Collagenase production by human mononuclear cells in culture: inhibition by gold containing compounds and other antirheumatic agents*

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SUMMARY  Human peripheral blood mononuclear cells in adherent cultures have been shown to synthesise and secrete collagenase. In the present study we have examined the modulation of collagenase production in these cultures by several antirheumatic agents. Incubation of monocytes in serum free medium with sodium aurothiomalate in concentrations varying from $7.7 \times 10^{-7}$ to $7.7 \times 10^{-3}$ mol/l resulted in marked dose dependent inhibition of the collagenase production. This inhibition was apparently selective in that total protein synthesis or the viability of the cells were not affected. Similar inhibition of the collagenase production was also noted with auranofin, aurothioglucose, and chloroauric acid. The inhibition with auranofin was achieved with a concentration as low as $7.4 \times 10^{-8}$ mol/l. To examine the mechanisms of the inhibition of the collagenase activity induced by sodium aurothiomalate the production of prostaglandin E$_2$ was also measured in the same cell cultures. Sodium aurothiomalate in concentrations $> 7.7 \times 10^{-4}$ mol/l significantly inhibited the prostaglandin E$_2$ production; the prostaglandin E$_2$ production was not inhibited, however, in $7.7 \times 10^{-5}$ mol/l concentration, while the collagenase production was reduced by 51.0%. Also, exogenous prostaglandin E$_2$ added to the cultures only slightly reversed the inhibition of the collagenase production by sodium aurothiomalate. Thus the inhibition of collagenase production by sodium aurothiomalate in human adherent mononuclear cell cultures appears to be independent of the inhibition of prostaglandin E$_2$ production. The inhibition of collagenase produced by monocyte-macrophages, as shown here in vitro, may contribute to the clinical efficacy of the compounds tested in the treatment of rheumatoid arthritis.

Key words: monocytes, sodium aurothiomalate, auranofin.

Monocyte-macrophages, the hallmark of chronic inflammatory lesions, have been shown to produce a variety of proteases, which are thought to mediate tissue destruction in disease. We have recently shown that adherent mononuclear cells cultured from human peripheral blood actively synthesise and secrete collagenase which is similar to other mammalian collagenases in that it cleaves native type I collagen in a single locus at a distance three-quarters from the amino terminus. The enzyme, which is secreted in an active form, requires calcium for its activity. The activity is enhanced by sulphydryl reagents such as N-ethylmaleimide. The enzyme production was shown to be stimulated by concanavalin A, and the total cumulative production in a 48 hour culture of the activated cells was shown to be of the same order of magnitude as the enzyme activity present in the same number of polymorphonuclear leucocytes. The enzyme production was shown to be exceptionally high in monocyte cultures established from three patients with haemochromatosis.

The destructive lesion of rheumatoid arthritis is characterised by degradation of the collagenous matrix in articular joints, leading to resorption of...
bone and cartilage. The degradation of collagen is conceivably mediated by the multiple collagenases produced by the cells present in the rheumatoid joint, including synovial cells, polymorphonuclear leucocytes, and monocyte-macrophages. Because the tissue macrophages are derived from circulating monocytes, cultures of peripheral blood mononuclear cells provide a system for studying the effects of various pharmacological agents on collagenase production by these cells. In the present study we have examined the effects of various agents which are in clinical use for the treatment of rheumatoid arthritis. The results indicate that some but not all antirheumatic agents inhibit the production of collagenase by cultured mononuclear cells.

Materials and methods

**Mononuclear cell cultures**

Peripheral blood mononuclear cells from healthy volunteers or from three patients with haemochromatosis, whose monocytes had been previously shown to be active in synthesising and secreting collagenase, were isolated by density gradient centrifugation on Ficoll-Hypaque (Histopaque-1077; Sigma Chemicals, St Louis, MO). The isolated mononuclear cells were pooled, washed in Hank's balanced salt solution (HBSS), and suspended in serum free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0-2% lactalbumin hydrolysate (DMEM-LH; Sigma Chemicals, St Louis, MO). To establish the monocyte cultures 1-2 x 10⁶ cells in 500 µl of DMEM-LH were plated in 24-well tissue culture plates (Corning, NY). The mononuclear cells were allowed to adhere for 120 min under 5% CO₂/95% air at 37°C. The medium containing the non-adhered cells was then removed, the adherent cells were washed with 500 µl of HBSS, and 500 µl of fresh, serum free DMEM-LH was added to the cultures. To enhance the production of collagenase the culture medium was supplemented with 20 µg/ml concanavalin A (con A; Difco, Detroit, MI); the test compounds (see below) were added at the same time. The incubations were then continued for varying time periods up to 72 hours at 37°C under 5% CO₂/95% air.

