Treatment of Collagen Induced Arthritis by Proteolytic Enzymes: Immunomodulatory and Disease Modifying Effects

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ABSTRACT. ***Objective.*** To investigate the efficacy of a novel therapy (proteases) in an animal model of rheumatoid arthritis, and to investigate the mechanisms of arthritogenesis.

Methods. We induced progressive arthritis in male DBA/1 mice by immunization and boosting with Type II collagen; groups of mice were treated orally twice daily with either ibuprofen or proteases, or were left untreated. After 2 weeks, joints were scored for clinical, radiographic, and histologic changes. In addition, we measured serum levels of IgG anti-collagen II, the glycosylation of circulating total and anti-collagen II IgG, and cytokine production by lymphocytes isolated from lymph nodes.

Results. Amelioration of joint inflammation, and accentuation of a prototypical Th2 cytokine (interleukin 5) were similar in the ibuprofen and protease treatment groups. However, protease treatment protects and preserves articular cartilage, normalizes the sialylation of IgG and anti-collagen antibody, and fully restores Th1 (interferon-γ) synthesis, distinct from ibuprofen.

Conclusion. Protease therapy has antiinflammatory efficacy in the early (inflammatory) phase of collagen induced arthritis, similar to ibuprofen. The immunomodulatory effects of proteases, not seen with ibuprofen, may underlie a correction of aberrant IgG glycosylation and/or contribute to the increased capacity of protease to delay or forestall erosive and destructive arthritis or ankylosis. Similar effects may apply to spontaneous RA in humans. (J Rheumatol 2001;28:2049–59)

Key Indexing Terms:
AUTOIMMUNITY RHEUMATOID ARTHRITIS CYTOKINES
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Rheumatoid arthritis (RA), a progressive polyarticular disease characterized by acute inflammation that occurs in roughly 1% of the worldwide population, continues to present therapeutic challenges, despite considerable recent progress[1-5]. Although definitive evidence is still lacking and specific mechanisms and recognition elements are controversial[6], most investigators consider RA to be an autoimmune disease instigated by autoreactive T cells and/or autoantibodies[1-5]. In rodents, a progressive and episodic arthritis that severely involves the carpal and tarsal joints can be instigated by immunization and boosting with type II collagen[7-13]. Such collagen induced experimental arthritis (CIA) has proved useful for study of several aspects of the clinical disease. In the murine model, restricted T cell receptor (TCR) usage in CIA, the linkage of genetic restriction of disease susceptibility to TCR and MHC genes, and the capacity to adoptively transfer T cells to induce CIA in otherwise unmanipulated nonimmunized recipients strongly implicate T cells as the causal autoreactive component of the immune system[8-13]. Recently, the role of specific T cell immune responses and especially the oligoclonal restriction of TCR usage reported in spontaneous human arthritis[14-20] has been called into question[6,21]. Nonetheless, many features of the murine model do correspond well to clinical RA. Among these, the effect of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) in humans[22-26] applies also to the murine model[27-29]. Similarly, the well documented alterations in Ig glycosylation in RA[30-34] are mirrored in the animal model[35,36].

Nonsteroidal antiinflammatory drugs (NSAID) form the cornerstone of therapy of RA. While these agents are typically effective in relieving pain and reducing inflammation, they are not always sufficient; moreover, much of the excess mortality observed in patients with RA derives from complications arising from the considerable toxic effects of such therapy[1-5,37-39]. Also, such agents have limited capacity to modify disease and thereby spare articular cartilage.

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These limitations have increasingly instigated the use of a variety of cytotoxic drugs and/or biologicals, so-called disease modifying agents, which themselves may carry significant toxicity[1-5,37-39]. The efficacy of NSAID in CIA is controversial; although some investigators reported reduced inflammation, others claimed no effect[40-54]. Part of the controversy arises from the different variables of disease measured or stressed, and/or the strain or species of experimental animal tested. Clearly, however, nonsteroidals do not influence the ankylosis or bony destruction that characterize the later phases of this disease. Therefore, the nonsteroidals are not generally considered to be disease modifying agents. Further, little or no information exists regarding the effects of either NSAID or cytotoxic therapy on the autoimmune response, cytokine production, or Ig glycosylation in RA, although such immunological variables may be central to pathogenesis of the disease[22-39].

There is a growing literature documenting the efficacy of proteases for therapy in a wide range of inflammatory diseases, especially glomerulonephritis and arthritis[55-62]. Preliminary clinical trials indicate efficacy of a commercial formulation of proteases with an antioxidant, Phlogenzym®, in RA[63-66]. Moreover, in addition to its antiinflammatory effects[67], this commercial formulation of proteases exerts considerable and significant effects upon several critical immune functions[68,69].

We assessed the efficacy of proteases in the early (inflammatory) phase of the CIA murine model of RA, and determined if such therapy modifies elements of the immune response in this model. In this latter regard, we focused upon the serum titer of antibody specific for allogeneic collagen II, Ig glycosylation, and T cell-derived cytokine production.

MATERIALS AND METHODS

Induction and treatment of arthritis. Collagen type II (Col II) purified from chicken sternal cartilage (Sigma, St. Louis, MO, USA) was dissolved at 2 mg/ml in 50 mM aqueous acetic acid by overnight incubation with agitation at 4˚C. In 3 independent experiments, a total of 57 male DBA/1 mice (Jackson Laboratory, Bar Harbor, ME, USA), 5 weeks old and weighing 18 g, were immunized by a subcutaneous injection at the base of the tail of 0.1 ml of a "hard" emulsion prepared from equal volumes of aqueous Col II and complete Freund's adjuvant (Gibco BRL/Life Technologies, Gaithersburg, MD, USA). On Day 21 post-priming, a "hard" emulsion was prepared from a fresh solution of 2 mg/ml Col II in 50 mM aqueous acetic acid and an equal volume of incomplete Freund's adjuvant (Gibco), and 0.1 ml of this emulsion was injected subcutaneously into the tail of each immunized mouse[7,8]. In each of the 3 experiments, another 5 or 6 male age matched mice, entirely unmanipulated, were maintained as a control group (total n = 17). All animals were given free access to food and water at all times, and were housed in AAALAC approved facilities.

