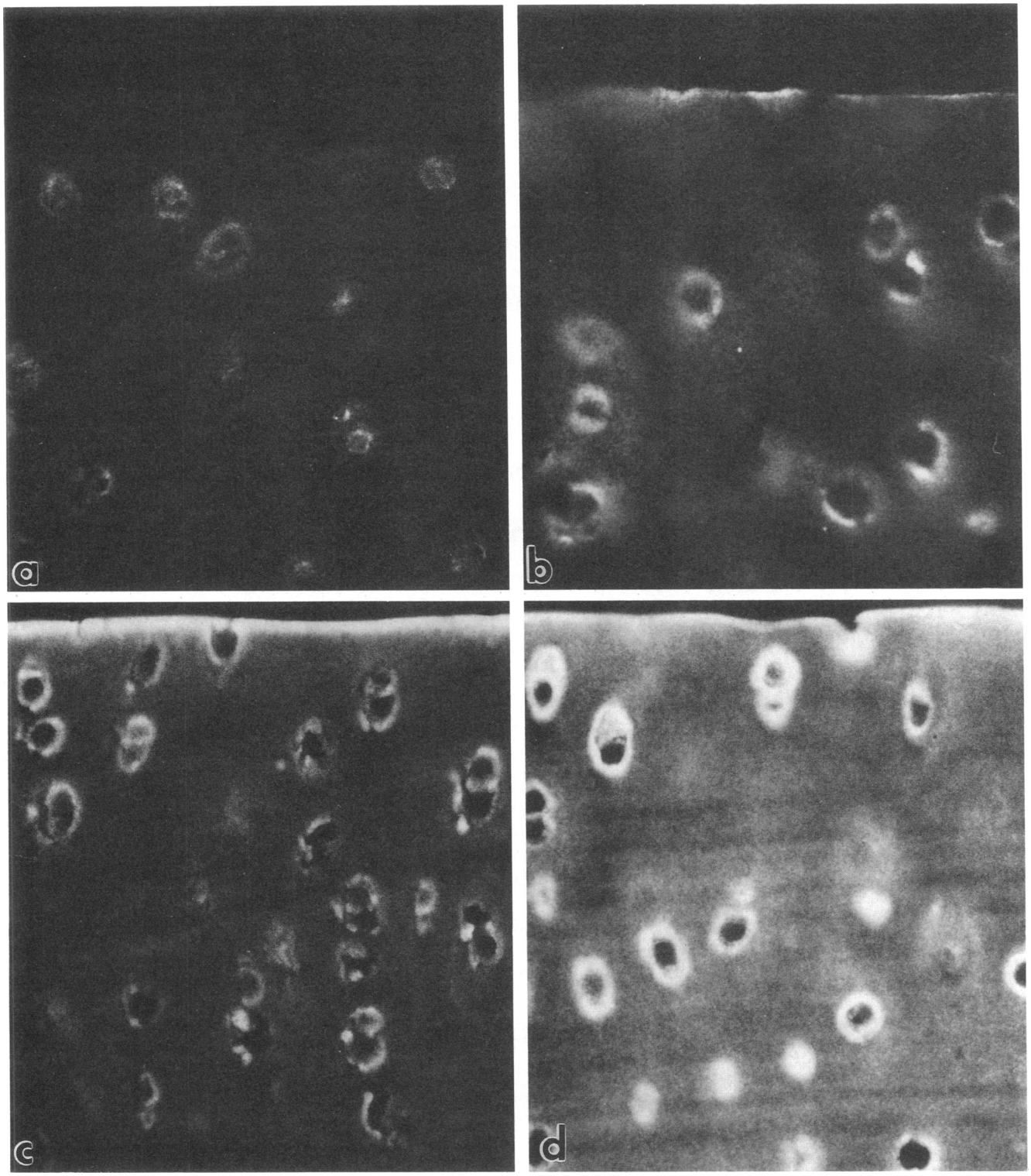

Figure 10. Immunohistochemical identification of type II collagen degradation in uncultured and cultured bovine articular cartilage. Immunofluorescent staining with immune R181 $F(ab')_2$ of bovine articular cartilage, (*b*) uncultured, (*c*) cultured for 5 d, (*d*) cultured with IL-1 for 5 d. As a control for the antibody, (*a*) tissue cultured for 5 d was stained with control (NRS) $F(ab')_2$ (×730).

throughout the superficial zone) and heavily stained in territorial sites around the chondrocytes in the deep zone.