**Test compounds**

The following agents were tested in the mononuclear cell cultures for their effects on collagenase production: sodium aurothiomalate (Merck, Sharp & Dohme, West Point, PA); chloroauric acid trihydrate (Sigma, St Louis, MO); aurothioglucose (Sigma, St Louis, MO); auranofin (S-triethylphosphine gold, 2,3,4,6-tetra-O-acetyl-l-thio-β-d-glycopyranoside; Smith, Kline & French Laboratories, Philadelphia, PA); indomethacin (Sigma, St Louis, MO); d-penicillamine (β,β-dimethylcysteine; Merck, Sharp & Dohme, West Point, PA); methylprednisolone sodium succinate (Abbott Laboratories, North Chicago, IL); prostaglandin E₂ (Sigma, St Louis, MO). The test compounds were dissolved in DMEM-LH and then added to the incubation media in final concentrations indicated in the 'Results'. Sodium aurothiomalate, auranofin, indomethacin, d-penicillamine, and prostaglandin E₂ were initially dissolved in absolute ethyl alcohol and further diluted with DMEM-LH; the final concentrations of ethyl alcohol in the incubation media varied from 0-0002% to 1-92% (v/v), and the parallel controls received the corresponding concentrations of ethanol. All test compounds in the concentrations used were soluble, with the exception of sodium aurothiomalate in 7-7 x 10⁻³ mol/l concentration which produced a visible precipitate.

For comparison, cultures consisting of polymorphonuclear leucocytes (PMNs) were also established. For this purpose PMNs were isolated from peripheral blood by a discontinuous Ficoll-Hypaque density gradient (combination of Histopaque-1077 and 1119; Sigma, St Louis, MO). The isolated PMNs were cultured in 24-well microtitre plates, 1 x 10⁶ cells/well, as described above.

**Collagenase assay**

At different time points of cell incubation aliquots of the serum free media were assayed for collagenase activity by a sensitive, specific, and convenient method. In these assays [³H]proline labelled, native, triple helical type I collagen with a specific activity of 2.6 x 10⁶ cpm/µg was used as substrate. Details of this assay procedure have been reported previously. Briefly, in a standard assay the substrate (3 x 10⁶ cpm in 20 µl of 0.01 M acetic acid) was mixed with 10 µl of 0.5 M TRIS-HCl (trometamol), pH 7.8, containing 2.0 M NaCl, 0.1 M CaCl₂, and 0.1 M N-ethyl-maleimide (NEM), and with 10 µl of a solution containing bovine serum albumin 0.2 mg/ml. Aliquots (60 µl) of the tissue culture supernatant to be assayed for collagenase activity were then added, and the incubations were continued for 120 min at 37°C. The collagenase digestion was terminated by the addition of 50 µl of 0.2 M disodium ethylenediamine tetra-acetate (Na₂EDTA). Unlabelled soluble type I collagen, trypsin, and α-chymotrypsin in final concentrations of 0.2, 0.16, and 0.16 mg/ml, respectively, and dissolved in 0.2 M NaCl and 50 mM TRIS-HCl, pH 7.5, were then added to give a final volume of...
400 μl; the incubations were continued for another 20 minutes at 37°C. An equal volume of 20% (w/v) ice cold trichloroacetic acid (TCA) was then added, and the undigested, [³H]proline labelled substrate was precipitated by centrifugation. The radioactivity in the supernatant, representing collagen degradation, was counted with a liquid scintillation counter (Beckman LS-7500). Collagenase activity was expressed in units (U), where 1 U equals 1 μg collagen degraded per minute.