On Day 28 post-priming, clinical scores were recorded for all mice (see below), and immunized mice were randomized into 4 groups. One of the groups of immunized mice was given 0.5 ml phosphate buffered saline (PBS), a second received 0.5 ml of 4 mg/ml ibuprofen (Whitehall Laboratories, Madison, NJ, USA) in PBS, and a third received 0.5 ml of 4 mg/ml of a commercial preparation of proteases with the antioxidant rutosid (Phlogenzym®, MUCOS Pharma GmbH, Geretsreid, Germany) in PBS, all delivered by orogastric intubation twice daily for 2 weeks (Days 28–41 post-priming). The remaining immunized mice and the nonimmunized controls were left untreated.

Evaluation of arthritis. At 28, 35, and 42 days after priming, each foot from each mouse was evaluated for swelling and redness to arrive at a clinical score, modified from previous reports[7-9,12]. Specifically, redness and swelling were each scored separately as 0 (not present), 1 (equivocal or weak), 2 (definitely present), or 3 (marked and severe) for each paw. The redness and swelling were generally, but not always, congruent. The sum of redness and swelling for each individual paw (range from 0 for normal to 6 for severely swollen and markedly erythematous) was added for all 4 paws to arrive at the "clinical score," which therefore ranged from 0 (normal) to 24 (severe redness and swelling in all 4 paws). In addition, selected feet from each mouse were measured in the transverse and sagittal planes with a tensioning micrometer (Fowler Instruments, Dearborn, MI, USA). One investigator, blinded to the treatment groups, performed all redness, swelling, and micrometer measurements. After sacrifice (Day 42 post-priming) by exsanguination under ether anesthesia and harvesting of serum samples from each mouse, the peripheral (axillary, brachial, cervical, and inguinal) lymph nodes were dissected from some (70%) randomly selected mice. In addition, after sacrifice, all mice were placed onto X-OMAT TL Ready-Pack film cassettes (Eastman Kodak, Rochester, NY, USA) and exposed to a 40 kV x-ray beam (Faxitron X-ray Corp., Buffalo, NY, USA) for 30 s. Finally, each limb from each mouse was disarticulated from the axial skeleton, and all skin and soft tissue was removed from each foot. The plantar and dorsal surfaces of each foot were scored 20 times each with a scalpel over the tarsus, metatarsal phalangeal, and interphalangeal joints with a scalpel to allow penetration of fixative. Similarly, the capsule overlying the femorotibial joint was nicked with the point of the scalpel both medially and laterally and scored on the extensor surface to promote fixation. All tissue samples were fixed in 10% buffered formalin for 24 h, rinsed in water for 30 min, and then decalcified (Decalcifier II®, Surgipath Medical Industries, Richmond, IL, USA) for ~60 min before embedding into paraffin (Paraplast®, Fisher Scientific). Paraffin blocks were step-sectioned at 4 μm on a microtome (American Optical, Buffalo, NY, USA) and stained with hematoxylin-eosin and a combined periodic acid-Schiff/Alcian blue; a few of the blocks were surface-decalcified by immersion of the blocks in Decal II for up to an additional 10 min, as required.

To prevent observer bias, radiographs and histologic sections were coded before examination. From radiographs examined under a magnifier, the joints among the carpal, metacarpal, and phalangeal bones, and the corresponding joints of the metatarsal bones of the hind limb, were scored for width of the joint space, loss of periarticular bone, formation of cysts within the bone, and irregularities in periarticular bone contour[27]. Histologic sections were graded[7-9,12,28] in blind fashion by 2 pathologists, including one with extensive experience in bone and joint diseases. The histologic score represented the arithmetic mean of the 2 pathologists' grading of the most severe change evident in at least 2 joints in each foot, with 1 representing inflammation, 2 denoting well developed pannus formation, 3 indicating erosion of cartilage without bony changes, 4 marking definite bony erosion and/or bone cyst formation, and 5 indicating destruction of the articulation. Morphometric evaluation of the articular cartilage employed a digital image analysis system, consisting of a Dage high resolution video camera (Dage MTI, Indianapolis, IN, USA) fitted to an Olympus BH-2 optical microscope (Olympus America, Rockleigh, NJ, USA). The camera signal was fed to a Centris 650 computer (Apple Computer, Cupertino, CA, USA) via a Video Spigot (SuperMac Technology, Sunnyvale, CA, USA) capture card. The area of the articular cartilage, the length of the free articular surface of the cartilage, and the length of the interface between the articular cartilage and the underlying bone were determined by the digital pantograph function of Image software (National Institutes of Health, Bethesda, MD). The area of cartilage divided by the length of the osteochondral junction was used as a normalized hyaline cartilage thickness, and the ratio of the length of cartilage at the

joint interface to that at the bone interface was recorded as a "contour index" to assess irregularities in the articular surface.

Levels and glycosylation patterns of total IgG and IgG anti-Col II antibodies in serum. Sandwich ELISA was used to quantify total IgG and antibodies specific for Col II in serum, as described[60,61,70]. Microwell plates (Immunoplate MaxiSorp®, Nunc, Roskilde, Denmark) were coated (10 μg/ml) with either a capture antibody (anti-mouse Fab' fragment) for assay of total IgG, or Col II antigen for assay of serum antibodies. After overnight incubation at 4°C, plates were blocked with 1% bovine serum albumin (BSA) in 0.01 M sodium phosphates, 0.15 M NaCl (PBS) for 90 min at room temperature, and washed 3 times with PBS. Next, samples of serum were applied at several dilutions, optimized in pilot experiments[70]. After 90 min at room temperature, plates were washed thrice with PBS, and anti-mouse IgG antibody conjugated to alkaline phosphatase was incubated for 1 h at room temperature. After another 3 washes with PBS, paranitrophenyl phosphate substrate was added (1 mg/ml in 50 mM glycine, 1 mM $MgCl_2$, pH 10.4). Hydrolysis of the chromogenic substrate was followed by monitoring optical density (OD) at 405 nm in a microplate reader (Cayman Chemical, Ann Arbor, MI, USA). The OD derived at the end of the linear phase of the reaction on plates coated with goat anti-mouse Fab' fragment antibody were interpolated into standard curves developed on the same plates using known concentrations of a highly purified mouse monoclonal antibody (Southern Biotechnology, Birmingham, AL, USA), and data are expressed as μg/ml. The antibody levels are expressed as relative units, based on the OD measurements. For all sera, the OD at all 3 concentrations of each serum were linear with the dilution applied to the plate.

