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EFFECTS OF ANTI-INFLAMMATORY AND OTHER COMPOUNDS ON THE RELEASE OF LYSOSOMAL ENZYMES FROM MACROPHAGES

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Abstract—The effects of anti-inflammatory drugs and other compounds on the selective release of lysosomal enzymes from cultured peritoneal-macrophages as a result of phagocytic uptake of zymosan particles were investigated. Two types of inhibitory effect were observed: (i) a biphasic effect which produced maximum inhibition at 3×10^{-7}–6×10^{-6} M for steroids, 3×10^{-5} M for guaiol and 10^{-5} M for phenylbutazone; and (ii) a linear log-dose effect with ED_{50}'s of 10^{-4} M for flufenamic acid and 3×10^{-7} M for concanavalin. Stimulation of zymosan-induced enzyme release was observed with chloroquine and indomethacin at concentrations of 10^{-4} M and greater. This effect was associated with cytotoxicity. Guaiol also stimulated hydrolase-release at 10^{-4} M, but only during short incubation times and the effect was not cytotoxic. All other non-steroidal anti-inflammatory compounds were without significant activity.

The involvement of lysosomal enzymes in acute inflammatory and chronic arthritic diseases is well documented [1, 2]. The initial degradation of articular-cartilage proteoglycan in osteo- and probably rheumatoid arthritis is due to lysosomal enzyme release either from constituent chondrocytes and phagocytic-synovial A-type cells, or from invading polymorpho-nuclear-leucocytes and macrophages. Rheumatoid arthritis may involve an autoimmune mechanism [3] whereby IgG antibody, altered by an initial excess of lysosomal protease due to an insufficiency of α_2 macroglobulin [4], forms IgM–IgG' complexes which are engulfed by macrophages at the site of inflammation. Excessive phagocytosis of this type in response to "foreign" material leads to a selective release of lysosomal enzymes either by fusion of perturbed primary lysosomes with the outer cell membrane, or by leakage of enzymes via secondary lysosomes with incompletely fused endocytic channels [5].

The effects of anti-inflammatory drugs on lysosomal enzyme release have previously been studied using isolated lysosomes but are confused owing to the great number of variables introduced into the cell-free system [6–14]. Accordingly indomethacin, phenylbutazone, aspirin and flufenamic acid have been reported to stabilise [10, 13, 15] as well as labilise lysosomal membranes in cell-free systems [5, 11]. The anti-inflammatory steroids and chloroquine however are usually reported as stabilisers [6, 10, 13, 15], but not always [7, 8, 9].

The effects of drugs on lysosomal enzyme release from cultured polymorphonuclear-leucocytes and macrophages as a result of pino- or phagocytosis are in greater agreement. Anti-inflammatory steroids reduce pinocytosis and hydrolase release from macrophages in response to heterologous serum [16, 17]. Compounds that increase intracellular cAMP (papaverine, prostaglandin E1) or disrupt microtubules (colchicine, vinblastine) also inhibit lysosomal enzyme release [5, 18–21], although an increase in cAMP levels is observed during phagocytosis [22]. In contrast extracellular cGMP stimulates enzyme release [21]. Consistent with this, autonomic agonists that are adrenergic (act via cAMP) inhibit induced hydrolase release whereas cholinergic agonists (act via cGMP) stimulate release [21, 23]. Cytochalasin [24], an inhibitor of microfilament function and concanavalin [25], a membrane disorganiser, inhibit phagocytosis; cytochalasin however enhances lysosomal enzyme release [26] but concanavalin stimulates phagocytic metabolism [27]. Chloroquine, in contrast to cell-free results, and aspirin, have little effect on hydrolase release from cultured phagocytes [5, 28].

Surprisingly little is known of the effect of most non-steroidal anti-inflammatory drugs on macrophage enzyme release. Work was therefore initiated to examine the actions of these compounds on whole cells during phagocytosis.

MATERIALS AND MEDIA

All tissue culture media and heat-inactivated foetal bovine sera were from Flow Laboratories, Irvine, Scotland. All enzyme substrates and zymosan were from Sigma (London) Chemical Co. Ltd., radiochemicals were from the Radiochemical Centre, Amersham, Bucks., England.

