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EFFECTS OF VARIOUS ANTI-INFLAMMATORY AND ANTI-RHEUMATIC AGENTS ON THE SYNTHESIS, SECRETION, AND ACTIVITY OF A CARTILAGE PROTEOGLYCAN-DEGRADING ENZYME AND OTHER MACROPHAGE ENZYMES

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Abstract—Enzymes released from the macrophage have been implicated in the connective tissue loss noted in a number of chronic inflammatory diseases. The effects of 2- and 3-day exposures to glucocorticosteroids, non-steroidal anti-inflammatory drugs and selected anti-rheumatic agents on release of a cartilage proteoglycan-degrading enzyme, and of β-glucuronidase, cathepsin D, and elastase from the thioglycollate-elicited mouse peritoneal macrophage were determined. The synthesis (and/or release) of the cartilage proteoglycan degrading enzyme was markedly inhibited following exposure to dexamethasone, flufenamic acid, chloroquine and gold sodium thiomalate. It was unaffected by exposure to aspirin, naproxen, indomethacin and *dl*-penicillamine, but was and stimulated by levamisole. Dexamethasone stimulated β-glucuronidase synthesis and release and inhibited elastase synthesis. The synthesis and release of β-glucuronidase, cathepsin D and elastase were not affected by non-toxic concentrations of indomethacin and chloroquine. These results demonstrate that certain anti-rheumatic and anti-inflammatory agents may derive at least a part of their therapeutic effects from the inhibition of enzyme release from the macrophage.

Enzymes derived from polymorphonuclear leukocytes (PMNs) and macrophages have been implicated in the connective tissue breakdown noted in a variety of diseases including rheumatoid arthritis [1–3], pulmonary emphysema [4–6], and periodontitis [7]. In rheumatoid arthritis, which is perhaps the most widely studied of these diseases, large numbers of PMNs are found free in the joint spaces; in addition, macrophage-like cells, located in granulation tissue termed pannus, are found adjacent to cartilage and bone [8, 9]. In animal models of arthritis and in patients, one finds evidence of tissue erosion, as noted both biochemically and morphologically, at the cartilage–pannus junction [10, 11]. These findings implicate macrophage-like cells as being, in part, responsible for this degradation. Enzymes derived from mononuclear cells isolated from rabbit bone marrow and mouse peritoneum are capable of degrading cartilage proteoglycan *in vitro*. The enzyme responsible is active at neutral pH, requires cations, and is not antagonized by serine protease or cathepsin D inhibitors [12, 13]. The enzyme is unable to degrade the collagen component of cartilage, hence is not a collagenase. It has some properties of an atypical elastase similar to that described in human rheumatoid synovial fluid [14].

The proteoglycan-degrading enzyme is but one of a large number of lytic enzymes present in the macrophage. The macrophage contains enzymes active at neutral pH, such as an elastase, collagenase and lysozyme, as well as those active at an acid pH [15–17]. To date, relatively few papers have appeared describing the effects of anti-inflammatory and anti-rheumatic agents on enzyme secretion from this cell, and none has appeared regarding the control of the proteoglycan-degrading enzyme secretion. In this paper we examine the effects of glucocorticosteroids, non-steroidal anti-inflammatory agents and anti-rheumatic drugs such as levamisole, penicillamine and gold sodium thiomalate, for their effects on the synthesis, secretion, and activity of selected macrophage enzymes.

METHODS

Cell culturing

Male or female Swiss–Webster mice, 20-25 g, were obtained from Simonsen Laboratories (Gilroy, CA). Peritoneal macrophages were harvested from mice injected i.p. 4 days previously with 1 ml thioglycollate medium (Difco, Detroit, MI). The cells, at a concentration of 2.5×10^6/ml, were incubated at 37° under 95% air/5% carbon dioxide in Medium 199 (Grand Island Biological Co., GIBCO, Madison, WI) supplemented with 10 I.U./ml sodium heparin (Sigma Chemical Co., St. Louis, MO), 2.5 μg/ml, fungazone (GIBCO), 0.5% gentamycin (Schering, Bloomfield, NJ), and 10 mM HEPES† buffer (GIBCO), plus 10% heat-inactivated fetal calf serum (GIBCO); after 2 hr, non-adherent cells were removed by vigorous washing and the cells were incubated a further 24 hr in the complete medium.

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†HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

At this time the medium was replaced with serumless medium (Neuman/Tytell, GIBCO) which contained 0.2% lactalbumin hydrolysate as a serum substitute [18], and drugs were added. Medium was collected at 2-day intervals for up to 6 days; drugs were replaced in the fresh medium. At the end of the incubation period the cells were lysed in saline containing 0.2% Triton X-100 and centrifuged, and the supernatant fraction was collected. Aliquots of the medium and lysate were assayed directly for β-glucuronidase, cathepsin D, and lactate dehydrogenase activity. The remainder were processed by dialyzing against distilled water, lyophilizing, and assaying for cartilage proteoglycan-degrading and elastase activity. Viability was estimated by trypan blue dye exclusion or lactic dehydrogenase leakage.

