RELEASE OF CARTILAGE MUCOPOLYSACCHARIDE-DEGRADING NEUTRAL PROTEASE FROM HUMAN LEUKOCYTES

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The granule fraction of human leukocytes contains neutral protease capable of degrading the noncollagenous, chondromucoprotein matrix of intact rabbit ear cartilage (1). In order to ascertain the mechanism by which these enzymes gain access to extracellular tissue, it is important to determine whether cells can release neutral protease under physiologic conditions. Human leukocytes have been reported to release lysosomal acid hydrolases during the process of phagocytosis without a concomitant loss of cell viability. Release has been observed when cells phagocytize latex particles, monosodium urate crystals, zymosan, and antigen/antibody complexes (2-5). This process is enhanced when cells attempt to phagocytose such substances fixed to a solid support (nonphagocytic enzyme release) (6).

In rheumatoid arthritis antigen/antibody complexes of the IgM and IgG anti-IgG type have been demonstrated in synovial fluid and as cytoplasmic inclusion in leukocytes which are chemotactically attracted to sites of inflammation (7-9). It is therefore possible that the cartilage destruction in rheumatoid arthritis could be a direct result of the release of deleterious enzymes during the process of phagocytosis of antigen/antibody complexes.

In this report we demonstrate that human leukocytes, in physiologic buffer, in the presence of either suspended or fixed aggregated human gamma globulin (AHGG), release neutral protease(s) that are capable of degrading the noncollagenous, chondromucoprotein matrix of cartilage. In addition, gold thiomalate and colchicine, both clinically active anti-inflammatory agents, were tested for their capacity to inhibit nonphagocytic release of human neutral protease and/or protease activity directly. Colchicine inhibited the nonphagocytic release of human neutral protease. Conversely, gold thiomalate and iso-

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1 Abbreviations used in this paper: AHGG, heat aggregated human gamma globulin (Fraction II); β-G, β-glucuronidase; Hanks' BSS, Hanks' balanced salt solution; HGG human gamma globulin (Fraction II); LDH, lactic dehydrogenase.

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logous human serum (10%) inhibited directly the protease activity without influencing enzyme release.

**Materials and Methods**

**Preparation of Viable Human Leukocytes and Leukocyte Granule Fraction.**—A leukocyte-enriched fraction, obtained from the peripheral blood of healthy rheumatoid factor negative (latex agglutination) donors, was isolated within 1 h of venipuncture, by dextran sedimentation as described previously (10). The mixed leukocyte suspension was washed once with Hanks' balanced salt solution (Hanks' BSS) containing 1% glucose and resuspended in the same medium. All incubations were started 3 h after collection of blood. The cells had a viability of >99% as judged by Trypan blue dye exclusion and on the average consisted of 40% neutrophils, 50% lymphocytes, 6% monocytes, 3% eosinophils, and 1% basophils. The granule fraction from human leukocytes was prepared as previously described (11). The granule fraction was resuspended in Hanks' BSS (pH 7.4) and subjected to 10 freeze-thaw cycles using a dry ice-acetone mixture. After the final thaw, the suspension was centrifuged at 15,000 X g for 25 min at 4°C and the supernatant was used immediately.

**Preparation of 35S-Labeled Rabbit Ear Cartilage.**—The preparation of rabbit ear cartilage labeled with 35S and the experiments defining the specificity of mucopolysaccharide labeling have been described in detail in a preceding report (1). Disks (3.0 mm diameter) of 35S-labeled cartilage were either used fresh or stored at --70°C for 1-3 wk. Frozen specimens were thawed at 4°C and used immediately. In each experiment cartilage from the same rabbit was used for both control and experimental conditions. Total 35S in the cartilage was determined by dissolving the tissue in 2.0 ml of hot concentrated hydrochloric acid and analyzing 0.1-0.2 ml aliquots for 35S activity in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.).

**Preparation of A HGG.**—Human gamma globulin (HGG) (Fraction II, Miles Laboratories Inc., Kankakee, Ill.) was heat aggregated by first dissolving 56 mg in 6.0 ml of phosphate-buffered saline (pH 6.4) and heating at 63°C for 20 min. An additional 100 mg HGG was dissolved and the incubation was continued for 20 min at 63°C. The precipitate (AHGG) was suspended in 2.5 ml of Hanks' BSS with glucose and a homogenous suspension was prepared with a Potter Elvehjem homogenizer (Potter Instrument Co. Inc., Meiville, N. Y.) equipped with a Teflon pestle. The final suspension contained 20 mg/ml of protein and was used immediately. Non heat-aggregated HGG was prepared by making a 2% solution of HGG in Hanks' BSS with glucose.