Lectin affinity assays, as described[70], were used to assess glycosylation of the total IgG and the specific IgG in each animal. Coating of microwell plates with anti-mouse Fab' fragment or Col II antigen followed the procedure just described; blocking with 1% BSA in PBS was maintained for 120 min. Samples of the IgG fraction of serum proteins eluted from affinity columns of protein G immobilized on Sepharose were incubated 120 min at room temperature, at 3 different IgG concentrations. After 3 washes, one of 2 biotinylated lectins, *Maackia amurensis* lectin II (MAC) or *Ricinus communis* I (RCA) (both from Vector Laboratories, Burlingame, CA, USA), was incubated 120 min at room temperature. After 3 more washes, alkaline phosphatase conjugated streptavidin (1 μg/ml) was added for 90 min at room temperature. Finally, after another 3 washes, chromogenic substrate was added as above. OD developed at the end of the linear phase of the reaction were recorded in a microplate photometer, and normalized to the values obtained from the same samples incubated in parallel plates with the same capture protein (anti-mouse Fab' fragment or Col II) but detected by anti-mouse IgG conjugated to alkaline phosphatase. As reported, the values obtained with lectin detection were systematically proportional to the values obtained with anti-IgG detection, and all controls were established as we described[70]. Moreover, in our previous work, independent monosaccharide analysis and oligosaccharide profiling and sequencing corroborated our lectin affinity data. Thus, we consider the relative binding to MAC and RCA to reflect terminal sialic acid and galactose levels on IgG or on the IgG fraction of anti-Col II.

Oral administration of the enzyme mixture used here results in detectable protease activity in the serum of experimental animals[55-58,62,67,68]. However, at the high dilutions of serum we used, enzymatic activity cannot be detected, even with synthetic chromogenic substrates. Accordingly, the determination of total IgG and IgG anti-collagen II antibody in ELISA of serum are not likely affected. Moreover, preparations of affinity purified IgG eluted from protein G columns to which the proteolytic enzymes do not bind produced values of total and collagen-specific IgG similar to those obtained using serum samples. Similarly, the lectin assays were performed on diluted samples eluted from protein G columns, and were thus not influenced by enzyme activity.

Cytokine production in vitro. Single-cell suspensions of peripheral (axillary, brachial, cervical, inguinal) lymph nodes were centrifuged, washed, and resuspended at 4×10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (all from BioWhittaker, Walkersville, MD, USA), 50 mM 2-mercaptoethanol, and 1 mM sodium pyruvate (both from Sigma). Duplicate cultures from each mouse were maintained for 48 h in a humidified 37°C environment with 5% CO_2, either with no addition or with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 300 ng/ml calcium ionophore A23187 (both from Sigma). Cytokine content in undiluted supernatants [or diluted 1:5 for mitogen stimulated interferon-γ (IFN-γ)] was assayed by appropriate sandwich ELISA. Both monoclonal anti-murine IL-5 antibodies (TRFK-4, TRFK-5) were kindly provided by Dr. Robert Coffmann (DNAX, Palo Alto, CA, USA), whereas the anti-murine IFN-γ antibodies were commercial preparations (Genzyme, Boston, MA, USA). In brief, samples were incubated 2 h at room temperature in blocked (1% BSA in PBS) microwell plates precoated with monoclonal antibodies specific for mouse IL-5 or IFN-γ; after 3 washes, biotinylated detection antibodies, specific for epitopes on murine IL-5 or IFN-γ distinct from those specified by the capture antibodies, were applied for 2 h at ambient temperature. Finally, after 3 additional washes, alkaline phosphatase conjugated streptavidin was added for 90 min at room temperature, followed by 3 more washes and addition of chromogenic substrate, as above. The OD developed at the end of the linear phase of the reaction were interpolated into parallel standard curves using purified recombinant mouse IL-5 or IFN-γ (Genzyme).

RESULTS

Evolution of arthritis. On Day 28 post-priming, prior to any therapeutic intervention, all immunized mice exhibited redness and swelling in at least 2, and typically 3, paws. Although only a few mice had objective arthritis in all 4 paws, the overall mean clinical score (12.2 ± 0.4) derived from a generally moderate involvement of 3 paws or severe involvement of 2 paws, and those few (~15%) mice with involvement of all 4 paws typically showed only mild redness and swelling in one or 2 paws. Rarely did control mice evidence redness or swelling of the feet, never more than mild; typically only redness in one or 2 paws was observed. By 2-way analysis of variance (ANOVA), there was no significant difference in the initial (pretreatment, Day 28 post-priming) clinical scores among the 3 experiments, and therefore the data were pooled. One-way ANOVA indicated that each of the groups of immunized mice differed significantly (all Dunnett's $t > 21$, all $p < 0.001$) from the nonimmunized mice, but there were no differences among the 4 groups of immunized mice initially (Table 1).

Effect of therapy on arthritis. After 2 weeks of therapy, both ibuprofen and proteases significantly reduced the signs of arthritis, represented as a clinical score, compared to the untreated mice immunized and boosted with Col II ($t > 8.9$, $p < 0.001$ for both treated groups vs untreated disease controls), as shown in Table 1. The effect was well in excess of that seen in mice treated with PBS (vehicle) alone (both $t > 8.3$, $p < 0.001$). However, both treated groups did have significantly more redness and swelling over the feet than did unmanipulated age matched healthy controls (both $t > 11.9$, $p < 0.001$), and there was no significant difference between the 2 treatments in terms of the clinical score. In general, the severity of involvement at Day 42 post-priming

Table 1. Synopsis of joint appearance.