Enzyme assays

Cartilage degradation-^{35}S release. Cartilage proteoglycan-degrading activity was ascertained using ^{35}S-labeled rabbit ear cartilage [19]. New Zealand rabbits (L.I.T. Rabbitry, Aptos, CA), 1 kg, were injected intravenously with 750 μCi $Na_2\,^{35}SO_4$ (New England Nuclear Corp., Boston, MA). The animals were killed 18 hr later, and the ears were removed and stripped of their adhering soft connective tissue. Discs, approximately 3 mm in diameter, were punched from the ears. They were incubated at 37° under 95% air/5% carbon dioxide in Medium 199 containing 0.2% lactalbumin hydrolysate with or without cell products, i.e. macrophage medium, macrophage lysates, or other agents. After incubation the radioactivity (expressed as cpm) in the medium and discs (following hydrolysis in hydrochloric acid) was determined with a Packard liquid scintillation spectrometer. The medium cpm was converted to a percentage of the total, i.e.

$$\frac{\text{cpm medium}}{\text{cpm medium + cpm discs}} \times 100.$$

In previous studies we demonstrated that the ^{35}S-release correlated directly with cartilage proteoglycan release as determined by a spectrophotometric alcian blue dye complexing assay [12].

β-Glucuronidase assay. β-Glucuronidase (EC 3.2.1.31) was assayed using phenolphthalein glucoronide (Sigma Chemical Co.) as substrate. The reaction mixture consisted of 200 μl of sample, 200 μl of 0.1 M sodium acetate buffer (pH 4.5), and 75 μl of 0.75 μM substrate. Following a 6-hr incubation at 37°, the reaction was halted with 525 μl alkaline reagent (0.1 M glycine–0.4 M sodium hydroxide, 1:25) and optical density was determined at 540 nm [20].

Cathepsin D assay. Cathepsin D (EC 3.4.4.23) was assayed using tritium-labeled hemoglobin (New England Nuclear Corp.), sp. act. 1.8×10^4 cpm/μg. The reaction mixture consisted of 20 μl sample, 20 μl substrate in 0.1 M sodium phosphate buffer, 0.14 M sodium chloride and 0.05 M sodium acetate, (pH 7.4), and 20 μl of 1.0 M formate buffer (pH 3.4). Following a 1-hr incubation at 37°, 50 μl of 3% (w/v) cold hemoglobin in 0.05 M phosphate buffer (pH 7.0) containing 1 M potassium chloride was added; 10 min later 100 μl of cold 6% (w/v) trichloroacetic acid was added and the mixture was incubated for 90 min. Following a 5-min centrifugation at 15,000 *g*, the radioactivity in a 100-μl sample of the supernatant fraction was determined using a Packard liquid scintillation spectrometer [21].

Elastase assay. Elastase was assayed using 1 mg sodium dodecyl sulfate (SDS)-conjugated ^{3}H-reduced elastin (New England Nuclear Corp.), sp. act. 1.1×10^5 cpm/μg, in 200 μl of 200 mM Tris buffer (pH 8.3) containing 2 mM calcium, and 100 μl of sample dissolved in the same buffer. After a 1-hr incubation at 37°, the mixture was centrifuged at 15,000 *g* for 5 min and the radioactivity of 200 μl of the supernatant fraction was determined [22]. Pellets were hydrolyzed by incubation with pancreatic elastase for 12 hr and total counts were determined to ensure uniform substrate aliquots. The total activity varied by less than 10 per cent per sample.

Lactic dehydrogenase assay. Lactic dehydrogenase (EC 1.1.1.27) activity was determined by measuring the initial rate of oxidation of reduced nicotinamide adenine dinucleotide (Sigma Chemical Co.) in 0.030 M sodium phosphate buffer, pH 7.4 [23]. Protein was determined by the procedure of Lowry *et al.* [24].

RESULTS

Cartilage proteoglycan-degrading enzyme

The substrate used in these studies, native rabbit ear cartilage pre-labeled with ^{35}S, released 15–25 per cent of the ^{35}S during the first 24-hr culture period. The culture medium in which 5×10^6 macrophages had been incubated for the first 2-day interval induced release of approximately 40 per cent ^{35}S in the cartilage discs during a 24-hr incubation period; discs incubated with culture medium, with or without trypsin plus soya bean trypsin inhibitor (SBTI), released between 10 and 20 per cent of the ^{35}S. Medium collected from macrophages maintained for a second 2-day interval induced release of 70–90 per cent of the ^{35}S. As shown previously, the enzyme could be activated by trypsin and was unaffected by serine protease inhibitors, such as phenylmethanesulfonyl fluoride, SBTI and elastatinal, and the cathepsin D inhibitor, pepstatin. It was inhibited by the chelating agents 1,10-phenanthroline and EDTA [12]. In the present studies, the culture medium collected at 2-day intervals was incubated with trypsin followed by SBTI prior to assay.

Effects of glucocorticosteroids on the secretion of the cartilage proteoglycan-degrading enzyme

The culture medium from cells exposed to dexamethasone (10^{-10} to 10^{-6} M for 2 days) contained 25–80 per cent less enzyme than medium from non-drug exposed controls. Following 4 days of exposure to 10^{-10} M dexamethasone, the extracellular enzyme activity was reduced by nearly 70 per cent; a maximal inhibition of 90 per cent was noted following a 4-day exposure to 10^{-6} M. Following exposure to 10^{-10} to 10^{-6} M dexamethasone, the intracellular activity was reduced by 36–90 per cent. Hydrocortisone was also an effective inhibitor; after 4 days of exposure to 10^{-6} M, the extracellular and intracellular enzyme activities were reduced by nearly 60 per cent (Fig. 1). Neither agent affected viability as estimated by trypan blue dye exclusion.