For assay of intracellular collagenase activity the medium was removed and the adherent monocytes were rinsed with phosphate buffered saline. DMEM-LH (500 μl) was added to each well, the cells were then detached by scraping with a rubber policeman, and homogenised with a Teflon-glass tissue homogeniser at 4°C. The homogenates were centrifuged at 18,000 g for 20 min, and the supernatants were assayed for collagenase activity as above.

**Other Assays**

Total protein synthesis by the cultured mononuclear cells was assayed by incubation of the cells with 30 μCi of [¹⁴C,²⁵³H]leucine (specific activity 151 Ci/mmol; Amersham) under conditions identical with those used for collagenase production. At the end of incubation protease inhibitors were added to the following final concentrations: 10 mmol/l Na₂EDTA, 10 mmol/l NEM, and 0-3 mmol/l phenylmethylsulphonyl fluoride. The adherent cells were detached by brief sonication, and the [³H]leucine labelled macromolecules in combined medium and cell fractions were precipitated by the addition of an equal volume of 20% TCA (w/v). The TCA precipitates were collected on Millipore prefilters (Millipore AP1502500; Millipore, Bedford, MA) on a vacuum manifold. The filters were air dried and counted in a Beckman LS-7500 liquid scintillation counter, as described previously.²

The viability of mononuclear cells was estimated by trypan blue dye exclusion test.¹² The concentration of prostaglandin E₂ was measured by a radioimmunoassay.¹³

**Statistical Analyses**

The results were evaluated for statistical significance by Student's *t* test.

**Results**

**Inhibition of Collagenase Production by Sodium Aurothiomalate**

The effects of antirheumatic agents on collagenase production were studied in adherent mononuclear cell cultures. We have shown that these cells synthesise and secrete collagenase in an active form, and the collagenase assays were performed in the presence of 10 mM NEM, thus allowing the determination of total enzyme activity.² In the first set of experiments sodium aurothiomalate was tested for its effect on collagenase production in adherent mononuclear cell cultures. Addition of sodium aurothiomalate in concentrations varying from 0.77 μmol/l to 7.7 μmol/l caused a significant reduction in collagenase production (Table 1 and Fig. 1a). This inhibition was not due to the salt form of the preparation, since sodium thiomalate in corresponding concentrations had no effect on the collagenase activity (Table 1).

To examine the possibility that sodium aurothiomalate might reduce the collagenase activity detectable in the cell culture media by directly inhibiting the collagenase assay, further in vitro experiments were performed. In these studies culture medium from the control cells was assayed for collagenase activity in the presence of sodium aurothiomalate in the same final concentrations as those present in the assays shown in Fig. 1. The addition of the gold compounds directly into the assay mixture had little, if any, effect on the collagenase activity (Fig. 1a).

In further experiments the activity of collagenase associated with the cell layer either in control cultures or in cultures incubated with 7.7 mM sodium aurothiomalate was assayed. As indicated previously,² very little intracellular enzyme activity was detectable in control monocyte cultures. In fact after 72 hours incubation the amount of enzyme activity detectable in the cell layer of control cells was less than 1% of that found in the medium. Similarly, essentially no activity was present in sodium aurothiomalate treated cells. This observation excludes the possibility that sodium aurothiomalate interferes with the secretion of collagenase and suggests that the inhibition takes place on the level of enzyme production. Additional experiments indicated that mixing of a 30 μl aliquot of control medium containing 29-6×10⁻² U of collagenase activity and 30 μl of cell extract with no detectable enzyme activity allowed the detection of 32-1×10⁻² U of enzyme activity. The latter result excludes the possibility that the cell extract contains an inhibitor for collagenase activity. Thus the reduction in collagenase activity present in the media of cultures treated with sodium aurothiomalate appears to result from reduced synthesis of the enzyme protein, rather than reflecting inhibition of the secretory process. The demonstration that sodium aurothiomalate minimally inhibited the con A stimulated release of PMN collagenase into the extracellular milieu also supported the latter suggestion. Specifically, sodium aurothiomalate in 77 μmol/l...
Table 1  Effects of sodium aurothiomalate, sodium thiomalate, aurothioglucose, and chloroauric acid on the production of collagenase in human monocyte cultures