Immunization and Boost	Treatment	n	Arthritis Scores†			
			Initial Clinical	Final Clinical	Radiographic	Histologic
Collagen II	None	16	12.9 ± 0.4	12.6 ± 0.6	6.2 ± 0.4	4.0 ± 0.2
Collagen II	Saline	11	12.0 ± 0.5	12.7 ± 0.4	5.7 ± 0.3	3.5 ± 0.3
Collagen II	Ibuprofen	15	12.5 ± 0.3	7.3 ± 0.3**	3.1 ± 0.4**	2.7 ± 0.2††
Collagen II	Proteases	15	12.9 ± 0.4	7.0 ± 0.3*	2.7 ± 0.5**	2.6 ± 0.2††
None	None	17	0.6 ± 0.3	0.1 ± 0.4	0.4 ± 0.1	0.2 ± 0.1

† Data are the mean ± SE scores for initial (Day 28 post-priming) and final (Day 42 post-priming) clinical scores, and the radiographic and histologic scores after sacrifice (Day 42 post-priming). In all cases, the results of 3 independent experiments are pooled; 2-way ANOVA indicated that no significant interexperimental variation occurred for any variable here. * Both treated groups differ from untreated mice ($F > 100$, Dunnett's $t > 8.9$, $p < 0.001$), from saline treated mice ($t > 8.3$, $p < 0.001$), and from normal mice ($t > 11.9$, $p < 0.001$). ** Both treated groups differ from untreated mice ($F > 50$, $t > 6.6$, $p < 0.001$), from saline treated mice ($t > 5.0$, $p < 0.01$), and from normal mice ($t > 5.0$, $p < 0.01$). †† Both treated groups differ from untreated mice ($F > 48$, $t > 4.1$, $p < 0.01$), from saline treated mice ($t > 2.4$, $p < 0.05$), and from normal mice ($t > 8.1$, $p < 0.001$).

was similar among all the paws in a particular mouse; indeed, there was significant correlation among the 4 paws of all mice (all Pearson correlation coefficients were significant, with all $p < 0.01$).

Radiographic evaluation proved practical only in the tarsal-metatarsal-phalangeal joint complex due to technical limitations in placing the mice onto the films. The metatarsal-phalangeal joints were less severely involved than the corresponding tarsal joints. Compared to normal controls (Figure 1A), untreated arthritic mice revealed marked periarticular osteopenia and bone resorption, reflected both by increased radiolucency and by rounding of condyles within each joint group (Figure 1B). Bone cyst formation was also evident in many of these mice. Arthritic mice treated with either agent showed significantly less osteopenia and blunting of condyles, and no cyst formation (Figure 1C). Indeed, the average radiographic score for untreated diseased mice was significantly higher than in the healthy controls; both therapeutic regimens resulted in significant (both $t > 6.6$, both $p < 0.001$) reductions in bone loss and radiographic joint abnormalities relative to untreated immunized mice (Table 1). Nonetheless, the typical rear paw in diseased mice treated with either agent appeared abnormal (Figure 1C) in comparison to age

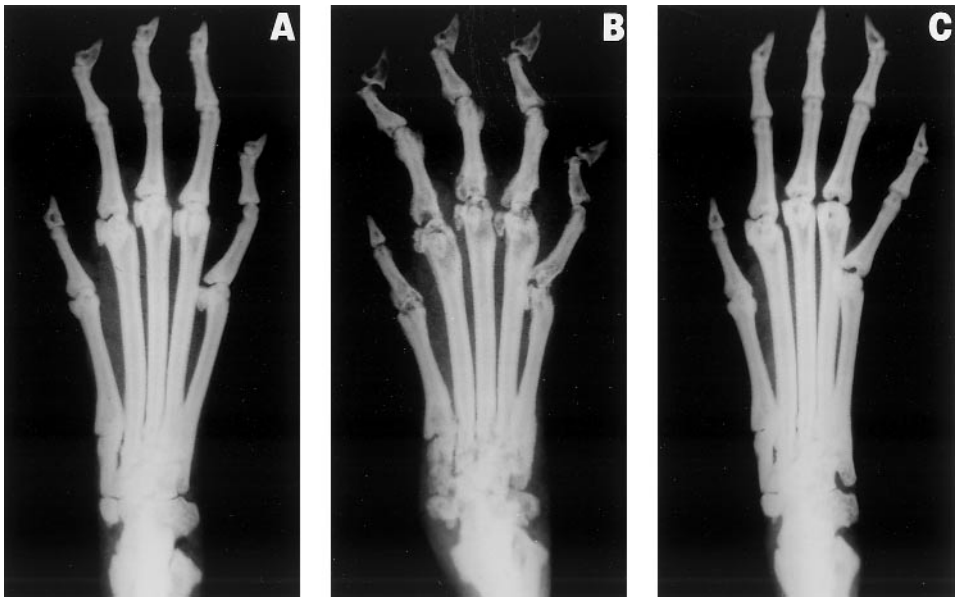

Figure 1. Portions of the whole-body radiographs of selected mice show the tarsal-metatarsal-phalangeal complex of the right hind paw. Compared to age matched normal mice (A), untreated immunized mice (B) show severe erosion and osteopenia of the tarsal bones, irregular articular contours, and formation of bone cysts. The metatarsal-phalangeal joints show similar, but somewhat less pronounced deformities. In a typical immunized mouse treated with proteases (C), osteopenia is much less pronounced, and more narrowly distributed; no cysts are evident. Immunized mice treated with ibuprofen (not shown) have a similar appearance.

matched healthy control mice (Figure 1A). The 2 treatment groups did not differ from each other in terms of the mean aggregate radiographic scores (Table 1).