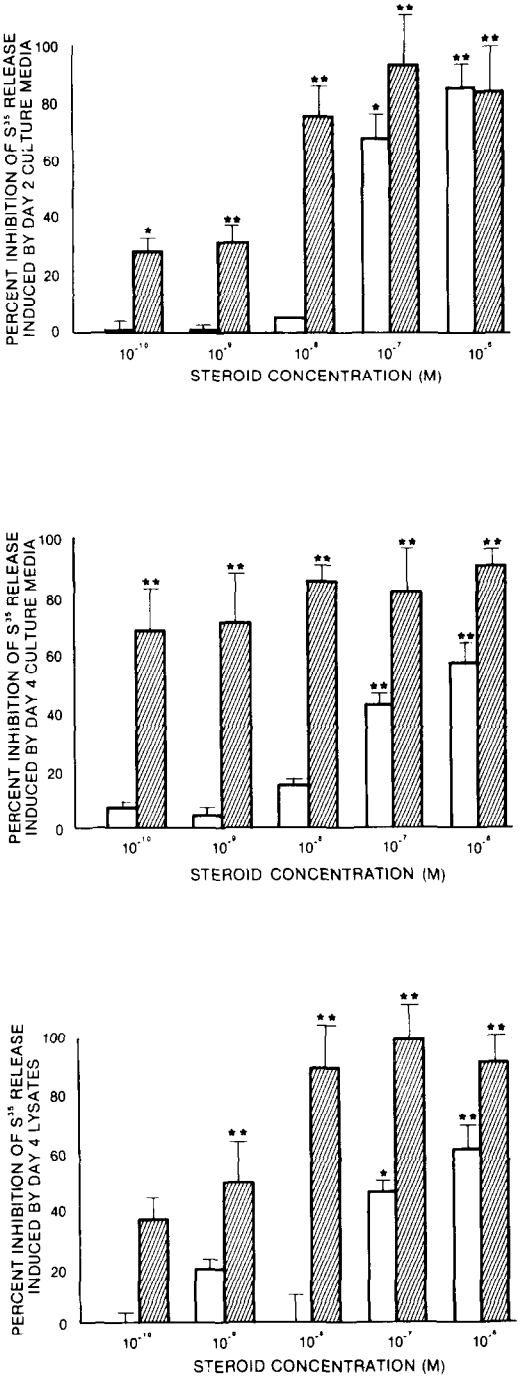

Fig. 1. Effects of hydrocortisone (clear) and dexamethasone (cross-hatched) on the medium and lysate activities of cartilage proteoglycan-degrading enzyme. Thioglycollate-elicited mouse peritoneal macrophages were incubated for 24 hr in the absence of drug and for two, 2-day intervals with drug. At the end of the second 2-day interval the cells were lysed, and the medium and lysate proteoglycan-degrading activities were determined. The processed culture medium from 5×10^6 cells, collected on days 2 and 4, induced 58.9 ± 4.3 and 82 ± 0.8 per cent ^{35}S release (mean ± S.D.; N = 4). Lysates of 5×10^6 cells collected after the second 2-day culture period induced 54.3 ± 9.3 per cent ^{35}S release (mean ± S.D.; N = 4). An asterisk (*) indicates $P < 0.05$; double asterisks (**) indicate $P < 0.01$.

Effects of non-steroidal anti-inflammatory agents on the secretion of the cartilage proteoglycan-degrading enzyme

Indomethacin and naproxen (10^{-6} to 10^{-4} M) and aspirin (10^{-6} to 10^{-3} M) did not significantly affect the secretion of the cartilage proteoglycan-degrading enzyme. Aspirin (10^{-4} M) slightly stimulated the intracellular activity. Flufenamic acid (10^{-4} M) significantly reduced the activity in the medium on days 2 and 4, and it reduced the intracellular activity by 83.9 ± 8.0 per cent (Fig. 2). No indication of toxicity, based on trypan blue dye exclusion, was noted. In

