Proc. Natl. Acad. Sci. USA
Vol. 76, No. 4, pp. 1873–1877, April 1979
Cell Biology

Inhibitors of membrane transport reduce lysosomal enzyme secretion from dogfish phagocytes and their killing of sea urchin eggs

(cytotoxicity/degranulation/ethacrynic acid/furosemide/phloretin)

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Communicated by James D. Ebert, January 24, 1979

ABSTRACT **Blood phagocytes of the dogfish *Mustelus canis* attack oocytes of the sea urchin *Arbacia punctulata,* first provoking a surrogate fertilization response and then killing the eggs. To test the hypothesis that secretion of lysosomal contents is critical in this model of phagocyte-mediated cell injury, we studied effects of agents that modify lysosomal enzyme secretion. Inhibitors of membrane transport (>0.1 mM) inhibited postphagocytic secretion of lysosomal β-glucuronidase from dogfish phagocytes: phloretin > ethacrynate > furosemide > 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid ≫ pyridoxal phosphate > ouabain. The same order of activity was found for inhibition by these agents of killing of *Arbacia* eggs by phagocytes. Cell activation (fertilization response) and cytotoxicity were quantitated both morphologically and by measurements of enzyme (β-glucuronidase, catalase) release. The agents neither inhibited fertilization responses of eggs to calcium ionophore A23187 nor impaired their viability. Vital staining demonstrated that ethacrynate prevented phagocytes from degranulating upon contact with zymosan particles. The data not only suggest that agents primarily known for their capacity to inhibit membrane transport systems can inhibit lysosomal enzyme secretion from phagocytes but also support the hypothesis that secretion of lysosomal contents mediates activation and killing of target cells in phagocyte-mediated tissue injury.**

Metchnikoff (1) described lysis of crustacean oocytes by masses of phagocytes from sponges. The phagocytes, too small to engulf the eggs, cluster around and eventually destroyed the target. In a similar, heterologous, cytolytic system, it has been shown (2) that blood phagocytes of the dogfish (*Mustelus canis*) can attack oocytes of the sea urchin (*Arbacia punctulata*); it was postulated that the phagocytes injured the target cells by discharging the contents of their lysosomes at the point of phagocyte–cell contact. In this model of phagocyte-mediated cell injury, the first response of the egg (30 min) was a kind of surrogate fertilization reaction: discharge of cortical granules, migration of echinochrome pigment granules to loci of phagocyte–egg attachment, and formation of the fertilization membrane. Subsequently (>60 min) the eggs were lysed, and degranulation in the phagocytes was apparent. Both activation (the surrogate fertilization response) and subsequent cytolysis were augmented if the eggs were first coated with dogfish immunoglobulin. Therefore it was suggested that this model was an example of "reverse endocytosis" (3) or "frustrated phagocytosis" (4), mechanisms whereby mammalian granulocytes (PMNs) secrete their lysosomal contents directly upon appropriately opsonized particles too large to be ingested. Tumor cells (5), avian erythrocytes (6), or even inert Sepharose beads (7) stimulate secretion of lysosomal enzymes from PMNs at zones of cell–particle contact.

Studies of mechanisms whereby lysosomal enzymes are secreted from PMNs have demonstrated that secretion may be modified by changes in the ionic environment of these cells (8–10). Moreover, various inhibitors of ion transport across cell membranes inhibit lysosomal enzyme secretion (11): the diuretics ethacrynic acid and furosemide, the anion transport inhibitors 4-acetamido-4′-isothiocyanostilbene-1,2′-disulfonic acid (SITS), pyridoxal phosphate, and probenecid, and other transport inhibitors such as phloretin (ouabain being essentially inactive). We therefore studied whether these agents can inhibit the postphagocytic secretion of lysosomal enzymes from blood phagocytes of *M. canis* and whether their order of activity in this regard parallels their order of activity in inhibiting phagocyte-mediated cytotoxicity of sea urchin eggs. Because dogfish phagocytes resemble the PMNs of mammals with respect to enzymic equipment (2, 12, 13), response to immunologic (2, 12) or nonimmunologic (13) stimulation, and capacity to provoke lysis of appropriate target cells (2), these studies should be relevant to modification of phagocyte-mediated injury in inflammatory states (14).