**Incubation Conditions.**—

**Nonphagocytic enzyme release:** A single layer of labeled cartilage disks was incubated in 5 ml Erlenmeyer flasks, containing excess AHGG or HGG (1.0 ml per 20 cartilage disks), at 37°C for 20 min. Each disc was then removed from the incubation medium with thumb forceps and rinsed (Hanks' BSS with glucose) free of excess gamma globulin. Depending upon the experiment, a single layer of 5-10 cartilage disks was placed in a 19 X 51 mm flat bottom vial which was incubated in an oscillating water bath (50 oscillations/min) at 37°C. In all experiments 1.5 ml of human leukocyte cell suspensions were added. The cell concentration and time of incubation were varied to obtain optimum conditions for enzyme release. The final method consisted of a 4 h incubation with five cartilage disks in 1.5 ml of cell suspension containing a total of 5 x 10^4 leukocytes. After incubation 0.5 ml aliquots of medium from each vial were removed and prepared for liquid scintillation counting as described previously (1). In most experiments, 0.2 ml aliquots of supernatant (obtained after centrifugation of medium at 900 X g for 10 min at 4°C) were assayed for β-glucuronidase (β-G) activity. At the termination of incubations, 98% of the cells layered at the bottom of the vials were viable as ascertained by dye exclusion. For additional confirmation of cell viability, lactic dehydrogenase (LDH) activity of supernatants was estimated. Triplicate samples were used for the control and experimental groups.
In certain experiments gold sodium thiomalate (Merck, Sharpe, and Dohme of Canada Ltd., Montreal, Canada), colchicine (Sigma Chemical Co., St. Louis, Mo.), and 10% isologous human serum were added to the incubation medium.

Phagocytic enzyme release: 5 million human leukocytes, suspended in 1.5 ml of Hanks' BSS with glucose, containing 2% AHGG, 2% HGG or buffer. After incubation for 2 h at 37°C in an oscillating water bath (50 oscillations/min), the cell suspension was sedimented by centrifugation (900 × g for 10 min) at 4°C. Aliquots of the supernatant were assayed for both LDH and β-G activity, and 1.0 - 1.5 ml of supernatant was incubated with five cartilage disks as described above. After an additional 4 h, 0.5 ml aliquots of medium were removed for 35S determination.

Enzyme Assays.—β-G and LDH assays were performed on the supernatants from experiments dealing with phagocytic and nonphagocytic enzyme release. β-G activity was assayed by the method of Gianetto and DeDuve using phenolphthalein glucuronide as substrate (12). Aliquots (0.2 ml) of supernatants were incubated in 3.0 ml of 0.1 M sodium citrate buffer (pH 4.8), containing 5 mg of substrate, at 37°C for 18 h. LDH assays were performed by the method described by Bergmeyer et al. (13) using β-diphosphopyridine nucleotide (disodium salt, reduced form) as substrate. Enzyme activity was quantitated by determining the average change in optical density (366 nm) per minute during 3 min of incubation.

RESULTS

Effect of Number of Leukocytes and Time of Incubation on Nonphagocytic Release of Enzymes from Human Leukocytes.—Experiments with varying numbers of human leukocytes were performed using a standard 4 h incubation (37°C) in Hanks' BSS (pH 7.4) containing 1% glucose. Triplicate determinations were made with each cell concentration overlying five cartilage disks. The results given in Fig. 1 indicate that, between concentrations of 0.5 × 10⁶ and 10 × 10⁶ leukocytes, there was a concentration-dependent release of both 35S and β-G into the incubation medium when cells were incubated with AHGG-coated cartilage. In other experiments, using up to 40 × 10⁶ cells, no further 35S release occurred. Essentially no release of 35S or β-G occurred when AHGG-coated cartilage disks were incubated in the absence of leukocytes. Although not shown in Fig. 1, less than 8% of total LDH activity was released with any of the cell concentrations used. In addition, dye exclusion studies indicated that, at all the cell concentrations used, greater than 96% of the cells were capable of excluding Trypan blue.