Drugs used in the investigation were obtained as follows: flufenamic and mefenamic acids from Parke–Davis, dexamethasone, hydrocortisone and prednisolone from Sigma and Merck, Sharp & Dohme; chloroquine and colchicine from Sigma; flurbiprofen and

ibufenac from Boots; phenylbutazone from Geigy; indomethacin from Merck, Sharp & Dohme; naproxen from Syntex; ketoprofen from May & Baker; concanavalin A from Boehringer; cytochalasin B from Serva; vinblastine from Lilly; pepstatin, aspirin and guaiol from Roche.

All compounds, which were not water soluble, were dissolved in dimethyl sulphoxide (DMSO) and diluted to 1% (v/v) in the culture medium. 1% (v/v) DMSO inhibited enzyme release 5–10 per cent relative to untreated cells but did not affect cell viability. Drug inhibitory values in Table 2 and Figs. 2 and 4 were corrected using 1% (v/v) DMSO treated controls. One per cent (v/v) ethanol was toxic to cells and enhanced enzyme release.

Medium A was used for routine maintenance of macrophage cultures [5] and contained TC 199 (plus phenol red), 50% (v/v) heat-inactivated foetal bovine serum, 100 units penicillin and streptomycin per ml, 50 units mycostatin per ml, 0·15% (w/v) bicarbonate and 2 mM glutamine. Medium B was the experimental medium used during drug and zymosan treatment and contained 10 g Eagles medium (Auto-Pow minus phenol red) per litre, 20% (v/v) heat-inactivated foetal bovine serum, non-essential aminoacids supplement, also penicillin, streptomycin, mycostatin, bicarbonate and glutamine as for medium A.

Zymosan suspensions (10 mg/ml i.e. 5×10^8 particles per ml) were boiled and washed according to the method of Weissmann *et al.* [5]. Immune complexes were formed by interacting bovine gamma-globulin (Sigma) with rabbit anti-cow serum (Wellcome) to the point of equivalence as determined by quantitative precipitation [29]. Monosodium urate (MSU) crystals were prepared according to the method of McCarty and Faires [30].

METHODS

Macrophage culture

To obtain pure macrophage cultures, 6-week-old male (CFW) mice were injected intraperitoneally with 1 ml sterile thioglycollate medium (Difco). Three days later, the mice were killed with ether and macrophages collected by washing the peritoneal cavity with 3 ml ice-cold TC 199 medium containing 5% (v/v) heat-inactivated foetal bovine serum, 100 units penicillin and streptomycin (Flow) per ml, and 50 units mycostatin (Squibb) and heparin (Pularin-Evans Medical) per ml. The pooled washings were adjusted to 50% (v/v) foetal bovine serum and a cell density of $1–2 \times 10^6$ nucleated cells per ml. Aliquots (1 or 2·5 ml) were distributed into either Biocult multidishes (10 cm^2 per dish) or Falcon tissue-culture flasks (25 cm^2) and incubated in an atmosphere of 5% CO_2 in air at 37° to allow surface adhesion of the monocytes. After 2 hr the cell sheet was washed four times with phosphate-buffered saline pH 7·0 at room temperature to remove unattached cells. Fresh medium A was added and incubation continued overnight. Ten to twenty hours after plating, cultures (>90% monocytes) were washed twice with phosphate-buffered saline and pre-incubated in medium B containing drug. After 1 hr pre-incubation, zymosan was added to give 2×10^7 particles/ml, and incubation continued. The culture medium was removed after the incubation times indicated (see Results), centrifuged and aliquots of the supernatant assayed for acid hydrolase and lactate dehydrogenase activities. Total enzyme activity was determined by addition of 0·2% Triton X-100 to a cell suspension or monolayer, followed by freeze-thawing.

Enzyme assays

All enzymes were assayed on the day of the experiment or stored at −20° overnight and assayed the following day owing to serum deactivation of certain enzyme activities.

(a) *β-Glucuronidase.* Culture supernatant (0·1–0·5 ml) was incubated with 0·5 ml 0·3 M acetate buffer (pH 4·5) and 0·5 ml 3 mM phenolphthalein β-D-glucuronide in a total volume of 1·5 ml at 37°. Incubation was stopped with 3 ml M Na_2CO_3 and the E_{555} determined.