MATERIALS AND METHODS

Cells. Phagocytes of *M. canis* and eggs from *A. punctulata* were obtained as described (2, 12). Phagocytes were suspended in elasmobranch Ringer's solution (ER) containing heparin (500 units/ml) and were distinguished by uptake of neutral red (1).

Secretion of Lysosomal Enzymes by Phagocytes. Release of β-glucuronidase was induced by exposing cells to zymosan. Cells suspended in ER/heparin with 10% fresh isologous plasma were incubated in mixtures (final volume, 1.74 ml) containing 5×10^7 leukocytes, of which 0.4×10^7 were phagocytes. After incubation at 23°C, cells were sedimented (10 min, 1600 rcf, clinical centrifuge) and the supernatant solutions were assayed. Results are expressed as percentage of total enzyme in cell pellets (Triton X-100 extracts, 0.2%, vol/vol).

Analysis of Enzyme Activities. β-Glucuronidase was measured as described (12), with phenolphthalein glucuronic acid as substrate (incubation at 23°C, 18 hr). Lactate dehydrogenase was measured as described (15) using Worthington Statzyme LDH (L-P) 16 (Worthington). Catalase was measured by the method of Bears and Siger (16); the initial rate of decrease of H_2O_2 concentration was measured at 250 nm. Appropriate corrections were made for inhibition of enzymes by drugs.

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Abbreviations: PMN, polymorphonuclear leukocyte; Me_2SO, dimethyl sulfoxide; ER, elasmobranch Ringer's solution; LDH, lactic dehydrogenase; $MeSO_4$, methyl sulfate; $EtSO_4$, ethyl sulfate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid.

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Cytotoxic Enzyme Release in Egg/Phagocyte Mixtures. These experiments were carried out as described (2); release of β-glucuronidase and catalase was measured.

Morphological Studies. Light micrographs were made by using a Zeiss Axiomat microscope equipped with Nomarski optics. In studies in phagocytosis of zymosan particles, the phagocytes were stained supravitally with neutral red (final concentration, ≈0.5%). Eggs and phagocytes were prepared for electron microscopy as described (13).

Activation of eggs (by phagocytes, sperm, or A23187) and attachment and attack on eggs by phagocytes were analyzed by light microscopy. Suspensions of eggs (2.0 ml) were mixed with sperm, phagocytes, or pharmacological agents in plastic dishes (35 × 10 mm) and evaluated ("blind") for: (*i*) activation (lifting of the fertilization membrane; peripheral migration of pigment granules); (*ii*) attachment of phagocytes (adherence during agitation); (*iii*) attack of phagocytes (furrowing and erosion of the surface of the egg subjacent to one or more phagocytes; lysis of the egg with extrusion of cytoplasm). In each observation, 200 eggs were scored.

Reagents. Phloretin, pyridoxal phosphate, ouabain, dimethyl sulfoxide (Me_2SO), and phenolphthalein glucuronic acid were obtained from Sigma; heparin (Na salt), from Sigma or from Fisher; zymosan and SITS, from ICN; neutral red, from Eastman Kodak; furosemide (Lasix), from Hoechst–Roussel Pharmaceuticals (Somerville, NJ); Na ethacrynate (Edecrin), from Merck Sharp & Dohme; the Ca ionophore A23187, from Lilly; hydrogen peroxide, from Fisher; Triton X-100, from New England Nuclear; choline chloride, from Syntex Agri-Business Inc. (Springfield, MO); and Na and K methyl sulfate ($MeSO_4$) and Ca ethyl sulfate ($EtSO_4$), from City Chemical Corp. (New York). Solutions of salts of choline, $MeSO_4$, and $EtSO_4$ were prepared fresh on the day of use.