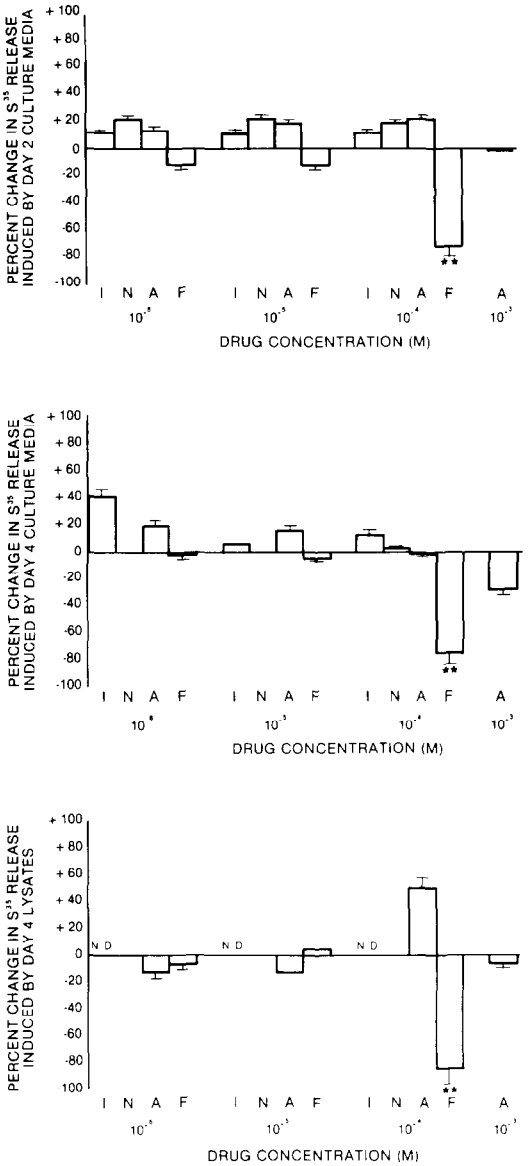

Fig. 2. Effects of indomethacin (I), naproxen (N), aspirin (A) and flufenamic acid (F) on medium and lysate activities of cartilage proteoglycan-degrading enzyme. Cells were incubated as described in Methods. Proteoglycan-degrading activity from non-drug exposed cells was comparable to that noted in the legend of Fig. 1. Double asterisks (**) indicate $P < 0.01$.

a separate experiment, flufenamic acid (10^{-5} and 10^{-4} M) was examined as a direct antagonist of the enzyme. When incubated with the products of 5×10^6 cells, it had no inhibitory activity (data not presented).

Effects of anti-rheumatics on the secretion of the cartilage proteoglycan-degrading enzyme

Gold sodium thiomalate (10^{-6} to 10^{-4} M) did not significantly affect the extracellular or intracellular activity of the proteoglycan-degrading enzyme. Following 2 and 4 days of exposure to 10^{-3} M, the extracellular activities were reduced by 39 and 29 per cent ($P < 0.01$), respectively, while the lysate activity and cell viability were unaffected (Table 1). At 10^{-3} M it had no direct effect on enzyme activity (data not presented). Cells exposed to 10^{-6} M levamisole had a 95 per cent increase ($P < 0.01$) in lysate activity on day 4; no significant effects were noted following exposure to 10^{-5} and 10^{-4} M levamisole (Table 1). Exposure to *dl*-penicillamine as well as to *d*-penicillamine (data not presented), 10^{-6} to 10^{-4} M, did not significantly affect extracellular or intracellular activity (Table 1). Exposure to chloroquine (10^{-5} and 10^{-4} M) led to reduced extracellular activity on days 2 and 4 intracellular activity was reduced by more than 92 per cent at 10^{-4} M and was unaffected by 10^{-5} M exposure (Fig. 3). Over 95 per cent of the cells exposed to 10^{-6} and 10^{-5} M were viable, whereas only 50 per cent of those exposed to 10^{-4} M were viable. Chloroquine (10^{-5} and 10^{-4} M) had no direct inhibitory effect on the enzyme (data not presented).

Effects of dexamethasone, indomethacin, and chloroquine on the secretion of β-glucuronidase, cathepsin D and elastase

In an effort to determine changes in enzymes other than the one capable of degrading the cartilage proteoglycan, selected agents were incubated with the peritoneal exudate cells for various periods of time, and the medium and lysate were assayed for β-glucuronidase, cathepsin D, and elastase activities as well as for the activity of lactic dehydrogenase, the cytoplasmic enzyme. Following exposure to dexamethasone (10^{-8} to 10^{-6} M) intra- and extracellular β-glucuronidase activities were consistently elevated. In each of three experiments, a 3-day exposure to 10^{-6} M dexamethasone led to 39, 39 and 97 per cent increases in extracellular β-glucuronidase activity. The intra- and extracellular cathepsin D activities were not consistently affected (Table 2). Release of lactic dehydrogenase was not affected (data not presented). In one experiment, aliquots from a common sample were used to assay the cartilage proteoglycan-degrading, and other, enzymes. Exposure to

Table 1. Effects of selected anti-rheumatic agents on release and/or activity of cartilage proteoglycan-degrading enzyme from thioglycollate-elicited mouse peritoneal macrophages*

Drug	Concn (M)	% change from non-drug-treated cultures†		
		Medium activity		Lysate activity
		Day 2	Day 4	Day 4
Gold sodium thiomalate‡	10^{-6}	−1 ± 0	9 ± 1	7 ± 2
	10^{-5}	14 ± 2	9 ± 1	12 ± 1
	10^{-4}	18 ± 1	5 ± 1	3 ± 0
	10^{-3}	−39 ± 12§	−29 ± 6§	5 ± 0
Levamisole‖	10^{-6}	46 ± 11	−10 ± +1	95 ± 7§
	10^{-5}	15 ± 2	− 2 ± +0	11 ± 7
	10^{-4}	16 ± 7	−11 ± +2	9 ± 2
di-Penicillamine‖	10^{-6}	16 ± 5	−2 ± 0	−16 ± 9
	10^{-5}	16 ± 4	−5 ± 1	−26 ± 10
	10^{-4}	−27 ± 5	−13 ± 2	−11 ± 1

* Cells were removed and incubated as described in Methods.