Intense pericellular, territorial and interterritorial matrix staining was detected in all rheumatoid articular cartilages examined. Shown in Fig. 13 is a photomicrograph of articular cartilage from patient A.D. stained with R181 $F(ab')_2$ (Fig. 13 *b*) and control NRS $F(ab')_2$ (Fig. 13 *a*). In contrast to the osteoarthritic cartilage shown, the most striking evidence for

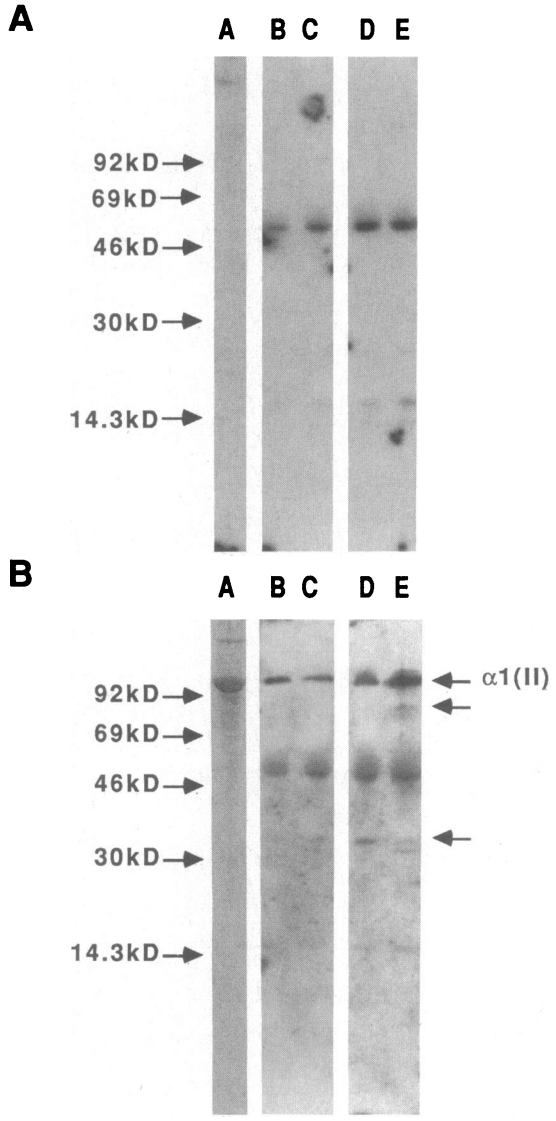

Figure 11. Immunochemical analyses of bovine cartilage extracts after culture with or without IL-1. Guanidine extracts of bovine articular cartilage were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. *A* was incubated with preimmune R181, panel *B* with immune R181. As a point of reference, 10 μg of purified bovine type II collagen was run in lane *A*. Lanes *B* and *C* are extracts of articular cartilage after 5 d in culture without (lane *B*) and with (lane *C*) IL-1. Lanes *D* and *E* are extracts of cartilage after 11 d of culture without (lane *D*) and with (lane *E*) IL-1. Each cartilage extract was loaded at 30 μl representing 20% of the total extract prepared as described in Methods. The position of R181-reactive fragments are indicated on the right by their relative molecular size (80 and 31 kD), along with the position of the intact α1(II) chain. Molecular weight standards were used as described and are indicated in kilodaltons to the left of *A* and *B*.

degradation was predominantly seen in the territorial and pericellular matrix of the deep zones and at the articular surface (Fig. 13, *b* and *d*). The staining was concentrated around the mid and deep zone chondrocytes with intense staining of the territorial matrix. This pattern could be observed in three of four of rheumatoid cartilages studied. Each tissue was unstained with the control nonimmune $F(ab')_2$. All rheumatoid cartilages were intensely stained for collagen degradation and the staining at the articular surface was much more intense than that seen in osteoarthritic cartilages. Moreover, one cartilage specimen (patient K.E.) was stained throughout the matrix, perhaps indicating the active state of the disease. Sections treated with nonimmune $F(ab')_2$ displayed very little staining (Fig. 13, *a* and *c*).