RESULTS

Enzyme secretion by phagocytes

Morphology. In dogfish phagocytes exposed to a dilute solution of neutral red, the dye became concentrated in lysosomes (Fig. 1 *a* and *b*). The cells readily took up zymosan particles by phagocytosis (Fig. 1*c*). Lysosomes fused with the phagosomes as they formed around the particles. This process (degranulation) was evident in Fig. 1*c* by the neutral red staining of the engulfed particles. Furthermore, lysosomes in cells containing particles were decreased in number or absent. The zymosan particles remaining unstained in cells were those most recently ingested. Pretreatment of phagocytes with ethacrynate prevented neither the staining of lysosomes nor phagocytosis of zymosan (Fig. 1 *d–f*). However, the drug did prevent degranulation as evidenced by the absence of staining of ingested particles and the persistence of stained lysosomes.

General Characteristics of Secretion. Fig. 2 shows dependence of secretion of β-glucuronidase on the concentration of zymosan (measured at 15 min) and the time course of secretion induced by zymosan at 80 mg/ml. The data show that neither of the conditions used routinely (15 min; 80 mg/ml) induces maximal release.

Pharmacological Modification of Secretion. Six drugs that affect membrane transport were tested for their effects on secretion of β-glucuronidase. Fig. 3 shows dose–response curves for inhibition of β-glucuronidase secretion. Phloretin was the most active and ouabain was the least.

Effect of Varying External Concentration of Inorganic Ions. Pazoles and Pollard (17) have reported that substituting a large organic anion for chloride inhibited "chemosmotic" secretion from chromaffin granules. We tested this possibility in dogfish phagocytes by substituting $MeSO_4$ (and $EtSO_4$) for Cl in the suspending solution ($MeSO_4$:$EtSO_4$, 29:1). After partial (≈66%) replacement of Cl^-, secretion of β-glucuronidase was inhibited about 50% (Table 1); more nearly complete substitution (≈95%) inhibited secretion completely, consistent with the findings of Pollard's group on secretion from parathyroid tissue (18) and release from chromaffin granules (17) (serotonin release from platelets was not inhibited by substituting organic anions for chloride) (19).

Cells in which secretion had been inhibited 50% by partial substitution of chloride retained their response to ethacrynate and SITS—i.e., the drugs diminished secretion by the same fraction in normal and low [Cl] cells. The anions substituted for Cl may, however, decrease Ca activity. $MeSO_4$ decreases Ca