† Each value indicates the mean ± S.D. from four replicates. A minus sign indicates an inhibition.

‡ Conditioned medium from 5×10^6 cells induced 79.8 ± 6.5 and 89.7 ± 1.7 per cent ^{35}S release on the first and second 2-day intervals; lysates of 5×10^6 cells induced 56.3 ± 2.6 per cent ^{35}S release (mean ± S.D.; N = 4). Cartilage autolysis was 17.5 ± 1.7 per cent ^{35}S release.

§ $P < 0.01$, as determined by Student's *t*-test, when compared with non-drug-treated control.

‖ Conditioned medium from 5×10^6 cells induced 37.4 ± 4.4 per cent and 79.5 ± 3.1 per cent ^{35}S release on the first and second 2-day intervals; lysates of 5×10^6 cells induced 33.5 per cent ^{35}S release. Cartilage autolysis was 19.3 ± 6.2 per cent ^{35}S release.

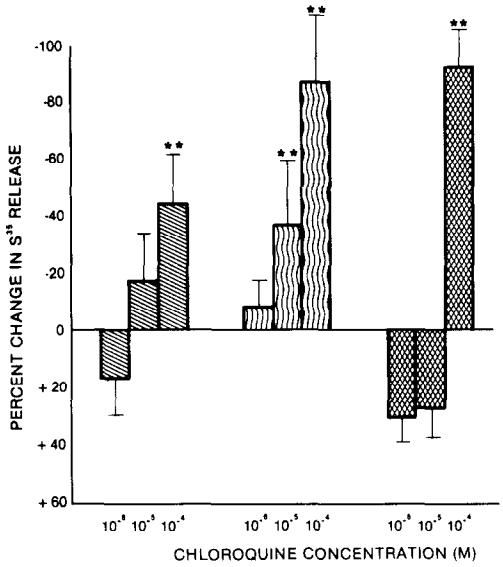

Fig. 3. Effects of chloroquine on the medium and lysate activities of the cartilage proteoglycan-degrading enzyme. Cells were incubated as described in Methods. Proteoglycan-degrading activity from non-drug exposed cells was comparable to that noted in the legend of Fig. 1. The diagonally lined areas indicate activities of day 2 media; the vertical-lined areas indicate day 4 media; and the cross-hatched areas indicate day 4 lysate activities. Double asterisks (**) indicate $P < 0.01$.

10^{-6} M dexamethasone, led to reduced extracellular activities of the proteoglycan-degrading enzyme and elastase, enhanced extracellular β-glucuronidase activity, and reduced intracellular cathepsin D activity (Table 3). Viability, as determined by LDH release and trypan blue dye exclusion, was not affected following 4 days of exposure to dexamethasone.

The amount of enzyme released from macrophages exposed to indomethacin for 3 days was extremely variable. In a series of five experiments, the extra- and intracellular β-glucuronidase and cathepsin D activities did not change by more than 10 per cent on the average following incubation with indomethacin (10^{-6} to 10^{-4} M). Exposure to indomethacin (10^{-6} and 10^{-5} M) for two 2-day intervals, led to little change in the extra- or intracellular activities of β-glucuronidase, cathepsin D and the proteoglycan-degrading enzyme (Table 4). Following 4 days of exposure to 10^{-4} M, approximately 25 per cent of the total lactic dehydrogenase was present extracellularly indicating significant toxicity. The diminished activities of β-glucuronidase and cathepsin D reflected this toxicity (Table 4).

Following exposure to chloroquine, the intracellular cathepsin D and elastase activities were reduced. In a series of three experiments, 10^{-5} M chloroquine reduced the total cathepsin D activities by 15, 24 and 28 per cent; elastase activity, which was found primarily extracellularly, was reduced by approximately 50 per cent. Following exposure to 10^{-4} M chloroquine, significant toxicity was noted as indicated by an elevated lactic dehydrogenase release; the cathepsin D activities were reduced by 19, 47 and 71 per cent in the three experiments. Total cellular protein decreased in a concentration-dependent fashion. Chloroquine appeared to have a paradoxical biphasic effect on β-glucuronidase activity; at low concentrations total enzyme activity tended to be decreased. At a toxic concentration (10^{-4} M), the total enzyme activity was elevated in two of three experiments, and all the enzyme was present extracellularly.

Table 2. Change in β-glucuronidase and cathepsin D secretion and in protein content of mouse peritoneal macrophages incubated with dexamethasone for 3 days*

	% Change in enzyme activity or protein content following exposure to dexamethasone†						
	β-Glucuronidase			Cathepsin D			Protein content
	Medium	Lysate	Total	Medium	Lysate	Total	
Dexamethasone (10^{-7} M)							
Expt. 1	7	61	26	17	−18	0	35
2	39	67	56	6	87	42	7
3	97	134	109	46	25	39	17
Dexamethasone (10^{-6} M)							
Expt. 1	39	61	47	50	−27	13	24
2	39	43	42	−36	59	6	7
3	97	110	101	−56	40	−25	7

* Thioglycollate-elicited peritoneal macrophages were incubated at 2×10^6 cells/2 ml in Medium 199 + 10% fetal cell serum for 24 hr. Non-adherent cells were removed and the remaining cells were cultured in Neuman/Tytell medium. Dexamethasone was added, and the cultures were maintained for a 3-day period, at which time the media and lysates from triplicate cultures were pooled and analyzed for β-glucuronidase, cathepsin D, and total protein.