(b) *Cathepsin D.* [^{3}H]acetyl haemoglobin ($2·5 \times 10^6$ cpm/mg) was used as substrate and was prepared according to the method of Hille *et al.* [31]; 0·02 ml [^{3}H]acetyl haemoglobin (5×10^5 cpm) was incubated with 0·1 ml culture supernatant and 0·1 ml 0·5 M sodium formate buffer (pH 2·8) at 45°. The incubation was stopped in ice by adding 1 ml 0·4% (w/v) haemoglobin and 0·8 ml 7·5% (w/v) trichloroacetic acid. In order to precipitate undigested labelled-haemoglobin incubation was continued at 45° for a further 20 min. The reaction mixture was cooled, centrifuged and a 0·5 ml aliquot of the supernatant mixed with 15 ml of a water-miscible scintillation fluid; 4 g BBOT/l toluene:Triton X-100 (2:1). The radioactivity was determined in a Packard scintillation counter.

(c) *Acid phosphatase.* Culture supernatant (0·1–0·5 ml) was incubated with 0·25 ml 8 mM *p*-nitrophenylphosphate and 0·25 ml 300 mM acetate buffer (pH 5·0) in a total volume of 1 ml at 37°. Incubation was stopped with 4 ml 0·1 NaOH and the E_{410} determined.

(d) *Aryl sulphatase.* This enzyme was assayed at pH 4·7 by the nitrocatechol sulphate/alkaline-quinol method described by Barrett [32].

(e) *Lactate dehydrogenase.* This enzyme was assayed by measuring the rate of decrease in E_{340} due to oxidation of 0·25 mM N NADH in the presence of 0·66 mM pyruvate and 0·1 M phosphate buffer (pH 7·5) at 25° as described in Sigma technical bulletin 340-UV.

(f) L-*Leucyl-naphthylamidase.* Diazo dye formation of β-naphthylamine liberated from L-leucyl-β naphthylamide at pH 7·1 was used in the assay as described in Sigma Technical Bulletin 251.

Measurement of phagocytosis

Particle uptake was measured by phase-contrast light microscopy. Cells containing three or more zymosan particles were counted as phagocytic.

Table 1. Effects of various agents on release of lysosomal (L) and cytoplasmic (C) enzymes from macrophages

		% Total enzyme activity 4 hr				
	Control	Zymosan	Bacteria	Ab/Ag	Vit. A	Urate Crystals
β Glucuronidase[L]	7·2 ± 0·7	21·3 ± 3·6*	14·7 ± 2·3*	12·1 ± 1·7*	27·3 ± 2·9*	32·2 ± 4·1*
Acid Phosphatase[L]	9·2 ± 0·8	15·7 ± 2·1*				
Cathepsin D[L]	5·3 ± 0·9	15·8 ± 4·5*				
Aryl Sulphatase[L]	1·4 ± 0·5	3·7 ± 0·7				
Lactate dehydrogenase[C]	2·1 ± 0·2	2·0 ± 0·7	2·3 ± 1·1	3·7 ± 2·1	28·9 ± 4·2*	29·1 ± 3·7*
L Leucine 2-	3·6 ± 1·2	3·8 ± 0·9				
		2×10^7 particles/ml	10^7 heat- killed Streptococci/ml	100 μg protein Ag/ml	50 μg retinol/ml	100 μg MSU/ml

* Significantly different from control enzyme release ($P < 0{\cdot}01$). Values are expressed as means ± S.E.M. of 10 observations for control and zymosan treated cultures and of four observations for other cultures.

Macrophages were cultured 18 hr in high-serum medium A (see Methods). The cells were washed and the incubation continued with low-serum medium B in the presence of enzyme-releasing agents as indicated. After 4 hr the enzyme activity in the culture supernatant was determined and expressed as a percentage of the total culture activity released by 0·2% Triton X-100. Total enzyme activities at the beginning of the 4 hr incubation were as follows: β-glucuronidase—49·7 ± 2·3 nmoles phenolphthalein/hr/culture; acid phosphatase—132·1 ± 7·1 nmoles *p*-nitrophenol/hr/culture; cathepsin D—3·9 ± 0·2 × 10^4[^{3}H] cpm/hr/culture (see Methods); aryl sulphatase—33·7 ± 2·7 nmoles nitrocatechol/hr/culture; lactate dehydrogenase—421 ± 8 absorbancy units/culture; Leucine naphthylamidase 281 ± 7·1 nmoles β naphthylamine/hr/culture.