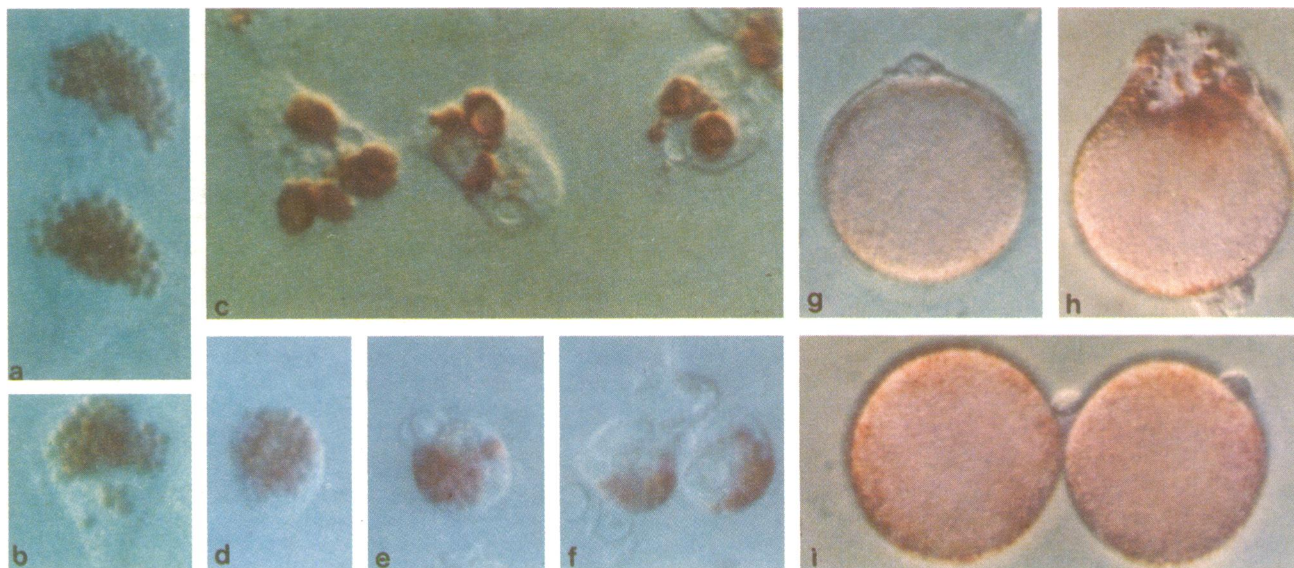

FIG. 1. Light micrographs (Nomarski optics) of dogfish phagocytes (*a–f*) or mixtures of phagocytes and sea urchin eggs (*g–i*). (*a–f*) Phagocytes adherent to glass slides were incubated 15 min with ER containing neutral red (0.5%); the cultures were then rinsed with ER. (*a* and *b*) Control phagocytes; (*c*) similar preparation 30 min after addition of zymosan particles; (*d*) phagocytes alone exposed throughout to 0.5 mM ethacrynate; (*e* and *f*) preparation similar to *d* 30 min after addition of zymosan. (*g–i*) Phagocytes mixed with eggs; (*g*) control mixture, 30 min; (*h*) control mixture, 2 hr; (*i*) mixture with ethacrynate (0.5 mM), 2 hr. (Nomanski optics; *a–f*, ×1900; *g–i*, ×380.)

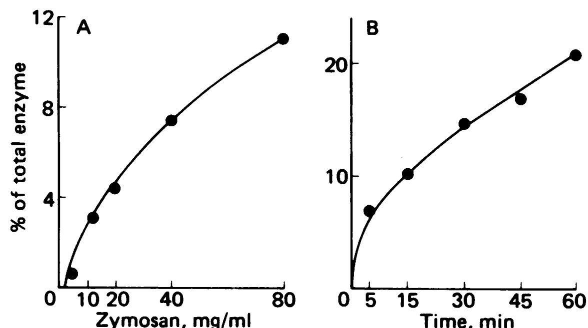

FIG. 2. Zymosan-induced release of β-glucuronidase by dogfish phagocytes. (*A*) Effect of varying concentration of zymosan (incubation time, 15 min). (*B*) Time course (zymosan concentration, 80 mg/ml). Enzyme release is expressed as percentage of total enzyme, determined on Triton X-100 extracts of cell pellets. Incubation mixtures contained $\approx 5 \times 10^7$ leukocytes; 0.4×10^7 were neutral red-staining phagocytes. Absolute activity of β-glucuronidase in the extracts was 1.4 μg of phenolphthalein released per hour per 10^7 cells. The points are means from single replicated experiments; the results of each experiment are representative of two others. Release of β-glucuronidase from phagocytes was determined in more than 50 experiments (15-min incubation) without and with zymosan (80 mg/ml). Means (±SEM) were 13.6% of total enzyme release with zymosan (± 1.4; n = 92) and 3.5% release in the control suspensions of phagocytes (± 0.3; n = 92).

activity less than most organic ions (20), but the effect of $EtSO_4$ is not known (although its concentration in the ER was less than 10 mM). The effect of varying Ca activity in enzyme secretion was determined by varying [Ca] (Table 1). Doubling [Ca] or decreasing it by 90% affected neither enzyme secretion nor its sensitivity to ethacrynate. By contrast, lowering [Na] in the external medium did not inhibit secretion; slight stimulation was observed.

Attack of eggs by phagocytes

Morphology. Light micrographs (Fig. 1 *g–i*) and electron micrographs (Fig. 4) illustrated attachment of phagocytes to eggs and subsequent injury to the eggs. Fig. 1*g* shows an early phase of attachment (30 min); the phagocyte has spread on the egg and has induced formation of a fertilization membrane. Fig. 1*h* shows an egg with phagocytes 2 hr after attachment. The egg has lysed, and the egg's pigment granules have accumulated at the locus of rupture. Fig. 4*a* shows an electron micrograph of the surface of an egg, with intact cortical granules before exposure to phagocytes. Fig. 4*b* shows a phagocyte in contact with an egg. Many of the cortical granules have discharged, leading to the formation of the fertilization membrane (not shown); the surface of the egg subjacent to the phagocyte is furrowed. Fig. 1*i* shows light micrographs of eggs and phagocytes after 2 hr in medium containing ethacrynate. A phagocyte is attached to an egg, but there is no indication of injury to the egg. Dose–response studies of inhibition by ethacrynate of cytotoxicity (5 min preincubation) showed 82% inhibition at 0.5 mM scored by morphology (no drug treatment = 77% of cells attacked).