† Each value is the percent change from non-drug-treated cultures. Control β-glucuronidase and cathepsin D activities and protein content in non-drug-treated cultures varied as follows: β-glucuronidase (media), 348–1800 μg/10^6 cells; β-glucuronidase (lysate), 150–3000 μg/10^6 cells; cathepsin D (media), 4–49 μg/10^6 cells; cathepsin D (lysate), 2–39 μg/10^6 cells; and protein, 75–661 μg/10^6 cells.

Table 3. Effect of dexamethasone on the release and content of selected enzymes from the mouse peritoneal macrophage*

	β-Glucuronidase (μg/10⁶ cells)			Cathepsin D (μg/10⁶ cells)			Elastase (ng/10⁶ cells)			Cartilage proteoglycan-degrading enzyme (% ^{35}S rel/10⁶ cells)		
	Medium	Lysate	Total	Medium	Lysate	Total	Medium	Lysate	Total	Medium	Lysate	Total
Day 2												
Control	317	137	454	58	120	178	1.0	0.2	1.2	11.0	2.3	13.3
Dexamethasone (10^{-6} M)	332	264	596	62	86	148	0.5	0.2	0.7	3.8	1.2	5.0
Day 4												
Control	316	179	495	82	130	212	3.0	0.4	3.4	14.7	4.9	19.6
Dexamethasone (10^{-6} M)	380	116	496	59	60	119	0.2	0.0	0.2	6.5	1.6	8.1

* Pooled cells were cultured as described in Methods. Aliquots of the medium, collected at the end of the first and second 2-day incubation periods, and of the lysate were assayed for β-glucuronidase and cathepsin D activities. The remainder was dialyzed, lyophilized, reconstituted, and assayed for elastase and cartilage proteoglycan-degrading activities. For details of effects, see Fig. 1. Media and lysates from four cultures were combined, and values indicate means of replicate determinations.

Table 4. Effect of indomethacin on the release and content of selected enzymes from peritoneal mouse macrophages*

	β-Glucuronidase (μg/10^6 cells)			Cathepsin D (μg/10^6 cells)			Elastase (ng/10^6 cells)			Cartilage proteoglycan-degrading enzyme (% ^{35}S rel/10^6 cells)		
	Medium	Lysate	Total	Medium	Lysate	Total	Medium	Lysate	Total	Medium	Lysate	Total
Day 2												
Control	532			11.5			2.2			11.0		
Indomethacin												
10^{-6} M	624			14.1			4.2			10.1		
10^{-5} M	624			14.0			3.4			10.3		
10^{-4} M	592			9.6			3.8			10.2		
Day 4												
Control	624	780	1404	8.2	25.6	33.8	2.8	1.0	3.8	15.6	6.3	21.9
Indomethacin												
10^{-6} M	908	908	1816	11.3	25.4	36.7	3.4	0	3.4	10.6	6.1	16.7
10^{-5} M	968	720	1688	10.0	23.0	33.0	3.8	0	3.8	14.9	4.4	19.3
10^{-4} M	564	280	844	5.1	14.0	19.1	4.2	0	4.2	14.4	5.4	19.8

* See Table 3 for general procedure followed.

DISCUSSION

The thioglycollate-elicited mouse peritoneal macrophage maintained in culture secretes a number of enzymes active at neutral and acid pH. Those active at neutral pH that were monitored in this study were an elastase and a cartilage proteoglycan-degrading enzyme; those active at acid pH included β-glucuronidase and cathepsin D. Continual secretion of macrophage neutral proteases, e.g. collagenase, elastase, lysozyme and plasminogen activator, has been reported by many investigators [15, 16, 18, 25]. The results obtained in the present study, especially with regard to the time course of neutral protease secretion, are consistent with these reports.

The nature of the cartilage proteoglycan-degrading enzyme is currently unknown. Its lack of activity in cleaving cartilage collagen indicates that it is not a collagenase [12]. Its inhibition profile, viz. inactivation by chelating agents but not by serine protease inhibitors, is similar to a macrophage elastase described by Dahlgren *et al.* [26]. Recent studies by Banda and Werb [27] are likewise consistent with this suggestion. Elastase derived from either porcine pancreas or human granulocytes is capable of degrading the proteoglycan moiety of cartilage [3]. Based on this inhibition profile [26] as well as on the finding that glucocorticosteroids and chloroquine antagonized the release of both the elastolytic and cartilage proteoglycan-degrading activities (see below), these enzymes may in fact be the same.

In the current studies, 33–75 per cent of the total β-glucuronidase was secreted into the culture medium during the 2-day intervals between medium changes (Tables 3 and 4). Cathepsin D was likewise actively secreted during the 2-day culture periods. Davies and co-workers [1], using resident macrophages, report that less than 10 per cent of the total β-glucuronidase was secreted into the culture medium over a 24-hr period. Our results are comparable to those reported by Schnyder and Baggilioni [28] and relate to the use of a thioglycollate-elicited, as opposed to a resident, cell population.