We examined four nonarthritic (normal) cartilages, all of which exhibited weak staining of the articular surface (some of which was also seen in controls treated with NRS $F(ab')_2$. There was limited pericellular staining observed at all levels (Fig. 14).

As a control for accessibility of antibody R181 in cartilage, human rheumatoid articular cartilage (patient A.D.) was stained for proteoglycan using the monoclonal antibody AN9P1 and its staining pattern compared to that of R181. Cartilage sections incubated with AN9P1 were intensely stained throughout the superficial, mid, and deep zones in a uniform pattern. Less staining for proteoglycan was observed in the superficial matrix than elsewhere. In comparison, the staining pattern seen with R181 was as previously described, concentrated at the articular surface and in the pericellular and territorial areas of the deep zone (data not shown). Therefore there is again no evidence to indicate that increased staining with R181 could be due to an increased accessibility of antibody as a result of a local loss of proteoglycan.

Discussion

One of the limiting factors in the study of cartilage degradation has been the inability to detect collagen breakdown in articular cartilage other than by detecting the release of hydroxyproline (36). By using a highly specific polyclonal antibody directed against fragmented and denatured type II collagen, we have developed methods for the detection of collagen degradation in situ produced both experimentally and in human arthritis. Based on earlier work on the immunology and antigenicity of collagens (13, 14, 37–39), a monospecific antiserum was prepared to antigenic determinants on the α chains of the type II collagen molecule which are only exposed upon unwinding of the triple helix (i.e., central determinants). This unwinding can result from cleavage of the collagen molecule either in the triple helix by collagenase (35) or by cleavage at the nonhelical telepeptide ends, such as can be produced by elastase (40).

To generate an antiserum for these studies we used as an immunogen, type II collagen peptides generated by cyanogen bromide cleavage of bovine type II tropocollagen which do not recombine to form helical collagen. Although the immunogenicity of tropocollagen (native or denatured) is often weak, selective antibody responses to central determinants have previously been observed (11).

Even though type II collagen has been identified as the major molecular form of collagen in cartilage (15), cartilage is now known to contain several collagen types. In addition to type II, Eyre and his colleagues have identified types V, VI, IX, and XI, each accounting for 1–2% of the total collagen (41). To rule out any possible cross-reaction with these minor collagens, antiserum R181 was tested in ELISA and shown not to react with the native or denatured forms of bovine type V, VI, IX, and XI collagens. The specificity of R181 for degraded type II collagen coupled with its demonstrated lack of reactivity with other cartilage molecules in bovine and human carti-