RESULTS

Selective release of lysosomal enzymes from macrophages. In order to determine which of the many reported methods for stimulating enzyme release from cultured macrophages was most suitable for studying drug action, the five stimulii described in Table 1 were used. Vitamin A [26] (a potent membrane labiliser) and monosodium urate crystals [19] (the physiological stimulus in gout) produced the greatest release of lysosomal enzymes during a 4-hr incubation. Their action,

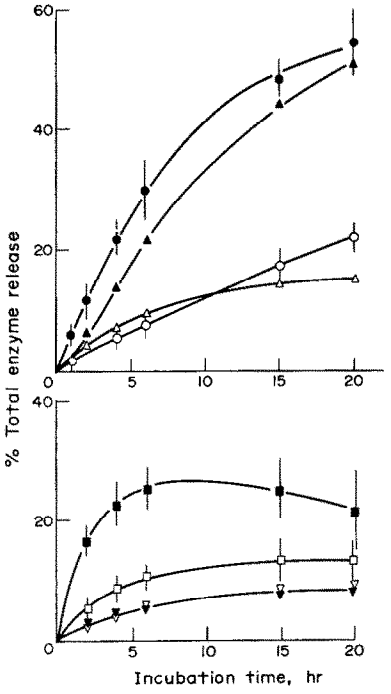


Fig. 1. Effect of zymosan particles on enzyme release from cultured macrophages. Macrophages were incubated in the presence and absence of zymosan for the times indicated and enzyme activities in the culture supernatant determined and expressed as a percentage of the total activity as described in Table 1. Each point is the mean ± S.E.M. of four experiments. Key: β-glucuronidase control (○), plus zymosan (●); acid phosphatase control (△), plus zymosan (▲); cathepsin D control (□), plus zymosan (■); Leu, naphthylamidase control (▽), plus zymosan (▼).

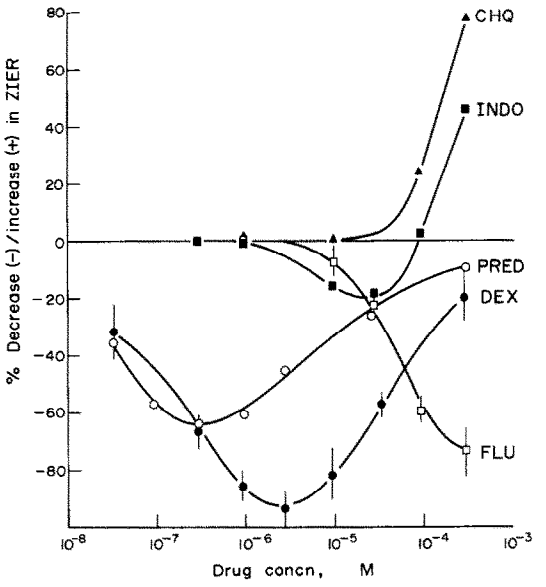


Fig. 2. Effects of anti-inflammatory drugs on zymosan-induced release of β-glucuronidase from cultured macrophages. Macrophage cultures were preincubated for 1 hr with chloroquine (▲), indomethacin (■), prednisolone (○), dexamethasone (●) and flufenamic acid (□) at the molarities indicated. Zymosan was added and incubation continued for 18 hr. β-Glucuronidase activities were determined on the culture supernatants and the percentage increase or decrease in ZIER calculated as described in Table 2. Each point is the mean ± S.E.M. (● and □) of four experiments.

however, was not selective and an almost equivalent release of cytoplasmic enzymes occurred. After 18 hr incubation, the cells became rounded and detached from the culture-vessel surface.

Zymosan (yeast cell-wall particles) [5], heat-killed streptococci and antibody–antigen complexes [33] produced a more selective response with no significant release of cytoplasmic enzymes. Zymosan increased β-glucuronidase and cathepsin D release threefold over the control values and this treatment was therefore chosen for all further studies. The macrophage monolayer remained 90–100 per cent viable for a minimum of 72 hr with the cells giving an enlarged and vacuolar appearance and the zymosan particles visible intracellularly (80–90 per cent cells phagocytic, see Methods). For the greatest reproducibility between experiments it

Table 2. Effects of anti-inflammatory drugs and other compounds on zymosan-induced release of β-glucuronidase from cultured macrophages

(a) Compounds exhibiting biphasic inhibitory effects

Compound	Max. biphasic inhib. % control ZIER	Molarity at max. inhib. after 18 hr
Dexamethasone	−93 ± 4·7 (6)	3×10^{-6}
Prednisolone	−64 ± 5·9 (5)	3×10^{-7}
Hydrocortisone	−31 ± 5·1 (4)	6×10^{-6}
Guaiol	−45 ± 4·1 (8)	3×10^{-5}
Phenylbutazone	−42 ± 9·3 (4)	1×10^{-5}
Indomethacin	−18 ± 3·5 (4)	3×10^{-5}

($P < 0.01$) Significantly different from control.
Values are expressed as means ± S.E.M. of the numbers of duplicate observations indicated in parenthesis.