The histologic appearance of all the joints examined differed sharply among the groups. Foremost, in contrast to normal joints (Figures 2A, 3A), immunized mice not given any therapy typically showed nearly total obliteration of tarsal (Figure 2B), metatarsal-phalangeal (Figure 3B), and femorotibial joints (not shown). Indeed, exuberant granulation tissue often filled the joint space and invaded the bone, with total effacement of cartilage in the untreated immunized mice. Foci of chondral and/or osseous metaplasia were evident in most joints, as was a pannus containing necrotic material. Sometimes granulation tissue extended into the marrow cavity of the phalanges, tibia, or femur. Interestingly, no lymphoid nodules were seen, although numerous activated lymphocytes were frequently observed within the synovium and within the granulation tissue filling the joint spaces. In contrast, joints from immunized mice treated with either proteases (Figures 2C, 3C) or ibuprofen (Figure 3D) were significantly ($t > 4.1$, $p < 0.01$) less severely damaged than the untreated immunized mice (Table 1). Rarely were the joints obliterated, although granulation tissue sometimes extended from the synovium into the joint space. Clearly, joints from immunized treated mice differed ($t > 8.1$, $p < 0.001$) from normal joints (Figures 2, 3), by virtue of synovial inflammation, irregularity, and thinning of the articular cartilage, but bony changes and invasive granulation tissue were markedly attenuated in immunized mice given either treatment in comparison to the untreated diseased controls.

As judged by the histologic score alone, there was little difference (Table 1, p = NS) between the 2 treatments (Figure 3C vs 3D). However, the histologic score was designed to assess the maximal severity of articular damage, rather than the overall number of joints involved or an average degree of injury. When preservation of articular (hyaline) cartilage was measured morphometrically, cartilage was preserved in the group treated with proteases relative to either immunized mice treated with ibuprofen or to immunized mice left untreated (Table 2). Indeed, among immunized mice, a majority of joints from those treated with ibuprofen had advanced attenuations of articular cartilage (Figure 3D), whereas most of the joints from mice treated with enzymes showed much better preservation of cartilage (Figure 3C).

Glycosylation of circulating total IgG and IgG anti-Col II antibody. IgG antibodies specific for Col II were readily detected in the serum of all immunized mice, but not in the unmanipulated controls; neither therapy had any effect on the level or apparent binding affinity of the circulating antibody (data not shown). Further, terminal sialylation (Figure 4) and galactosylation (data not shown) of total IgG (i.e., IgG fraction purified from serum and captured by antibody specific for mouse Fab' fragment) were significantly ($F > 13.9$, all $t > 3.9$, all $p < 0.001$) lower in all immunized groups compared to age matched controls; protease treatment partially corrected such defective glycosylation of total IgG ($t > 2.1$, $p < 0.05$ vs untreated and ibuprofen groups), whereas ibuprofen had no effect (Figure 4). Specific (anti-Col II) IgG purified from the serum of immunized untreated mice was also profoundly hypoglycosylated ($F > 8.55$, $t > 5.2$, $p < 0.001$). Protease treatment fully restored the sialylation (Figure 4) and partially restored the galactosylation (data not shown) of the specific IgG towards normal ($t > 3.3$, $p < 0.01$ vs untreated mice), whereas

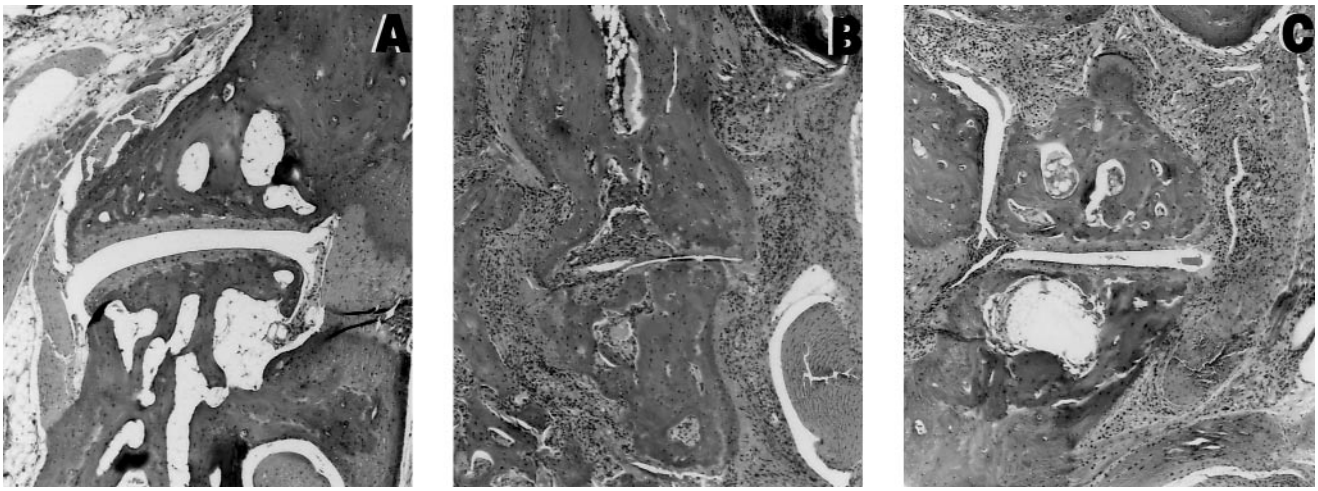

Figure 2. Representative tarsal-metatarsal joints are compared among normal age matched controls (A), immunized mice left untreated (B), and immunized mice treated with proteases (C). Complete destruction of joints, with necrotic pannus formation, a lymphocytic inflammatory infiltrate with invasive granulation tissue, effacement of articular cartilage, and severe erosion of articular bone is typically seen in immunized mice left untreated (B). Some erosion of articular cartilage and subchondral bone is present in treated immunized mice (C), but the articulations are generally well preserved and include appreciable hyaline cartilage despite conspicuous synovial inflammation and granulation tissue; destruction of joints in these mice is rare. Note open joint space and a thin clear synovium, with a thick smooth layer of articular cartilage, in the normal joint (A). All stained with H&E, original magnification 150×.