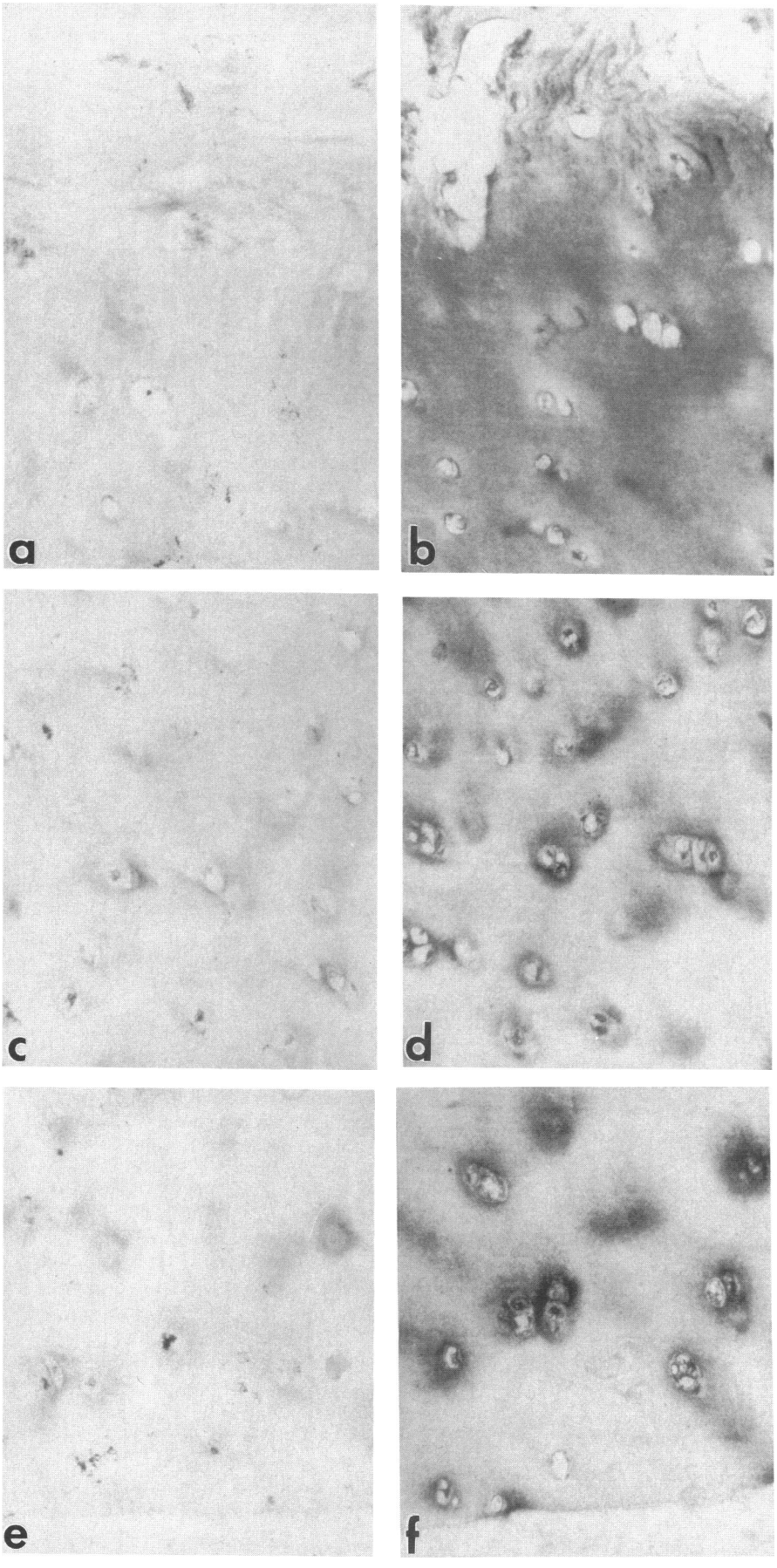

Figure 12. Immunohistochemical identification of type II collagen degradation in human osteoarthritic articular cartilage. Cryostat sections of femoral condylar articular cartilage from a 69-yr-old male (patient P.S.) were stained with NRS $F(ab')_2$ (*a, c, e*) or immune R181 $F(ab')_2$ (*b, d, f*) and with the streptavidin-gold, as described. The superficial zone with a fibrillated articular surface (*a, b*), mid (*c, d*), and deep zones (*e, f*) are shown (×300).