(b) Anti-inflammatory compounds exhibiting linear log-dose-related effects.

Compound	% Inhibition (−)/stimulation (+) of control ZIER 4 hr	18 hr
Anti-inflammatory drugs (10^{-4} M)		
Flufenamic acid	−27 ± 4·1	−58 ± 5·6 (5)
Mefenamic acid	−19 ± 2·1	−38 ± 4·7 (4)
Aspirin	−19 ± 1·3	−26 ± 1·2 (4)
Chloroquine	+21 ± 4·2	+24 ± 2·4 (4)

$P < 0.01$
Meclofenamic acid, ketoprofen, ibufenac, penicillamine, flurbiprofen, naproxen and aminocaproic acid were inactive.

(c) Compounds acting via cAMP, microtubules, microfilaments and membrane receptors

Compound	4 hr	18 hr
Concanavalin A (10^{-5} M)	> 100	> −100*
Vinblastine (10^{-6} M)	−12	+47 (3)
Colchicine (10^{-4} M)	−27	+63 (3)
Cytochalasin (2×10^{-5} M)	+33	+21 (3)
Prostaglandin E1 (10^{-4} M)	−16	−23 (3)
Theophylline & cAMP (10^{-3} M)	−15	−25 (3)
Papaverine (3×10^{-5} M)	−24	−37 (3)

* See Fig. 3, concanavalin ED_{50} at 4 hr, 3×10^{-7} M and at 18 hr, 10^{-6} M assuming mol. wt of 71,000 (35).
Macrophages were preincubated with compound for 1 hr. Zymosan was added and incubation continued for a further 4 and 18 hr. β-Glucuronidase activity was determined in the culture supernatants and the results expressed as % decrease (−)/increase (+) in zymosan-induced stimulation of enzyme release (ZIER) of the control.

$$\text{decrease}(-) = \frac{\text{control ZIER} - \text{drug ZIER}}{\text{control ZIER} - \text{control non-induced enzyme release}}$$

$$\text{increase}(+) = \frac{\text{drug ZIER} - \text{control ZIER}}{\text{control ZIER} - \text{control non-induced enzyme release}}$$

Control ZIER—control non-induced enzyme release = 5·1 nmoles after 4 hr and 21·7 nmoles after 18 hr phenolphthalein/hr/culture.

was important to use the same batch of zymosan and to follow exactly the boiling and washing procedure described by Weissmann *et al.* [5].

Zymosan-induced release of β-glucuronidase, as shown in Fig. 1, was approximately 3 times control value after 4 and 18 hr incubation. The induced release after 18 hr was 50–60 per cent total enzyme activity in cells at zero time. The ratio of induced:control acid-phosphatase release however increased with time whereas induced cathepsin D activity decreased slightly after 6 hr incubation with zymosan, probably due to serum deactivation [4]. The cytoplasmic enzymes lactate dehydrogenase and leucine-naphthylamidase showed only a small increase in released activity over the 20-hr incubation and no significant additional increase due to zymosan. However detection of low amounts of these unstable cytoplasmic enzymes in the presence of non-specific activities in the serum was difficult and not always a reliable guide to selectivity of release [26]. Dye exclusion and the observation of cell morphology and surface-adhesion were more reliable cytotoxic indications. Interpretation of time-course experiments with enzyme release must be treated cautiously owing to the low recovery of activities from complex serum-containing media.