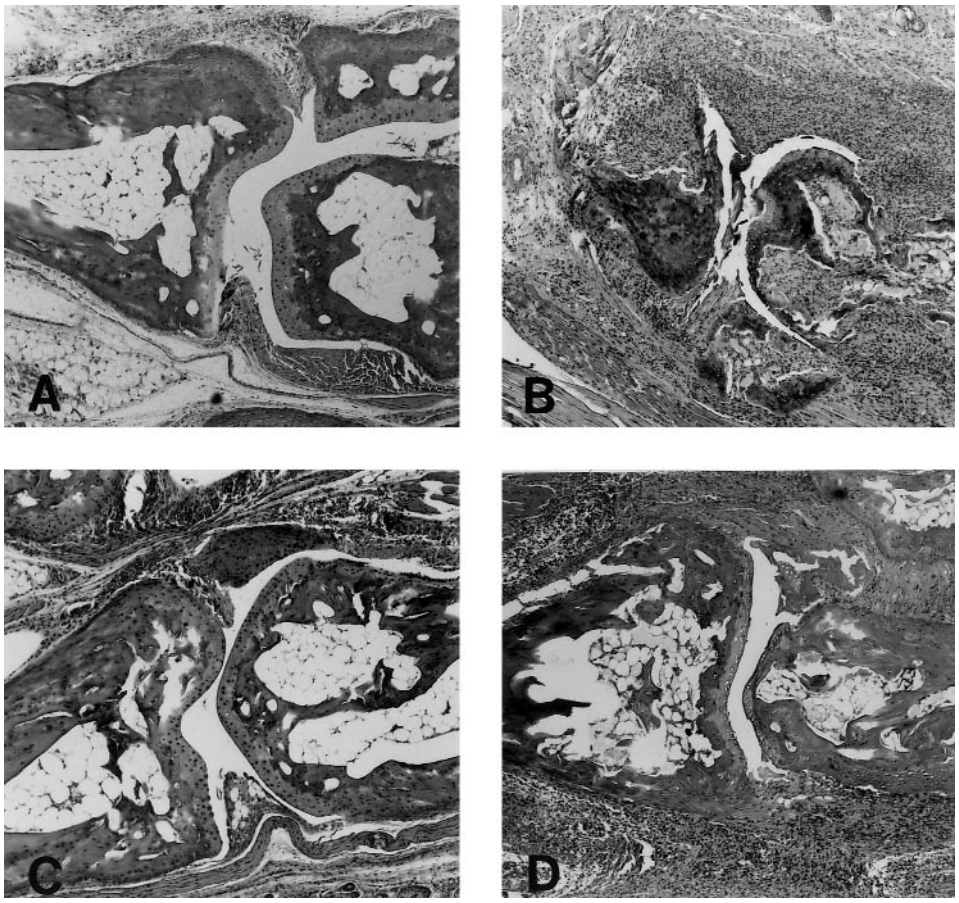

Figure 3. Representative metatarsal-phalangeal joints are compared among normal age matched controls (A), immunized mice left untreated (B), immunized mice treated with proteases (C), and immunized mice treated with ibuprofen (D). In the untreated immunized mice (B), severe invasive pannus totally effaces articular cartilage and replaces much of the articular bone, so that the joint is barely identifiable as such. There is more articular cartilage in the typical joint in an immunized mouse treated with proteases (C) compared to the joint of an immunized mouse treated with ibuprofen (D). Bony erosion is minor in both treatment groups, and synovitis with granulation tissue, although often well developed, is not so extensive or invasive as it is in untreated immunized mice (B). The differences in preservation of articular cartilage between the 2 treatment modalities are consistent over all the mice examined (see Table 2). All stained with H&E, original magnification 225×.

ibuprofen had only an equivocal effect on sialylation and no effect on galactosylation.

Cytokine production in vitro. No IFN-γ or IL-5 was detected in supernatants from lymphocytes cultured under basal conditions without addition of the mitogenic stimulus (PMA plus A23187). A deficient IFN-γ response to mitogen is seen in lymphocytes isolated from untreated arthritic mice ($F = 7.9$, $t > 3.1$, $p < 0.01$), compared to lymphocytes isolated from normal mice (Figure 5). Protease treatment restores the production of IFN-γ to levels seen in control mice ($t = 4.2$,

Table 2. Morphometric measurement of articular cartilage.

Immunization and Boost	Treatment	Mean Cartilage Thickness, μm			
		Femur at Knee	Tibia at Knee	Tarsus at 1st TMT	Metatarsal at 1st TMT
Collagen II	None	17 ± 14	31 ± 22	4.2 ± 5	4.3 ± 8
Collagen II	Ibuprofen	28 ± 18*	56 ± 19*	16 ± 9*	24 ± 15*
Collagen II	Proteases	47 ± 23*†	72 ± 28*†	25 ± 9*†	38 ± 14*†
None	None	60 ± 14	120 ± 23	34 ± 8	53 ± 12

* Significantly greater ($F = 12$, $p < 0.001$) than immunized untreated mice, and significantly less than normal controls. † Significantly greater ($F = 12$, $p < 0.002$) than immunized mice treated with ibuprofen. TMT: tarsometatarsal.

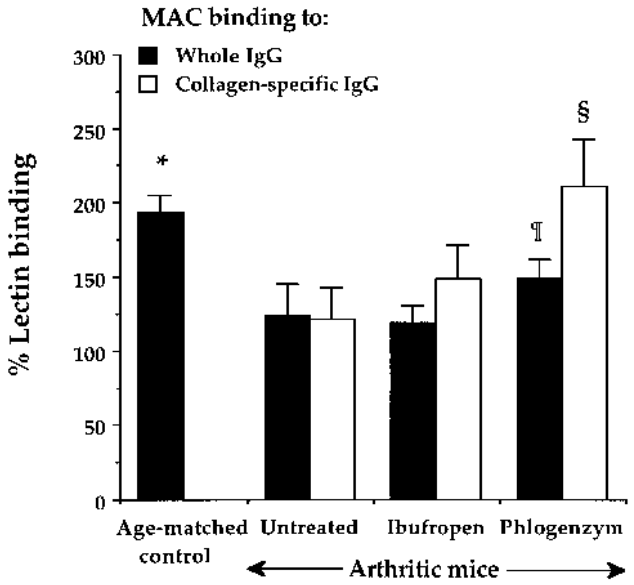

Figure 4. Sialylation of IgG in the sera of mice is measured by binding of *Maackia amurensis* lectin II (MAC). Compared to age matched controls, all groups of arthritic mice show a reduction (*F = 15.3, $t \geq 3.9$, $p < 0.001$ for control vs all other groups) in terminal α2-3 sialic acid on whole IgG (black bars). Ibuprofen shows no difference in the sialylation of total IgG compared to untreated mice, but proteases increase total IgG sialylation to 77% of the control value (¶$t \geq 2.1$, $p < 0.05$ vs all other groups). Moreover, protease treatment restores the glycosylation of specific IgG to normal (§F = 11.5, $t \geq 3.4$, $p < 0.005$ vs other arthritic groups), whereas ibuprofen has a modest effect that is not significantly different from untreated arthritic mice.