Release of 35S with 10 × 10⁶ cells represented approximately 58% (9,454 cpm) of the total available 35S, since HCl hydrolysis of five cartilage pieces released 16,300 cpm. The β-G released from the same number of cells (10 × 10⁶) accounted for 40% of the total enzyme activity in the cell lysate (legend, Fig. 1). Although there was a parallelism between the absolute amount of 35S and β-G release, the percentage of the total 35S released from cartilage increased with cell number, whereas the percentage of total β-G decreased as cell numbers increased. Release of 35S was cumulative indicating that the enzyme causing 35S release remained active and available to degrade substrate. When the time of incubation was varied under conditions where the number of leukocytes (5 × 10⁶) and the amount of cartilage remained constant, maximum marker enzyme release (β-G) occurred at 2 h, whereas, there was a linear increase in 35S release.
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Fig. 1. Relationship between the number of human leukocytes and nonphagocytic enzyme release. Five cartilage disks were incubated at 37°C with varying numbers of human leukocytes for 4 h at pH 7.4. Release of \( ^{35}S \) cpm is a measure of neutral protease activity and OD values (540 nm) define \( \beta \)-G activity. Total \( ^{35}S \) per sample was 16,000 cpm. \( \beta \)-G release from cartilage coated with AHGG during a 4 h incubation was 0.01 OD U, while \( ^{35}S \) release was 320 cpm. Total \( \beta \)-G activity in cell lysates of 0.5, 1.0, 5.0, and 10.0 \( \times 10^6 \) cells was 0.06, 0.11, 0.52, and 1.0 OD U, respectively. Each point represents the Mean ± SEM of three separate determinations. Symbols: ▲—▲, release of \( \beta \)-G from intact cells in the presence of AHGG-coated cartilage; ●——●, release of \( ^{35}S \) from intact cells in the presence of AHGG-coated cartilage.

through 4 h (Fig. 2). During the course of these experiments there was no significant release of LDH into the incubation medium.

Analysis of the Specificity of Enzyme Release.—In the experiments illustrated in Figs. 1 and 2, the cartilage was preincubated with AHGG. In order to define the specificity of AHGG in causing nonphagocytic enzyme release, cartilage was also preincubated with buffer, 2% HGG or 2% AHGG (HGG protein). The results in Fig. 3 indicate that 5 \( \times 10^6 \) leukocytes caused the release of significant amounts of \( ^{35}S \) and \( \beta \)-G only when cartilage was precoated with AHGG. Uncoated cartilage and cartilage coated with HGG released 3,000–4,500 cpm. In contrast to this, the human leukocytes provoked the release of 13,300 cpm from cartilage coated with AHGG. In all instances, release of \( \beta \)-G activity paralleled release of \( ^{35}S \). Less than 8% of the total LDH contained in 5 \( \times 10^6 \) leukocytes was released into the incubation medium (Fig. 3).

Three concentrations of gold thiomalate and colchicine in triplicate tubes were included in the incubation media of 5 \( \times 10^6 \) leukocytes and opsonized (AHGG) \( ^{35}S \)-labeled cartilage disks. In Table I it can be seen that release of
Fig. 2. Relationship of incubation time to nonphagocytic enzyme release from human leukocytes. Leukocytes (5 x 10^6) were incubated with five cartilage disks for 4 h (37°C) at pH 7.4. Total 35S per sample was 16,800 cpm, and release of 35S by lysates of 5 x 10^6 cells was 9,447 cpm. Total β-G activity in the lysate of 5 x 10^6 cells was 0.55 OD U (540 nm). The lysates also caused a change in OD/min (366 nm) for lactic dehydrogenase of 0.331 OD U. Each point represents the Mean ± SEM of three separate determinations. Symbols: ▲--▲, release of β-G from intact cells in the presence of AHGG-coated cartilage; ○--○, release of 35S from cartilage coated with AHGG (no cells); ●--●, 35S release in the presence of 5 x 10^6 human leukocytes. ▲----▲, release of LDH from intact cells in the presence of AHGG-coated cartilage.