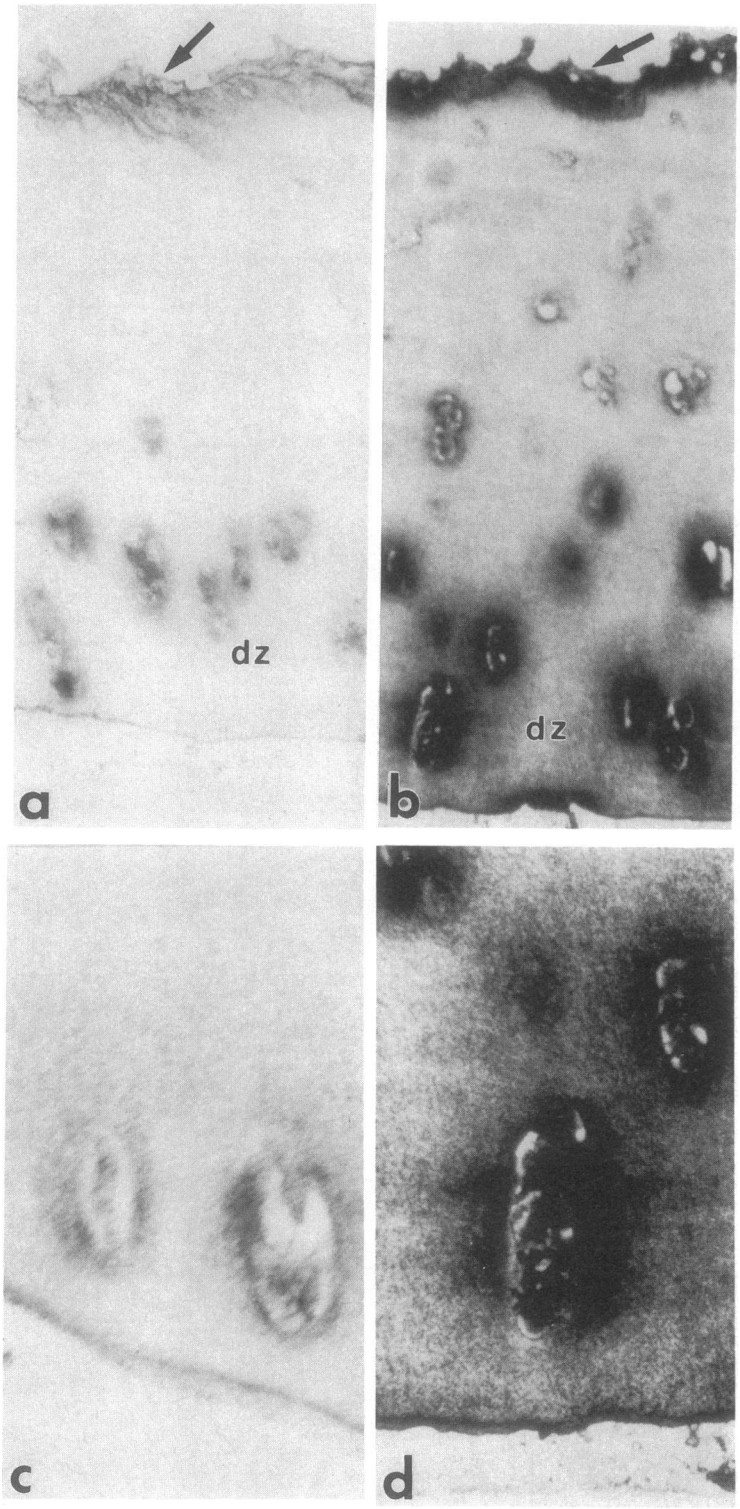

Figure 13. Immunohistochemical identification of type II collagen degradation in human rheumatoid articular cartilage. Cryostat sections of femoral condylar articular cartilage from a 60-yr-old male (patient A.D.) stained with control NRS $F(ab')_2$ (*a*) or immune R181 $F(ab')_2$ (*b*) are shown (×300). *c* and *d* are high power magnification (×730) micrographs of the deep zone cartilage of *a* and *b*, respectively. Antibody binding was as described in Fig. 9. The articular surface (*arrows*) and deep zones (DZ) are indicated.

lage makes it possible to detect specifically the degradation of type II collagen.