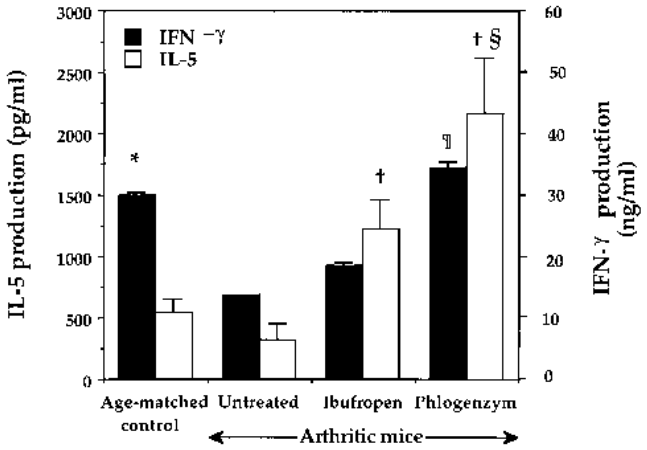

Figure 5. In vitro production of IFN-γ (a Th1 cytokine) and IL-5 (a Th2 cytokine) elicited by mitogen and measured by ELISA. Normal mouse lymph node suspensions produce significantly (*F = 7.9, both $t \geq 2.4$, $p < 0.05$) more IFN-γ (black bars) than lymphocytes from untreated or ibuprofen treated arthritic mice; protease treatment restores IFN-γ production to normal (¶p = NS vs control, $t \geq 3.5$, $p < 0.01$ vs untreated and ibuprofen treated arthritic mice). Whereas untreated arthritic mice exhibit a minor decrease in the IL-5 (white bars) response to mitogen stimulation relative to normal (p = NS), ibuprofen and proteases increase IL-5 production to levels that exceed normal (†F = 6.2, $t \geq 2$, $p < 0.05$ vs untreated arthritic mice). Further, the effect of proteases on IL-5 production significantly (§$t = 2.2$, $p < 0.05$) exceeds that of ibuprofen, eliciting IL-5 that far surpasses (§$t \geq 3.1$, $p < 0.01$) the production by cells from age matched control mice or untreated arthritic mice.

$p < 0.001$ vs untreated, p = NS vs control), but ibuprofen has no significant effect. Indeed, similar effects are seen in peripheral blood lymphocytes isolated from patients with rheumatoid arthritis[65,66]. Both ibuprofen and protease treatment increase the production of IL-5 ($F = 6.2$, $t > 3.5$, $p < 0.01$) to levels above those seen in age matched control mice (Figure 5).

DISCUSSION

Our observations confirm that experimental arthritis induced in DBA/1 mice by immunization and boosting with emulsions of Col II is a progressive disease, characterized by inflammation of the small joints, ultimately with progressive erosion of cartilage and bone, as reported[7-13,27,28]. Treatment of the mice with the potent nonsteroidal antiinflammatory agent ibuprofen, often used for therapy of RA in clinical settings[1-5,37-39], ameliorates the outward signs of redness and swelling over the exposed joints, and the early (inflammatory) changes in the joints recognized histologically and radiographically. Phlogenzym®, a proprietary formulation of bromelain, trypsin, and the antioxidant rutosid, shows equally potent antiinflammatory activity. In addition, this new therapeutic modality better preserves the integrity of the articular cartilage, and has potentially salutary effects on the glycosylation of circulating total IgG and IgG anti-Col II antibody. Further, although both therapies increase the production of IL-5 by lymphocytes from arthritic mice to levels above those released by normal lymphocytes, only protease treatment elicits commensurate and balanced increases in IFN–γ.

The use of formulations of proteolytic enzymes as therapy of immune mediated disease began more than 10 years ago, and has expanded, both in experimental models and in clinical trials[55-69]. Initially, proteases were employed in glomerulonephritides and arthritis initiated by immune complexes[55-61]. The main effect of the proteases was ascribed to their capacity to cleave Ig molecules, especially in the tuftsin-like region in the second constant heavy chain (CH2) domain. Such proteolysis of the Ig component(s) of an immune complex would, in principle, solubilize the complex, and indeed this effect on immune complexes was documented *in vivo* and *in vitro*. More recently, additional effects of the proteases on other Ig superfamily members have become recognized[68,69]. Some of the antiinflammatory activity of the protease formulations undoubtedly derives from cleavage of adhesion molecules such as CD54 and CD44[68,69], known to be critical factors for leukocyte immigration and activation[71]. Indeed, cellular adhesion molecules, and especially CD54, CD44, and CD106, are recognized as important contributors to inflammation in RA

in both humans and in rodent models[25,26,72-75]. However, the salutary effects of proteases transcend those of ibuprofen, even though there is little or no difference in the amelioration of joint inflammation by the 2 agents. This observation suggests that other properties of the protease formulation, apparently unrelated to the antiinflammatory properties, may be important. There is growing appreciation of the immunomodulatory effects of protease, likely derived in part from cleavage of adhesion molecules critical to T cell activation, such as CD4, CD80, and CD86, in addition to cleavage of the aforementioned adhesion molecules applicable to leukocytes in general[68,69]. Moreover, given the powerful influence of transforming growth factor-ß (TGF-ß) upon chondrocyte viability and function, proteases may ameliorate cartilage injury by modulating bioactive TGF-ß. Although this property is speculative at present, initial reports do indicate that proteases can influence the secretion and activity of TGF-ß[62,66,67]. More research of mechanistic detail is warranted.