β-G was unaffected by concentrations of 10^-4M, 10^-5M, or 10^-6M gold, whereas a concentration-dependent inhibition of cartilage degradation occurred (85%, 55%, and 33%, respectively). In contrast to this, colchicine, at 10^-4M and 10^-5M, inhibited the release of both β-G and 35S. In order to analyze the differential effects of gold and colchicine, another series of experiments was performed in which gold or colchicine was incubated with lysates from granules of 5 x 10^6 human leukocytes. The results of these experiments are given in Table II. Under these conditions colchicine, even at a concentration as high as 10^-4M, was ineffective in preventing the granule lysates from causing the release of 35S from labeled cartilage. Gold, however, at 10^-4M and 10^-3M, inhibited significantly cartilage destruction (79% and 63%, respectively) induced by human granule lysates.

Comparison of Phagocytic and Nonphagocytic Release of Enzymes and the Effect of Serum.—The supernatant from cells preincubated with a suspension of AHGG (phagocytic enzyme release) for 2 h at 37°C (after centrifugation) was
Fig. 3. Requirement of AHGG for the selective nonphagocytic release of neutral protease and $\beta$-G from human leukocytes. 10 cartilage disks were incubated with $5 \times 10^6$ human leukocytes for 4 h at 37°C (pH 7.4). Cartilage coated with either AHGG or nonaggregated HGG. Total $^{35}$S in 10 cartilage disks equals 29,120 cpm. Lysates of $5 \times 10^6$ cells contained 0.625 OD U of $\beta$-G activity and yielded a change in OD/min (366 nm) for LDH of 0.44. Each point is the Mean ± SEM of three separate determinations.

Recent reports have illustrated two important phenomena relating to the role of human leukocytes in the pathogenesis of tissue damage secondary to
tissue localization of antigen/antibody complexes. Firstly, human leukocytes contain enzymes, active at neutral pH, capable of destroying the chondromucoprotein matrix of cartilage (1, 14) and collagen fibrils (15, 16). Secondly, lysosomal constituents are released when human leukocytes phagocytose, or attempt to phagocytose, immune complexes (2, 3, 6, 17-19). The purpose of the

| TABLE I |
|------|
| Effect of Gold Sodium Thiomalate and Colchicine on Nonphagocytic Release of Neutral Protease and β-Glucuronidase from Human Leukocytes |

| Experimental condition | Neutral protease (3S; cpm) | β-glucuronidase (OD, 540 nm) |
|-----------------------|---------------------------|-----------------------------|
| Cartilage alone‡      | 786 ± 10                  | —                           |
| Cartilage + cells     | 7,116 ± 95                | 0.382 ± 0.007               |
| Cartilage + cells + gold (10⁻⁴ M) | 1,140 ± 25 | 0.391 ± 0.010               |
| Cartilage + cells + gold (10⁻⁵ M) | 2,934 ± 40 | 0.373 ± 0.008               |
| Cartilage + cells + gold (10⁻⁶ M) | 4,845 ± 90 | 0.360 ± 0.008               |
| Cartilage + cells + colchicine (10⁻⁴ M) | 3,625 ± 50 | 0.175 ± 0.001               |
| Cartilage + cells + colchicine (10⁻⁵ M) | 5,430 ± 105 | 0.200 ± 0.010               |
| Cartilage + cells + colchicine (10⁻⁶ M) | 7,100 ± 95 | 0.340 ± 0.008               |

* 5 X 10⁶ human leukocytes incubated with cartilage for 4 h at 37°C in Hanks' BSS + 1% glucose. Cartilage coated with AHGG.
† Five cartilage disks/vial = 13,200 cpm.

| TABLE II |
|------|
| Effect of Gold and Colchicine on Release of 3S from Labeled Cartilage by Granule Lysates of Human Leukocytes |

| Experimental* condition | 3S cpm† |
|------------------------|---------|
| Cartilage alone        | 459 ± 10 |
| Cartilage + granule lysate | 14,727 ± 150 |
| Cartilage + granule lysate + colchicine (10⁻³ M) | 13,845 ± 100 |
| Cartilage + granule lysate + gold (10⁻⁴ M) | 3,126 ± 20 |
| Cartilage + granule lysate + gold (10⁻⁵ M) | 5,580 ± 85 |