Action of standard anti-inflammatory drugs on zymosan-induced release of lysosomal enzymes. Dose–response curves for clinically-active drugs are shown in Fig. 2. Three types of effect were observed. The first was a biphasic inhibitory effect exhibited by the anti-inflammatory steroids (dexamethasone, prednisolone and hydrocortisone), phenylbutazone and to a lesser extent by indomethacin. On increasing the drug concentration from 10^{-8} M an increase in inhibition was observed until an inhibitory optimum was reached (see Table 2a). Further increase in concentration resulted in a decrease in inhibition and in the case of indomethacin, a stimulation of enzyme release associated with a loss in cell viability. Dexamethasone produced the greatest inhibition although the optimum concentration of prednisolone was at a lower molarity. In all cases maximal inhibition was greater and optimum molarity lower after longer incubation times (18 hr).

The second type of effect was a typical linear log-dose related response. Flufenamic acid and to a lesser degree mefenamic acid and aspirin exhibited this effect which again was greater after longer incubation times (see Table 2b). Much greater drug concentrations were required for inhibition by this effect as compared with the biphasic drugs.

The third type of effect was stimulatory and usually occurred as a direct result of cytotoxicity at concentrations greater than 10^{-4} M, e.g. chloroquine and indomethacin. Cytochalasin and, under certain conditions, guaiol (see later) stimulated selective enzyme-release without cytotoxicity.

None of the active compounds described above inhibited the phagocytic process (75–85 per cent cells phagocytic). In nearly all cases, inhibition of acid-phosphatase and cathepsin D release paralleled inhibition of β-glucuronidase release after 18 hr. Inhibition of acid-phosphatase release was sometimes greater but subject to greater variability than β-glucuronidase release. After short incubation times (4 hr) inhibitory effects on β-glucuronidase release were significantly more pronounced. The following anti-inflammatory drugs had no significant effect on zymosan-induced enzyme release (ZIER) at 10^{-6}–10^{-4} M; meclofenamic acid, ketoprofen, ibufenac, penicillamine, flurbiprofen, naproxen and aminocaproic acid.

Pepstatin [34] at 10 ng/ml ($1{\cdot}5 \times 10^{-9}$ M) completely inhibited all cathepsin D activity released from the macrophage cultures. Concentrations up to 10 μg/ml however failed to have reproducible effects on release of the other enzymes although the occasional inhibition was observed. *In vivo* pepstatin administration (i.p. 10 mg/kg) 5 hr prior to thioglycollate injection of mice significantly lowered the yield of peritoneal macrophages and lowered their response to zymosan (unpublished observations).

Oral administration of steroids was also tried (100 mg/kg). The yield of macrophages and response to zymosan was unaffected. Macrophage spreading on the culture-vessel surface was however enhanced (see guaiol results), (unpublished observations).

Actions of compounds acting via cAMP, microtubules, microfilaments and membrane receptors. Vinblastine, colchicine, prostaglandin E1, theophylline, cAMP and

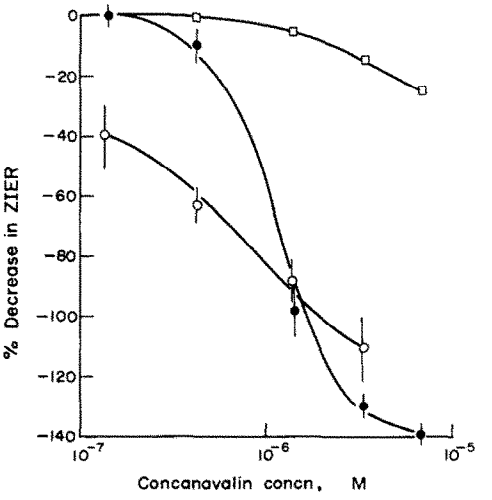
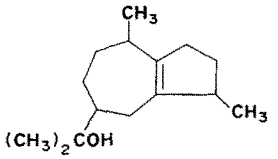


Fig. 3. Inhibitory effect of concanavalin on zymosan-induced release of β-glucuronidase from cultured macrophages and its reversal by maltose. Macrophage cultures were preincubated for 1 hr with concanavalin A at the molarities indicated. Zymosan was added and incubation continued for 4 hr (○) and 18 hr (●). β-Glucuronidase activities were determined on the culture supernatants and the percentage decrease in ZIER calculated as described in Table 2. Macrophages were also pretreated with 60 mM maltose (□) as described in Ref. 24, and incubated with concanavalin and zymosan for 4 hr. Each point (○ and ●) is the mean ± S.E.M. of six experiments. Maltose pretreatment points (□) are the mean of two experiments.

papaverine all inhibited lysosomal enzyme release after short incubation times although none had more than low activity (Table 2c). Vinblastine and colchicine were toxic to macrophages after 18 hr incubation. 2×10^{-5} M cytochalasin B enhanced zymosan-induced lysosomal-enzyme release without increasing cytoplasmic-enzyme release.