The mechanism(s) of the nonsteroidals remains poorly understood; although interference with arachidonate metabolism is certainly part of the effect, different NSAID have quite disparate effects on various forms of inflammation. Indeed, some argue that different drugs are best suited for particular circumstances of inflammation[1-5,37-39]. The effect of nonsteroidals in CIA remains controversial, but dual inhibition of both the cyclooxygenase and lipoxygenase pathways seems critical for any antiinflammatory properties[40-54]. Cartilage and bone sparing properties of the nonsteroidal drugs seem to be minor or absent in CIA. The proteases have considerable effect on arachidonate mediated inflammation[67], but it is not clear whether the effect derives from altered synthesis of some or all eicosanoids, interference with appropriate eicosanoid receptors, both mechanisms, or neither. For example, antioxidant agents have moderated the severity of CIA[76], and both the proteases and especially the rutosid component of the preparation used here may act in part through antioxidant effects. In addition, the rutosid component may alter the pattern of eicosanoids produced at inflammatory sites. Likely, the major antiinflammatory effect of these proteolytic enzyme formulations resides outside the arachidonate cascade[53,54].

We observed 3 elements that distinguish protease therapy of CIA from ibuprofen. Protease therapy preserves articular cartilage, increases both Th1 and Th2 responses to mitogenic stimulation of lymph node suspensions from diseased mice in balance, and selectively shifts the glycosylation of IgG anticollagen II antibody towards normal. These features may be independent of each other, and any or all 3 may be irrelevant to the salutary effects of protease therapy on this model of RA. Perhaps most interesting among these is the potential for protease therapy to spare articular cartilage in the CIA model, and more cogently in RA. The changes we observed represent the early, inflammatory lesions of CIA. In the later stages, loss of cartilage, bony erosion, and ankylosis ensue. Palliation of pain, perhaps with attendant reduction but not abrogation of inflammation, must be distinguished from alteration of the longterm course of a chronic and progressive disease such as CIA or RA. If protease therapy can support the preservation of articular cartilage over the long term, and thereby forestall bone loss and fusion or obliteration of the joints, this would represent a significant step in the management of RA. The data available at present are, of course, too limited to establish disease modifying effect of the protease therapy, but do suggest such potential. The mechanism(s) of such effect would also be of significant interest. For example, proteases might interfere with cytokine cascades. Although any effect of proteases on IL-1 and TNF and their receptors remains unknown, these cytokines are increasingly considered to be important elements in joint injury in the CIA model[27-29] and in RA[22-26]. If protease therapy acts in other ways, such as via modulation of TGF-ß response in chondrocytes, then protease therapy might synergize with therapy targeted at IL-1 and TNF-α. Longterm experiments to determine the efficacy and mechanism of protease therapy for disease modification are clearly required in this respect.

The immunomodulatory effects of proteolytic enzymes are even more obscure, and yet these effects may represent the most powerful and crucial benefits of these formulations for patients with autoimmune disease. Often in autoimmune disease, the depth and breadth of immunologic injury expands until the target organ(s) are completely destroyed via a phenomenon known as "epitope spreading." The initial injury promotes amplification of the autoimmune response[77,78]. Redirection of immune responses away from these autoantigenic targets offers the potential for durable therapy[79,80]. Our recent observations[70] surrounding the effects of cytokines derived from T cells, and especially of Th2 and Th1 cytokines, upon glycosylation of several products of B cells *in vitro*, including secreted Ig, suggest that the effects of protease treatment on the glycosylation of IgG anti-Col II antibody and its effects on cytokine production by lymphocytes isolated from diseased mice are causally related. Altered glycosylation of IgG in RA is now established[30-34] and parallel changes in the glycosylation of IgG in CIA are recognized[35,36]. Although the pathogenic role of such aberrant glycosylation in RA remains questionable, the mechanistic basis of abnormal IgG glycosylation apparently resides in deficiency in the content and/or activity of galactosyl transferase in patients' B cells[35,36]. Deficient B cell galactosyl transferase activity, with consequent diminished IgA galactosylation, is also observed in patients with IgA nephropathy, a form of glomerulonephritis in which aberrant Th2 cytokine production is seen[70]. We recently reported that Th2 cytokines, but not other stimuli of B cells, lead to aberrant glycosylation of IgA produced *in vitro*[70]. These effects, apparently due to diminished galactosyl transferase activity,

are offset by IFN-γ. The role of Th1 versus Th2 cytokines in RA and CIA remains controversial in several areas[6,39,81-84], but if the data on IgA glycosylation applies to IgG glycosylation as well, the reduced production of IFN-γ in untreated arthritic mice reported here may in fact underlie the glycosylation defects. The disproportionate reduction in Th1 (IFN-γ) relative to Th2 (IL-5) in untreated arthritic mice is not offset by ibuprofen treatment; indeed, the IFN-γ to IL-5 ratio is decreased further in diseased mice treated with ibuprofen. In contrast, protease therapy restores IFN-γ to normal, and the ratio of IFN-γ to IL-5 is closer to normal and may exceed a critical threshold. If altered glycosylation of IgG is indeed an important element in the genesis or progression of RA[30-34], then the selective effects of the protease formulation on the expression or activity of glycosyl transferases may represent a linchpin for the efficacy of proteases in the treatment of this disease. Obviously, more work on the mechanistic basis of protease effects is warranted.

Regardless of the causal links between the effects of proteases and the sparing of articular cartilage, proteases appear to offer potential as a disease modifying therapy, and are clearly better than NSAID in this respect. The safety of protease therapy in general is well established, since the formulation used here and other similar formulations have had widespread use in a variety of clinical settings[63-66]. Use of proteases for those with NSAID intolerance or toxicity, or combination of proteases with NSAID to gain synergistic effect, may offer considerable clinical advantages. Moreover, proteases may synergize with the evolving drugs that interfere with IL-1 or TNF-α, and thereby contribute to very powerful therapy for RA. The immunomodulatory properties of the proteases, while offering tantalizing prospects for salutary effect, also commend cautious application until the mechanistic basis for the protease effect, and a fuller appreciation of the effects of protease on the autoimmune response, become established.

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