Glucocorticosteroids

Exposure to concentrations of dexamethasone as low as 10^{-10} M led to significant reductions in the synthesis and release of the cartilage proteoglycan-degrading enzyme; at 10^{-7} M the inhibition was more than 80 per cent. As mentioned above, the secretion of elastase was likewise inhibited to approximately the same extent as the cartilage proteoglycan-degrading enzyme (Table 3), suggesting that one enzyme may have been cleaving both the proteoglycan and elastin.

To date, a number of investigators have reported that secretion of neutral proteases, such as collagenase, elastase and plasminogen activator, is inhibited by exposure to glucocorticosteroids [29, 30]. Lysozyme secretion from the macrophage is not affected by dexamethasone exposure [29]. In an elegant series of experiments, Vassali *et al.* [31] demonstrated that the inhibitory effect of dexamethasone was completely blocked by concomitant exposure to actinomycin D, indicating that the glucocorticoid was acting to block plasminogen activator synthesis. In the present studies, the intracellular activities, as well as the medium contents were reduced, suggesting an effect on synthesis. The glucocorticoid effect on synthesis appears to be selective for the enzymes active at neutral pH; intra- and extracellular β-glucuronidase concentrations were consistently elevated following glucocorticosteroid exposure (Table 2). The amount of β-glucuronidase present extracellularly was not enhanced to the same extent as that present intracellularly, suggesting that some inhibition of secretion was occurring. Short-term exposure to glucocorticosteroids is reported to reduce zymosan-induced release of β-glucuronidase from both mouse and guinea pig peritoneal macrophages [30, 32].

Non-steroidal anti-inflammatory agents

Most of the non-steroidal anti-inflammatory agents had little or no effect on the release or synthesis of the cartilage proteoglycan-degrading enzyme. Non-toxic concentrations of indomethacin likewise had minimal effects on the other enzymes under study. According to most investigators, the non-steroidal anti-inflammatory agents play a relatively minor role in affecting secretion of neutral proteases from macrophages. Exposure to indomethacin, for example, does not alter release of either plasminogen activator or elastase from the mouse peritoneal macrophage [26, 31]. Indomethacin, however, does inhibit release of collagenase from the guinea pig peritoneal macrophage [30, 33]. β-Glucuronidase release was likewise reported to be minimally affected by most non-steroidal anti-inflammatory agents [32].

Flufenamic acid was the only non-steroidal agent that antagonized the release of the cartilage proteoglycan-degrading enzyme. This agent inhibited the collagenase activity of a lysosomal preparation *in vitro*, as well as complement [34, 35]. We found that it did not affect the activity of the cartilage proteoglycan-degrading enzyme, and inhibited enzyme synthesis.

Anti-rheumatic agents

Of the four anti-rheumatic agents under study, exposure to 10^{-3} M gold sodium thiomalate or 10^{-5} M chloroquine inhibited release and/or synthesis of the cartilage proteoglycan-degrading enzyme; exposure to levamisole stimulated release, whereas *dl*-penicillamine was without effect. Gold sodium thiomalate is a therapeutically effective agent that may be active either as a modulator of immune responsiveness or as an inhibitor of one or more of the inflammatory steps occurring in the disease [36–38]. In the current studies, it appeared to inhibit the secretion of the cartilage proteoglycan-degrading enzyme without affecting synthesis (Table 1) or enzyme activity. Gold has been shown to accumulate in lymph nodes and synovial macrophages in patients undergoing therapy and may inhibit a number of macrophage functions, including phagocytosis and protease secretion [39–41].

Levamisole is an anthelmintic agent effective as an immunopotentiator, e.g. it stimulates lymphocyte blast response to mitogens, and phagocytosis by the mouse macrophage [42, 43]. Cruchaud *et al.* [44] recently reported that 1- and 22-hr exposures to 1–100 μM levamisole, had not effect on β-glucuroni-

dase or cathepsin D release from mouse macrophages. A 3-day exposure led to a decreased enzyme release [44]. In the present studies, we noted stimulation of release; to date, no others have reported the effects of long-term exposure to levamisole on the release of enzymes having a neutral pH optimum.

The most potent of the anti-rheumatics was chloroquine. Following exposure to 10^{-5} M, the secretion of the proteoglycan-degrading enzyme was reduced, whereas the synthesis and activity were unaffected (Fig. 3). The amount of elastase secreted was also reduced in a concentration-dependent fashion. Chloroquine reportedly localizes in the macrophage lysosomes following *in vitro* exposure and interferes with protein degradation [45, 46].

In summary, a system for assaying the pharmacologic effects of anti-inflammatory and anti-rheumatic agents on protease secretion from macrophages has been described. Glucocorticosteroids, flufenamic acid, gold sodium thiomalate and chloroquine may derive at least some of their therapeutic effects from the inhibition of enzyme release from macrophages.