* Buffer in all experiments was Hanks' BSS containing 1% glucose; 5 X 10⁶ human leukocytes.
† 10 cartilage disks/sample coated with AHGG = 25,200 cpm total (HCl).
TABLE III

Nonphagocytic and Phagocytic Release of Neutral Protease and \( \beta \)-Glucuronidase from Human Leukocytes

| Experimental condition | Nonphagocytic release* | Phagocytic release† | Preincubation condition |
|------------------------|------------------------|---------------------|------------------------|
|                        | Marker                  | No serum            | 10%* serum             | Marker                  | No serum            | 10% serum            |
| Cartilage (coated with HGG) | \( ^{35} \)S (cpm) | 390                  | 441                    | Cells + buffer          | \( ^{35} \)S (cpm) | 492                  | 542                    |
|                        | \( \beta \)-G (OD)      | 0.050                | 0.062                  | \( \beta \)-G (OD)      | 0.021                | 0.044                |
| Cartilage (coated with HGG) + cells | \( ^{35} \)S (cpm) | 9,960                | 1,911                  | Cells + AHGG            | \( ^{35} \)S (cpm) | 2,373                | 440                    |
|                        | \( \beta \)-G (OD)      | 0.305                | 0.318                  | \( \beta \)-G (OD)      | 0.160                | 0.179                |
| Cell lysate + cartilage | \( ^{35} \)S (cpm) | 15,348               | 3,132                  |                         |                      |                      |
|                        | \( \beta \)-G (OD)      | 0.655                | 0.657                  |                         |                      |                      |

* Buffer in all experiments was Hank's BSS containing 1% glucose, with or without 10% isologous serum: \( 5 \times 10^6 \) human leukocytes were used.
† After 2 h incubation supernatant was incubated with five \( ^{35} \)S cartilage disks for another 4 h.
§ Filters cartilage disks/sample \( = 15,000 \) cpm total (HCl).

The present studies was to relate the above findings to a common pathogenic mechanism in a simple in vitro model where neutral protease released from viable leukocytes specifically degrade protein mucopolysaccharide matrix from intact cartilage.

In a previous report, evidence was presented that release or solubilization of \( ^{35} \)S-labeled anionic material from rabbit ear cartilage was a measure of enzymatic degradation of the noncollagenous chondromucoprotein matrix (1). Such degradation could be achieved with enzymes obtained from granule lysates of human but not guinea pig or rabbit leukocytes. We now demonstrate that human leukocytes overlying cartilage to which aggregated human IgG is bound, have the capacity to release neutral protease that subsequently degrade the protein mucopolysaccharide matrix. Cells overlying noncoated cartilage or cartilage coated with nonaggregated IgG do not release proteases and, therefore, no cartilage degradation is observed. Thus, enzyme release appears to be specific for aggregated IgG. A similar, but no greater, effect is achieved by coating cartilage with washed precipitates of aggregated IgG preincubated with high titer human rheumatoid factor serum.2 Several lines of evidence indicate that the viability of leukocytes was maintained during the process of enzyme release and cartilage degradation. During the course of a 5 h incubation, the cells excluded Trypan blue dye. Noncoated cartilage, and HGG-coated cartilage were not degraded in the presence of overlying viable cells, and LDH determinations indicated that cytoplasmic components were not released into the incubation medium. Finally, the kinetics of the dose-response experiments indicated that maximum degradation occurred with \( 10 \times 10^6 \) cells and a

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further increase in cell number did not adversely affect the cartilage in spite of the fact that lysates of the same number of cells induced two to three times greater destruction of matrix.

When incubation time was varied, release of $\beta$-G from cells began immediately but was essentially complete at 2 h. These data confirm previous observations that maximal enzyme release occurs 1–2 h after initiation of phagocytosis (20). Cartilage matrix degradation (release of $^{35}$S) continued in a linear fashion up to at least 4 h, indicating that the neutral protease was relatively stable after being released and remained active under the experimental conditions.