The identification of the location of the reactive epitopes on the collagen alpha chains was made possible using CNBr-derived peptides, immunoblotting and microsequencing. The major immunoreactive peptides, α1(II)CB8 and α1(II)CB11, are located well within the helical region and are adjacent to the region of the molecule with the collagenase cleavage site (Fig. 7), where the initial disruption of the collagen molecule is thought to occur. The higher molecular weight fragment of type II collagen α chain identified in extracts of bovine cartilage undergoing collagen degradation induced by IL-1 which is similar in size to the three-fourths length collagenase cleavage product (35) implicates more directly mammalian collagenase in type II collagen degradation.

The experimental studies of cartilage degradation induced by IL-1 served to demonstrate for the first time the degradation of type II collagen in situ. We have shown that treatment of

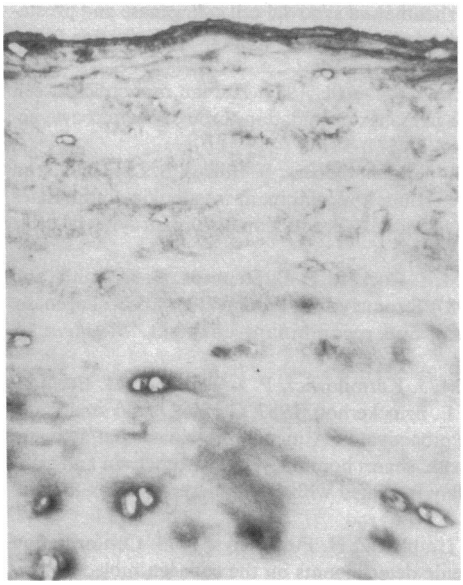

Figure 14. Immunohistochemical identification of type II collagen degradation in normal (nonarthritic) articular cartilage from the femoral condyle of a nonarthritic 73-yr-old male. Shown is a photomicrograph of cartilage reacted with R181 as described in Fig. 9 (×300).

chondrocytes with IL-1 (in explant culture) stimulates the release of collagenase and that this is accompanied by collagen degradation, mainly around chondrocytes.

Both normal bovine and human adult articular cartilage exhibit low level staining for degraded collagen particularly around chondrocytes in pericellular sites. Since cartilage, like most connective tissues, is constantly undergoing turnover and remodeling for proteoglycans and one would presume the pericellular staining may reflect basal collagen turnover, or represents the persistence of previously degraded collagen. In all four normal human cartilages examined, the matrix in the superficial zone is also lightly stained. This suggests that there may be a more active process of collagen remodeling of the articular surface or that collagen degradation per se has taken place at or near the articular surface.

A reduction in staining is seen in bovine cartilage explants treated with IL-1 at and after 9 d and suggests that further degradation is accompanied by a loss of detectable reactive epitopes. This may be due to epitope destruction (by other proteases) or a loss of reactive epitope on α chain fragments into the culture medium. The observation, however, that immunoreactive fragments of collagen are detectable in extracts of cartilage at 11 d of culture, but not at 5 d when immunohistochemical staining was most intense, demonstrates that the intensity of staining does not necessarily reflect the amount of collagen degradation. This must reflect changes in the accessibility of epitopes to antibody in situ, probably as a result of matrix remodeling during degradation.

Our studies of collagen degradation in human cartilages revealed that arthritic cartilages exhibit much more evidence of collagen degradation than the corresponding normal adult cartilages. The staining of degraded collagen observed in arthritic cartilage did not correspond to any loss of staining observed for proteoglycan even though loss of proteoglycan may coincidentally occur under these conditions. Also, staining with R181 for degraded collagen was independent of a loss of proteoglycans or glycosaminoglycans and there was no evidence for staining of intact helical collagen.

In the human cartilages examined, differences in staining of degraded collagen were not only in intensity, but also in distribution. In three of the four osteoarthritic cartilages examined, staining was observed mainly in the superficial and mid-zones, often where fibrillation is occurring, with intense staining in both pericellular sites and moderate staining in territorial sites that may vary in location depending upon the joint pathology of each patient. The pericellular and strong diffuse staining associated with fibrillated superficial cartilage clearly indicates collagen degradation in these sites and explains in part the appearance of fibrillated cartilage.