Concanavalin A was by far the most active inhibitor of all compounds studied in this investigation, but because of its large molecular weight [35], comparatively high concentrations were used. Fifty per cent inhibition of β-glucuronidase (also cathepsin D) release was at 3×10^{-7} M (20 μg/ml) after 4 hr and 10^{-6} M after 18 hr (see Fig. 3). The response was not biphasic and at concentrations greater than 2×10^{-6} M the unstimulated release of all tested enzymes from control cells was inhibited. Pre-incubation of the cultured cells with 60 mM maltose [25] almost completely blocked concanavalin action after 4 hr, but had little effect after 18 hr. Phagocytic uptake of zymosan particles was blocked by concanavalin at concentrations greater than 10^{-6} M (25–40 per cent cells phagocytic) but the cells still had an enlarged and vacuolar appearance and were 100 per cent viable after 72 hr. α-Methyl-glucose reversed concanavalin action after 4 and 18 hr, (unpublished observations).



Guaiol

Action of guaiol. Guaiol is a component of guaiac resin which was used as an old cure for rheumatoid arthritis and inflammation. After 18 hr incubation with cells in the presence of zymosan particles a biphasic curve similar to that described for dexamethasone was obtained (Fig. 4) with an inhibitory optimum at 5×10^{-5} M (Table 2a). Exposure of cells to concentrations of guaiol in excess of 10^{-4} M for short time intervals however stimulated lysosomal-enzyme release without affecting viability or causing release of cytoplasmic enzymes. The morphology of guaiol-treated cells resembled to some degree cells treated with steroid. The culture was predominantly composed of cells with unusually long thin processes which only fattened out slightly after zymosan-treatment. Phagocytic uptake of zymosan particles was only partially inhibited, (60–80 per cent cells phagocytic).

None of the active compounds described above, with the exception of 10^{-3} M phenylbutazone, directly inhibited the activities of β-glucuronidase, acid phosphatase or cathepsin D at the concentrations used.

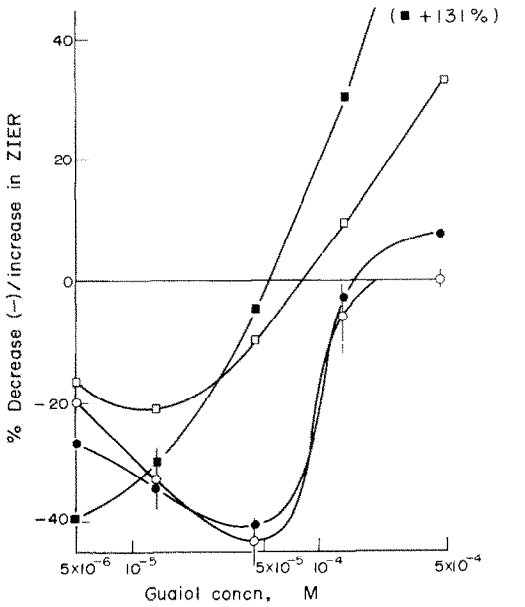


Fig. 4. Inhibitory and stimulatory effects of guaiol (Ro 20-2951) on zymosan-induced release of β-glucuronidase from cultured macrophages. Macrophage cultures were preincubated for 1 hr with guaiol at the molarities indicated. Zymosan was added and incubation continued for 1 hr (■), 3 hr (□), 6 hr (●) and 18 hr (○). β-Glucuronidase activities were determined on the culture supernatants and the percentage increase or decrease in ZIER calculated as described in Table 2. Each point is the mean ± S.E.M. (○) of four experiments.