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REFERENCES

1. C. J. Cardella, P. Davies and A. C. Allison, *Nature Lond.* **247**, 46 (1974).
2. J. C. Cayhill and D. A. Pitkeathly, *Ann. rheum. Dis.* **25**, 137 (1966).
3. C. J. Malemud and A. Janoff, *Arthritis Rheum.* **18**, 361 (1975).
4. J. Lieberman, *Chest* **70**, 62 (1976).
5. M. Galdston, A. Janoff and A. L. Davis, *Am. Rev. resp. Dis.* **107**, 718 (1973).
6. M-L. Blue and A. Janoff, *Am. Rev. resp. Dis.* **117**, 317 (1978).
7. R. C. Page, P. Davies and A. C. Allison, *Archs oral Biol.* **18**, 1481 (1973).
8. D. R. Robinson, A. H. Tashjian and L. Levine *J. clin. Invest.* **56**, 1181 (1975).
9. N. J. Zvaifler, *J. exp. Med.* **134**, 276s (1971).
10. I. Kobayashi and M. Ziff, *Arthritis Rheum.* **18**, 475 (1975).
11. N. R. Ackerman, W. H. Rooks, L. Shott, H. Genant, P. Maloney and E. West, *Arthritis Rheum.* **22**, 1365 (1979).
12. N. Ackerman, S. Jubb, B. Trimble and B. Yoxall, *Agents Actions* **9**, 488 (1979).
13. P. Hauser and G. Vaes, *Biochem. J.* **172**, 275 (1978).
14. J. Saklatvala, *J. clin. Invest.* **59**, 794 (1977).
15. Z. Werb and S. Gordon, *J. exp. Med.* **142**, 346 (1975).
16. S. Gordon, J. O. Todd and Z. A. Cohn, *J. exp. Med.* **134**, 2228 (1974).
17. P. Davies and A. C. Allison, in *Immunobiology of the Macrophage* (Ed. D. J. Nelson), p. 427. Academic Press, New York (1976).
18. Z. Werb and S. Gordon, *J. exp. Med.* **142**, 361 (1975).
19. L. J. Ignarro, A. C. Oronsky and R. Perper, *Clin. Immun. Immunopath.* **2**, 35 (1973).
20. W. H. Fishman, B. Springer and R. Brunetti, *J. biol. Chem.* **173**, 449 (1948).
21. N. Levine, V. B. Hatcher and G. S. Lazarus, *Biochim. biophys. Acta* **452**, 458 (1976).
22. S. Takahashi, S. Seiffer and F. C. Yang, *Biochim. biophys. Acta* **327**, 138 (1973).
23. H. V. Bergmeyer, E. Berrot and B. Hess, in *Methods of Enzymatic Analysis* (Ed. H. V. Bergmeyer), p. 736. Academic Press, New York (1965).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. J. C. Unkeless, S. Gordon and E. Reich, *J. exp. Med.* **139**, 834 (1974).
26. M. E. Dahlgren, P. Davies and R. J. Bonney, *Biochim. biophys. Acta* **630**, 338 (1980).
27. M. J. Banda and Z. Werb, *Fedn. Proc.* **38**, 1339 (1979).
28. J. Schnyder and M. Baggiolini, *J. exp. Med.* **147**, 435 (1978).
29. Z. Werb, *J. exp. Med.* **147**, 1695 (1978).
30. T. Okimura, H. Ohmori, Y. Kubota and I. Yamamoto, *Biochem. Pharmac.* **28**, 2729 (1979).
31. J-D. Vassali, J. Hamilton and E. Reich, *Cell* **8**, 271 (1976).
32. P. J. Ringrose, M. A. Parr and M. McLaren, *Biochem. Pharmac.* **24**, 607 (1975).
33. L. M. Wahl, C. E. Olsen, A. L. Sandberg and S. E. Mergenhagen, *Proc. Natn. Acad. Sci. U.S.A.* **74**, 4955 (1977).
34. A. J. Anderson, *Biochem. J.* **113**, 457 (1969).
35. T. Di Perry and A. Autery, in *Future Trends in Inflammation* (Eds. D. A. Willoughby, J. P. Giroud and G. P. Velo), p. 215. Piccin Medical Books, London (1974).
36. J. D. Jessop and M. L. F. Currey, *Ann. rheum. Dis.* **27**, 577 (1968).
37. J. W. Sigler, G. B. Bluhm, H. Duncan, J. T. Sharp, D. C. Gusign and W. R. McCrum, *Ann. intern. Med.* **80**, 21 (1974).
38. R. N. P. Cahill, *Experientia* **27**, 913 (1971).
39. R. M. Persellin and M. Ziff, *Arthritis Rheum.* **9**, 57 (1966).
40. J. D. Jessop, B. Vernon-Roberts and J. Harris, *Ann. rheum. Dis.* **32**, 294 (1973).
41. K. Ugai, M. Ziff and P. E. Lipsky, *Arthritis Rheum.* **22**, 1352 (1979).
42. M. S. Al-Ibrahim, R. S. Holzman and H. S. Lawrence, *J. infect. Dis.* **135**, 517 (1977).
43. J. W. Hadden, R. G. Coffey, E. M. Hadden, E. Lopez-Corrales and G. H. Sunshine, *Cell. Immun.* **20**, 98 (1975).
44. A. Cruchaud, M. Berney and H. D. Welscher, *Int. J. Immunopharmac.* **1**, 49 (1979).
45. M. E. Fedorro, J. G. Hirsch and Z. A. Cohn, *J. Cell Biol.* **38**, 377 (1968).
46. J. L. Goldstein, G. Y. Brunschede and M. S. Brown, *J. biol. Chem.* **250**, 7854 (1975).