The release of $\beta$-G and neutral protease was compared under phagocytic and nonphagocytic experimental conditions. Release of enzymes was two to four times greater during nonphagocytic release, which is in agreement with the data of Henson (6) who showed that complexes fixed to nonphagocytosable surfaces were more effective in inducing enzyme release from leukocytes than when such complexes are in suspension (21). Although complete evidence is lacking as to the fate of all antigen/antibody complexes in joint fluid from rheumatoid arthritics, some are removed by phagocytosing synovial lining cells and cells suspended in the synovial fluid (22, 23). In addition, it is also possible that some complex becomes fixed to the surface of the articular cartilage. In support of this is the observation that in experimentally-induced synovitis in rabbits, antigen/antibody complexes are retained preferentially in the intra-articular space and probably bound to articular cartilage (24). It is reasonable to suspect, from these observations, that in human arthritic disease immune complexes could bind to articular cartilagenous structures. The cartilage would then be susceptible to the degradative action of neutral proteases released from leukocytes under nonphagocytic enzyme release conditions.

Release of lysosomal enzymes cannot be attributed to the presence of platelets since these cells did not release detectable amounts of $\beta$-G or neutral protease under the experimental conditions described. Phagocytic and nonphagocytic release of lysosomal enzymes did not require the intact complement system since enzyme release was observed with or without fresh isologous serum. Similar findings were reported by Weissmann et al. (2).

By the use of appropriate inhibitors it was possible to distinguish two events comprising the process by which viable cells, adhering to cartilage, destroy the protein mucopolysaccharide matrix. Both gold sodium thiomalate and colchicine inhibited cartilage destruction during nonphagocytic enzyme release by human leukocytes. However, the mechanisms of action of these two compounds were completely different. Colchicine inhibited the release of enzymes (neutral protease, $\beta$-G) but not the activity of the released enzymes, since it was virtually ineffective in preventing the cartilage destruction by cell lysates. In contrast, gold inhibited the activity of the released neutral protease without affecting the release process, as evidenced by the finding that gold inhibited cartilage destruction without affecting the release of $\beta$-G. These results might explain
the therapeutic effect of gold salts in the treatment of rheumatoid arthritis since it has been shown that gold salts, when administered parenterally, accumulate in the granules of both synovial lining cells and infiltrating leukocytes (25, 26).

Isologous human serum (10%), like gold, inhibited neutral protease activity without affecting the enzyme release process. Perhaps this might argue against the relevance of neutral protease activity in vivo since vascular permeability is increased in most inflammatory arthritides, and at least some plasma constituents would be expected to contaminate the joint fluid. However, it should be noted that a high degree of variability exists with regard to the concentrations of proteins and other constituents in joint fluids under pathological as well as normal conditions (27). It therefore would be important to conduct various experiments designed to examine the influence of synovial fluids on the release and activity of enzymes from human leukocytes. Experiments of this type are presently under way in our laboratories.

SUMMARY

The granule fraction of human leukocytes contains neutral protease capable of degrading the noncollagenous protein mucopolysaccharide matrix of cartilage at neutral pH in physiological salt solution. Cartilage degradation was monitored by quantitating the release of 35S from labeled rabbit ear cartilage. Degradation of cartilage matrix occurs when intact viable human leukocytes are incubated with cartilage opsonized with aggregated human gamma globulin (AHGG). During a similar 4 h incubation period cells did not degrade uncoated cartilage or cartilage coated with nonaggregated gamma globulin. Cells remain viable during the enzyme release process as evidenced by the absence of a cytoplasmic enzyme marker (lactic dehydrogenase) in the supernatant and dye exclusion studies. The release of 35S from labeled cartilage by human leukocytes in the presence of cartilage coated with AHGG (nonphagocytic enzyme release) was compared with the cartilage degrading activity of the supernatant from the same number of cells preincubated with a suspension of AHGG (phagocytic enzyme release). Nonphagocytic enzyme release by 5 × 10^6 cells provoked two to four times more 35S and β-glucuronidase (β-G) release from cartilage than phagocytic enzyme release conditions. β-glucuronidase was used as an indicator of the release of lysosomal granule enzymes.

By the use of selected pharmacological agents it was possible to dissociate the enzyme release process from intrinsic enzyme (neutral protease) activity. Neutral protease and β-G release by human cells in the presence of AHGG-coated cartilage was inhibited by 10^{-2} M colchicine, whereas the protease activity, but not the release process, was inhibited by 10^{-4} M gold thiomalate and 10% human serum. It is suggested that the release of a cartilage degrading neutral protease by viable human cells when exposed to AHGG might be a relevant model for the study of cartilage destruction as it occurs in rheumatoid arthritis.
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