DISCUSSION

Cultured mouse peritoneal macrophages were stimulated to release lysosomal enzymes with zymosan particles and the actions of drugs on this process were investigated. Zymosan was selected as phagocytic inducer since it produced maximal selective release of lysosomal hydrolases compared to vitamin A, urate crystals, bacteria and immune complexes. Inhibitors of the stimulated enzyme release gave either biphasic or linear responses. The anti-inflammatory steroids, phenylbutazone and guaiol were the most active "biphasic" inhibitors. Flufenamic acid, and concanavalin A were the most active "linear" response inhibitors. All other non-steroidal anti-inflammatory drugs had little activity with the exception of chloroquine and indomethacin which enhanced the stimulated release at concentrations greater than 10^{-4} M owing to cytotoxicity. After short incubation times guaiol also enhanced release, but this effect was not cytotoxic and was peculiar to this compound since longer incubation produced a biphasic inhibitory response characteristic of steroid action. The effects of all active compounds were found to be completely reversible at least up to 4 hr with the exception of cytotoxic drugs (unpublished observations). The only compounds examined in detail in the literature using this method have been vinblastine, colchicine, cytochalasin, cyclic nucleotide mediators, chloroquine [5, 21], hydrocortisone [17] and aspirin [28].

There has been much discussion [16] as to the mechanism of action of steroids on membranes and it would appear that an active stabilizing molecule must have a coplanar ring system, be capable of hydrophilic, hydrophobic and hydrogen bonding, and possess charge-transfer properties [36]. However, most planar steroids are biphasic [14] and their membrane action is sometimes dependent on the osmolarity of the medium [16]. Guaiol, although structurally dissimilar from steroids, may resemble the physical properties of the steroid ring system sufficiently for its action on membranes to be related.

Concanavalin A binds reversibly to glycoprotein receptor sites on the membrane causing clustering of protein molecules normally dispersed in the lipid [25]. It has been reported that this macromolecule inhibits phagocytosis [24] but stimulates oxidative metabolism which normally occurs during phagocytosis [26]. It was also found in the present studies that concanavalin was a potent dose-related inhibitor of hydrolase-release both from control cells and phagocytosing cells, and that this effect was characteristically reversed by maltose. Phytohaemagglutinin (a plant lectin similar to concanavalin) was only slightly active at equivalent concentrations (unpublished observations). With the exception of concanavalin and to a lesser degree guaiol, all compounds found active in inhibiting hydrolase release had no effect on the phagocytic uptake of zymosan particles. The steroids and guaiol however enhanced cytoplasmic spreading and spindling of macrophages.

Activity of a pharmaceutical compound in repressing lysosomal enzyme release during phagocytosis would indicate a mechanism of action distinct from that proposed for most of the non-steroidal anti-inflammatory drugs [37], (i.e. inhibition of prostaglandin synthesis). Recent results of Perper and Oronsky [38] have indicated that enzyme release from human leucocytes in contact with immobilized immunologic reactants is suppressed by phenylbutazone, indomethacin, colchicine, aspirin and hydrocortisone. Gold and chloroquine produced effects by directly inhibiting neural protease activity.

In summary, the macrophage plays a key role in prolonging the degradative processes in chronic arthritic lesions. The macrophage however is also an essential component of the reticuloendothelial system, phagocytosing invading microorganisms, "processing" antigens for B and T lymphocyte function and acting as a specific cytotoxic agent against tumour cells [39]. During inflammation the complement components C_{3_a} and C_{5_a} enhance lysosomal enzyme release [40], as do prostaglandins E_2, B_2, $F_{1\beta}$ and $F_{2\alpha}$ [18]. The naturally produced tetrapeptide, tuftsin [41] also stimulates phagocytosis. Other prostaglandins however inhibit hydrolase release (PGE_1 and A_2) [18, 42], as do fatty acid hydroperoxides [43]. It is proposed by Weissmann [40] that certain prostaglandins may first act as activators of enzyme release but, in response to the consequent increase in phospholipase activity and hence prostaglandin precursors, eventually act as "local feedback inhibitors". The results described in this paper indicate that certain non-steroidal anti-inflammatory drugs may influence lysosomal enzyme release but only to a small degree and have an action quite distinct from that observed for steroids. Concanavalin and guaiol, although possessing interesting *in vitro* activities, were inactive in carrageenin oedema (unpublished observations).

In the interpretation of these results, comparison is difficult with results in the literature obtained with peripheral polymorphonucleocytes which play a more important role in the acute stages of inflammation. The peritoneal macrophages used in this work were obtained under stimulatory conditions and may be unrelated to the phagocytic cell types at the site of chronic inflammation. They behave quite differently to polymorphonucleocytes in culture and may possess different enzyme populations. In addition the preponderance of work on acid-hydrolases should not obscure the growing interest in neutral proteases [38, 42, 44] and their involvement in the degradative stages of rheumatoid arthritis and osteoarthrosis.

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