Table 1. Effects of varying concentrations of Cl, Ca, and Na in ER on secretion of β-glucuronidase by dogfish phagocytes

		% inhibition of release	
mM	β-Glucuronidase, % release	Ethacrynate (0.5 mM)	SITS (1.0 mM)
Varying [Cl]			
310 (normal)	15.2	53	33
≈100	7.9	61	29
310	8.1	67	10
17	0.0	NFI	NFI
Varying [Ca]			
20	7.8	47	ND
10 (normal)	8.7	42	ND
≈1	8.2	49	ND
Varying [Na]			
285 (normal)	10.4	50	ND
38	12.9	51	ND

ER was prepared with 12 mM KCl, 4.5 mM $NaHCO_3$, 10 mM dextrose, and 360 mM urea. Hepes (5 mM) was used as buffer rather than $Na(PO_4)_x$. To vary [Ca], $CaCl_2$ was either omitted or substituted for NaCl. To vary [Na], choline Cl was substituted for NaCl. To vary [Cl], the Ringer solution was prepared with $NaMeSO_4$, $KMeSO_4$, and $CaEtSO_4$ intead of the chloride salts. Incubation time, 15 min; zymosan, 80 mg/ml. ND, not determined; NFI, no further inhibition.

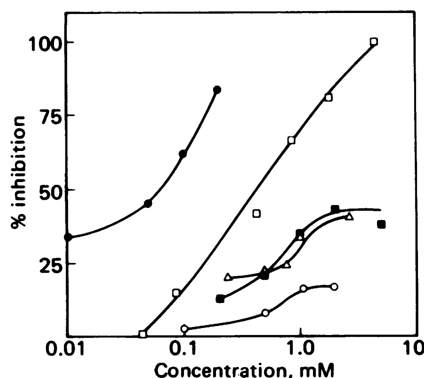

FIG. 3. Inhibition of secretion of β-glucuronidase by dogfish phagocytes. Dose–response curves for five drugs: ●, phloretin in 0.1–1% Me_2SO; □, ethacrynate; ■, furosemide; △, SITS; ○, ouabain. Results are from single replicated experiments with the exception of SITS for which the points represent means from two to six experiments. Percentage inhibition was calculated as percentage of release with zymosan in the absence of drug. In all experiments, incubation time was 15 min and zymosan concentration was 80 mg/ml. Release in the presence of phloretin was corrected for control samples incubated with Me_2SO (0.1–1.0%, vol/vol).

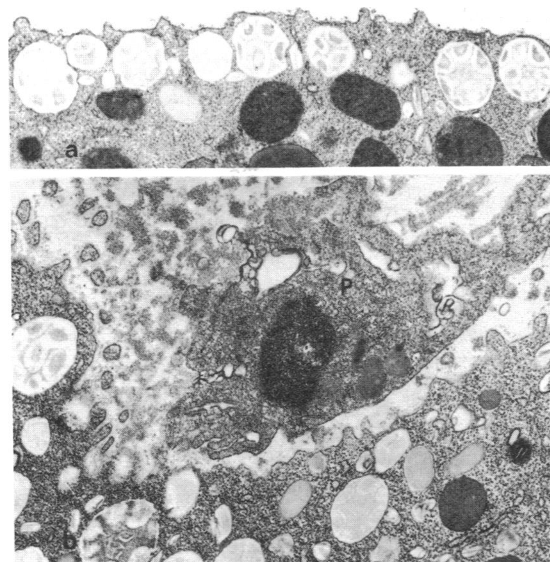

FIG. 4. Electron micrographs. (*a*) Surface of an untreated sea urchin egg. (*b*) Surface of an egg with attached dogfish phagocyte (P), 30 min after mixing of the two cell types. (×11,900.)