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>Concentration (mol/l)</th>
<th>Collagenase production</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U x 10^-1/10^6 cells</td>
<td>% of control</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>20.8±0.6</td>
<td>100.0</td>
</tr>
<tr>
<td>Ethanol alone (0.1%)</td>
<td>—</td>
<td>22.0±0.2</td>
<td>105.8</td>
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<td>Sodium aurothiomalate</td>
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<td>7.9±0.4</td>
<td>35.9</td>
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<tr>
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<td>15.5</td>
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<td>2.9±0.2</td>
<td>3.2</td>
</tr>
<tr>
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<td>7.7 x 10^-7</td>
<td>21.7±0.5</td>
<td>104.3</td>
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<td>22.7±0.8</td>
<td>109.1</td>
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<tr>
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<td>21.8±0.4</td>
<td>104.8</td>
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<tr>
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<td>11.3±0.4</td>
<td>54.3</td>
</tr>
<tr>
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</tr>
<tr>
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<td>10.6</td>
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<tr>
<td>Chloroauric acid</td>
<td>1.0 x 10^-4</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Adherent mononuclear cell cultures were established from peripheral blood, and the cells were incubated in serum free medium for 72 hours with or without the test compounds; collagenase activity in the culture media was then assayed as indicated in "Materials and methods".

The values are mean±SEM of four parallel cultures.

Calculated as a percentage from the corresponding control either with or without 0.1% (v/v) ethanol used as solvent for sodium aurothiomalate.

Calculated by Student's t test; NS=statistically not significant.

Fig. 1  Inhibition of monocyte collagenase production by sodium aurothiomalate (gold sodium thiomalate, GST), auranofin, and indomethacin. Adherent mononuclear cells isolated from human peripheral blood were incubated in serum free DMEM for 48 hours with the test compounds in concentrations indicated. Aliquots of the media were assayed for collagenase activity (—), as indicated in the text. Culture media from the control cells were also assayed for collagenase activity in the presence of each of the test compounds in the same final concentrations as were present in the assays described above (— — —). Collagenase activity was expressed in units (U), where 1 U=1 µg collagen degraded per minute. The values represent mean±SEM from two parallel cultures each assayed in duplicate.
concentration inhibited such release of the enzyme only by 16-1%.

Further experiments indicated that the viability of cells was not affected by the incubation with sodium aurothiomalate. In control cultures 92.8% of the cells excluded trypan blue, indicating viability; the corresponding viability values in cultures incubated with 77 μM, 0.77 mM, or 7.7 mM sodium aurothiomalate were 94.8%, 92.7%, and 95.9%, respectively.

To assess the selectivity of the sodium aurothiomalate induced inhibition of the monocyte collagenase production, total protein synthesis was determined. For this purpose mononuclear cells were preincubated with 77 μM sodium aurothiomalate for 30 min, 23 hours, or 47 hours and pulse labelled with [3H]leucine for 60 min; the incorporation of radioactivity into TCA precipitable macromolecules was then determined. The results indicated that at all three time points examined the total protein synthesis in sodium aurothiomalate treated cultures was the same as that in the control cultures containing the corresponding concentration of ethanol (3×10⁻⁴%) (v/v) used as solvent (results not shown). Thus the reduction in collagenase production by sodium aurothiomalate is not a reflection of generalised inhibition of total protein synthesis.

Finally, mixing experiments were performed to exclude the possibility that sodium aurothiomalate rather than inhibiting collagenase production, induced the synthesis of an inhibitor of the enzyme activity. Mixing of equal volumes of control medium with medium from cell cultures incubated with 0.77 mM sodium aurothiomalate (which contained collagenase activity of about 4.9% of that detectable in control medium) resulted in a value average between the original samples (Fig. 2). Thus no evidence for induction of a collagenase inhibitor, in excess of the active collagenase, in the presence of sodium aurothiomalate was observed.