In contrast, in rheumatoid cartilages there was very intense staining of the articular surface. This collagen degradation may be caused by proteolytic enzymes originating from the synovial fluid which are capable of degrading collagen. Obvious candidates are the collagen-degrading proteases, elastase and cathepsin G (40, 42), which can be released by polymorphonuclear leukocytes upon attachment to articular cartilage which results in the degradation of the more superficial articular cartilage (43, 44). In one specimen (K.E.), degradation was detected throughout the matrix. In the others it was concentrated in the deep and mid-zones. Degraded type II collagen was detected around chondrocytes in pericellular and territorial sites in the deep zone close to the calcified cartilage. Most rheumatoid cartilage was examined from sites at or close to the cartilage-synovial junction. The data indicate that not only may type II collagen degradation occur at the cartilage-synovial junction, associated with mononuclear cell infiltration and pannus formation (3, 45–47), but that it also occurs around the chondrocytes more remote from the articular surface and remote from the synovium and synovial fluid. Earlier, ultramorphological studies of rheumatoid articular cartilage, using cationic dyes, also showed that proteoglycans were lost and collagen was damaged in the deep zone cartilage (4). Since much of the degradation in human cartilage was centered around cells, the chondrocyte is clearly implicated as the primary source of enzymes degrading the type II collagen, as in bovine cartilage.

We have shown in bovine cartilage that molecules such as IL-1 can diffuse into bovine cartilage and activate chondrocytes to degrade the collagen matrix throughout the matrix. The degradation of collagen around and between cells appear to result from chondrocyte activation resulting from cytokines, such as IL-1. In addition to such cytokines originating from synovium, pannus, and synovial fluid (48), they could also arise from the inflamed subchondral bone marrow. Inflammatory changes are well recognized in subchondral sites in rheumatoid arthritis and are associated with bone resorption and loss which can result from the action of cytokines such as IL-1 and tumor necrosis factor (49–53). Cellular infiltration from subchondral bone into cartilage has also been observed in rheumatoid arthritis (54–56). Earlier studies by Fell and co-workers demonstrated that stimulation of subchondral bone marrow with complement sufficient antiserum can cause degradation of the adjacent articular cartilage (57, 58). Our observations thus indicate that degradative changes occurring in the deep zone cartilage may be initiated by the release of cytokines released from inflammatory cells not only in the joint cavity but also from underlying inflamed subchondral marrow cavities.

Degradation of type II collagen can lead to the exposure of α chains or fragments thereof previously hidden or sequestered from the immune system. These exposed or fragmented α chains may be recognized by the immune system, in particular by T cells (48). We and others have already demonstrated that denatured type II collagen or fragments thereof are preferentially recognized as autoantigens by T cells over helical collagen by patients with rheumatoid arthritis (59, 60). Moreover, denatured type II collagen injected into rats and mice can elicit the development of erosive inflammatory joint disease resembling rheumatoid arthritis (48, 61, 62). This involves the expression of T cell mediated immunity to the collagen as well as antibody production. Injection of T cell clones that recognize both helical and denatured type II collagen can induce a rheumatoid-like polyarthritis (63). T cell mediated autoimmunity to type II collagen, in particular to the degraded molecule could therefore arise in patients with rheumatoid arthritis as a result of the release of immunologically active collagen fragments produced by collagen degradation and may play a pivotal role in the immunopathology of these diseases.

There is a large body of evidence that would link both collagen and proteoglycan-degrading enzymes to the loss of integrity of articular cartilage in arthritic diseases (48). The study described here has permitted the identification of cartilage collagen degradation in experimental and human pathology and has produced important observations and raised questions concerning the pathology of these diseases. Further studies utilizing these techniques should help to elucidate the destructive process involved and the mediators that regulate the control of cartilage destruction.

Acknowledgments

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