Effects of Ethacrynate on Attack and Attachment. It remained possible that the primary effect of ethacrynate on cytotoxicity was inhibition of attachment of phagocytes to eggs. Indeed, ethacrynate-treated phagocytes appeared to be more rounded than control cells (Fig. 1), and spreading and attachment are probably related. Accordingly, attack and attachment of phagocytes to eggs, in both control and ethacrynate-treated mixtures, were scored morphologically. In four experiments (400 eggs examined in each), the drug strongly inhibited attack but had no effect on attachment. In controls, 38 ± 2% of eggs were attacked; phagocytes attached to 42 ± 9%. In mixtures exposed to 0.5 mM ethacrynate, 4 ± 1% of eggs were attacked, but phagocytes remained attached to 39 ± 9%.

Activation of Eggs by Sperm and Ca Ionophore: Effects of Drugs. It remained possible that inhibition of cytotoxicity by drugs was due to impairment of eggs' ability to respond to *any* activating stimulus. Therefore, drug-treated eggs were exposed sequentially to sperm and ionophore A23187 (21). Eggs were preincubated for 5 min with drugs; after sperm were added, activation was scored (Table 2). Furosemide at 5 mM had no effect on activation in this system, but ethacrynate, SITS, and pyridoxal phosphate (1 mM each) all were completely inhibitory. However, inhibition was completely reversible by subsequent addition of A23187. To determine whether the drugs were acting on sperm or eggs, each was preincubated with drug for 5 min and then removed from the suspending medium by centrifugation. Eggs treated with drug in this manner were fully activated by sperm. The sperm pretreated with ethacrynate were unable to activate untreated eggs, but the eggs were fully activated by ionophore.

Pattern of Drugs Inhibition of Cytotoxicity and Enzyme Secretion. To test further the hypothesis that inhibition of secretion is causally related to inhibition of cytotoxicity, we compared patterns of inhibition of six drugs on both processes

Table 2. Effect of drugs on activation of sea urchin eggs by sperm and by Ca ionophore A23187

	% activation	
	First addition: sperm (t = 0)	Second addition: A23187 (t = 5 min)
Control		
Eggs + sperm	100	—
Eggs preincubated with drug		
Eggs_{EA} + sperm	97	—
$\text{Eggs}_{\text{SITS}}$ + sperm	100	—
Eggs_{PYR} + sperm	98	—
Sperm preincubated with drug		
Eggs + sperm_{EA}	0	99
Eggs + $\text{sperm}_{\text{SITS}}$	74	98
Eggs + $\text{sperm}_{\text{PYR}}$	100	—
Drug present throughout		
$(\text{Eggs + sperm})_{\text{EA}}$	0	99
$(\text{Eggs + sperm})_{\text{SITS}}$	0	100
$(\text{Eggs + sperm})_{\text{PYR}}$	0	99

Eggs and sperm in sea water were preincubated for 5 min with ethacrynate, SITS, or pyridoxal phosphate (all at 1 mM). The cells (both with and without drug) were centrifuged and resuspended in fresh sea water. One drop of sperm suspension was added to eggs (t = 0) and 100 eggs were scored for activation (t = 5 min). For control, cells were centrifuged but not treated with drug. EA, ethacrynate; SITS, SITS; PYR, pyridoxal phosphate. When eggs and sperm had been pretreated, the drugs were present after t = 0. In conditions such that sperm activated <90% of eggs, A23187 was added (t = 5 min; 17 μM), and activation was scored again after an additional 2 min (t = 7 min).