**INHIBITION OF COLLAGENASE PRODUCTION BY OTHER GOLD CONTAINING COMPOUNDS**

Several additional compounds were tested in mononuclear cell cultures. Addition of auranofin in concentrations varying from 7.4×10⁻⁸ to 7.4×10⁻⁴ mol/l significantly inhibited the collagenase production (Fig. 1b). Again, addition of auranofin directly into the assay system in concentration present during the enzyme measurements from media containing 7.4×10⁻⁶ mol/l auranofin had little, if any, effect on the activity of control enzyme (Fig. 1b). The viability of cells incubated with auranofin in concentrations up to 7.4×10⁻⁶ mol/l was unaffected. The viability of cells incubated with 7.4×10⁻⁵ or 7.4×10⁻⁴ mol/l auranofin, however, was slightly reduced when tested by trypan blue exclusion (89.2 and 71.1%, respectively, v 92.8% of cells viable in control cultures). Nevertheless, the incorporation of radioactive leucine, as an index of total protein synthesis, was not reduced in the presence of 7.4×10⁻⁷ mol/l of auranofin, as compared with controls (not shown).

Two additional gold containing compounds, aurothioglucose and chloroauric acid, also proved inhibitory for collagenase production (Table 1).

**D-PENICILLAMINE AND METHYLPREDNISOLONE DO NOT AFFECT COLLAGENASE PRODUCTION**

In further experiments two additional agents which
production of parallel determinations. In previous studies results indicated that the addition of PGE₂ alone in \(10^{-9}\) or \(10^{-7}\) mol/l concentration had little, if any, effect on the collagenase activity in mononuclear cell cultures (Fig. 4a). The results further indicated that the inhibition noted in the presence of \(7.7 \times 10^{-5}\) mol/l sodium aurothiomalate could be only partially reversed by the addition of exogenous PGE₂ (Fig. 4b).

In parallel experiments the production of PGE₂ was inhibited by incubation of cells with indomethacin. As expected, indomethacin in \(10^{-5}\) and \(10^{-4}\) mol/l concentrations completely inhibited the production of PGE₂ (not shown). In the same cultures the production of collagenase was reduced by 18.1% and 53.2%, respectively (Fig. 1c).

are in clinical use were tested for their effects on collagenase production. D-Penicillamine in 25 and 100 \(\mu\)g/ml had no effect on collagenase production in mononuclear cell cultures. Similarly, incubation of the cells for 48 hours with methylprednisolone in concentrations varying from \(10^{-9}\) to \(10^{-7}\) mol/l had no effect on the enzyme activity (results not shown).

**Fig. 3** Effect of sodium aurothiomalate (GST) on the production of prostaglandin E₂ (PGE₂) in adherent mononuclear cell cultures. PGE₂ production was determined by a radioimmunoassay in the same cultures as shown in Fig. 1a. The individual values are means of two parallel determinations.

**Fig. 4** Effects of exogenous prostaglandin E₂ (PGE₂) on sodium aurothiomalate induced inhibition of collagenase production in adherent mononuclear cell cultures. The cells were incubated with prostaglandin E₂ and sodium aurothiomalate (GST) in the concentrations indicated. Aliquots of the media were tested for collagenase activity, as described in the text. The values are mean ± SEM from four parallel determinations.
Discussion

In this study we have shown that several gold containing compounds readily inhibit the production of collagenase by adherent mononuclear cells in culture. These cells were activated by incubation with con A, and they actively synthesised and secreted collagenase. Two of the gold containing compounds, — namely, sodium aurothiomalate and auranofin, are in clinical use for treatment of rheumatoid arthritis. The inhibition of monocyte collagenase production by sodium aurothiomalate was achieved in vitro with concentrations which correspond to tissue levels clinically obtainable.\textsuperscript{17} Specifically, previous studies have detected a concentration as high as $3 \times 10^{-5}$ mol/l in synovial tissue after sodium aurothiomalate administration.\textsuperscript{18} Also, serum concentrations of $2-5 \times 10^{-5}$ mol/l can be attained and levels of $1-5 \times 10^{-5}$ mol/l are maintained during sodium aurothiomalate therapy.\textsuperscript{18} These values are clearly within the inhibitory range noted in our study. It is conceivable, therefore, that the inhibition of collagenase production, as observed in monocyte cultures, may contribute to the clinical efficacy of these compounds in vivo. It should be noted that the gold containing compounds can have multiple, independent effects on other macrophage functions, including chemotactic responsiveness,\textsuperscript{20} phagocytic activity,\textsuperscript{21} 22 and secretion of other macrophage enzymes.\textsuperscript{23} All these effects can contribute to the clinical response achieved by these compounds.

The mechanism of the inhibition of collagenase production in mononuclear cell cultures appears to involve inhibition of the enzyme protein synthesis. In support of this conclusion were several observations: Firstly, little, if any, intracellular enzyme could be detected in the cultures in which the collagenase production was inhibited, indicating that the inhibition was not due to a block in the secretion of the newly synthesised collagenase. Secondly, the compounds had no direct effect on the collagenase activity when added directly in vitro into the assay used for the enzyme determinations. Thirdly, the compounds had no effect on cell viability, as determined by the trypan blue exclusion test. Thus the inhibitory compounds appear to exert their effects on the level of collagenase gene expression. It is currently unknown, however, whether they affect the collagenase production by interfering with the rate of transcription of the collagenase gene or whether they alter the translational activity of the messenger ribonucleic acid (RNA) molecules corresponding to the collagenase protein. The mechanistic details of the inhibition will be addressed in further studies using collagenase specific cDNA probes which are currently being developed.\textsuperscript{24} It is of interest that two other pharmacological compounds clinically used for the treatment of rheumatoid arthritis, D-penicillamine and methylprednisolone, were not effective inhibitors of collagenase at the concentration levels which are conceivably achievable in vivo. These observations indicate that the beneficial effects of D-penicillamine in rheumatoid arthritis are attributable to other mechanisms which may involve modulation of the immunological and metabolic aspects of this disease.\textsuperscript{25} Previously it has been shown that the secretion of collagenase by mouse peritoneal macrophages is decreased by dexamethasone, cortisol, and triamcinolone acetonide.\textsuperscript{26} In the present study, however, methylprednisolone was not found to be an effective inhibitor of the production of collagenase by human mononuclear cells when tested in concentrations up to $10^{-7}$ mol/l.

Previous studies have suggested that collagenase production in a variety of cell systems may involve the prostaglandin pathway.\textsuperscript{27} For example, production of collagenase by synovial cells was decreased with inhibition of PGE\textsubscript{2} by the addition of indomethacin. Furthermore, indomethacin-induced reduction of the level of collagenase could be restored by PGE\textsubscript{2}. Similarly, peritoneal macrophages activated by endotoxin produced less collagenase in the presence of indomethacin;\textsuperscript{14} this inhibition could also be overcome by the addition of exogenous PGE\textsubscript{2}. These observations thus suggest an association between the collagenase production and prostaglandin pathway but do not prove a dependent relationship. In the present study sodium aurothiomalate markedly inhibited the production of PGE\textsubscript{2}. Sodium aurothiomalate in a concentration of $7.7 \times 10^{-5}$ mol/l, however, had no effect on PGE\textsubscript{2} production, while the collagenase production in the same cells was markedly reduced. At the same time, addition of exogenous PGE\textsubscript{2} failed to increase the collagenase production in mononuclear cell cultures or had only a small reversing effect on the sodium aurothiomalate induced inhibition of the collagenase production. It appears, therefore, that the inhibition of the collagenase production by sodium aurothiomalate is independent of the modulation of PGE\textsubscript{2} in monocyte cultures. This observation does not exclude the possibility that other mediators of the prostaglandin pathway, such as PGE\textsubscript{2a}, might participate in the collagenase modulation.

Rheumatoid arthritis is a complex connective tissue disorder in which several different immunological and biochemical mechanisms contribute to the disease process.\textsuperscript{28} 30 In an affected rheumatoid joint, collagen metabolism is altered at least at two
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different levels. Firstly, the active proliferative phase of pannus formation is characterised by increased biosynthesis of collagen and activated fibroblast proliferation, resulting in excessive deposition of connective tissue. At the same time there is degradation of collagen, resulting in destruction of the cartilage and ligaments, and resorption of the bones. The degradation of collagen is apparently mediated by a variety of collagenases which can originate from the synovial cells, fibroblasts, polymorphonuclear leukocytes, and monocyte-macrophages. Thus the inhibition of collagenase production by gold containing compounds, including aurothiomalate and auranofin, as shown here in monocyte cultures, may contribute to the clinical efficacy of these compounds in vivo.

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