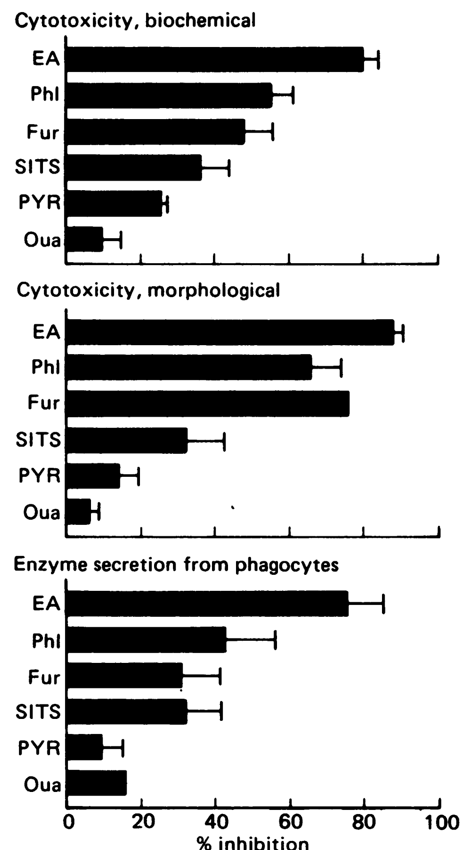

FIG. 5. Inhibition by drugs of phagocyte-mediated cytotoxicity, and of secretion of enzymes from phagocytes. Cytotoxicity (3–5 hr) was measured biochemically (by release of β-glucuronidase from damaged eggs) and morphologically. Secretion of β-glucuronidase from phagocytes was induced by zymosan (80 mg/ml; 15 min). Bars show means; SEM is indicated when $n > 4$. Drugs: EA, ethacrynate, 1.0 mM; Phl, phloretin, 0.1 mM; Fur, furosemide, 5.0 mM; SITS, 1.0 mM; PYR, pyridoxal phosphate, 1.0 mM; Oua, ouabain, 0.5 mM.

(Fig. 5). It has been demonstrated (2) that lysis of immunoglobulin-coated or uncoated eggs caused by phagocytes could be quantitated by measurement of enzymes released from eggs. In the present experiments we dispensed with coating the eggs and we increased the number of phagocytes to enhance lysis. Six drugs were tested for their capacity to inhibit cytotoxic release of β-glucuronidase from mixtures of eggs and phagocytes. The pattern of inhibition (Fig. 5 *top*) closely resembled that for cytotoxicity measured morphologically (Fig. 5 *middle*) and that for zymosan-induced secretion of enzyme from phagocytes (Fig. 5 *bottom*). Appropriate calculations (details are given in ref. 2) showed that β-glucuronidase released from phagocytes (rather than eggs) could have comprised no more than 15% of the total activity recovered in the supernatant solutions.

In two experiments on cytotoxicity, release of catalase from sea urchin eggs was also measured. This enzyme is undetectable in dogfish phagocytes (2). Percentage inhibition of catalase release from cytotoxic mixtures paralleled the inhibition of β-glucuronidase release shown in Fig. 5 *top*: ethacrynate, 78%; phloretin, 40%; SITS, 15%; pyridoxal phosphate, 18.5%; and ouabain, 19% (n = 2). Inhibition by furosemide was not determined; it absorbs strongly at 240 nm, interfering with assays of catalase.

Measurement of release of cytoplasmic lactic dehydrogenase is used to distinguish cell lysis from selective secretion of lysosomal enzymes (3, 14). In eight experiments in which inhibition of secretion was measured for phloretin, pyridoxal phosphate, and furosemide, lactic dehydrogenase release was also measured

(data not shown). By this criterion, the drugs were not cytotoxic.

DISCUSSION

Degranulation of dogfish phagocytes and secretion of lysosomal enzymes were inhibited by several agents usually used to inhibit membrane transport of ions or other solutes. In this respect, dogfish phagocytes resemble human PMNs, the secretion of enzymes from which is also inhibited in the order: phloretin > ethacrynate > furosemide > SITS ≫ pyridoxal phosphate > ouabain (11). When secretion from dogfish cells was inhibited, so was phagocyte-mediated cytotoxicity, and the order of activity of these agents was nearly the same for both types of measurements. Consequently, these experiments provide strong support for the previous suggestion (2) that secretion of lysosomal contents from phagocytes is responsible both for activation (the fertilization response) and for cytolysis of target eggs. Additional support for this hypothesis is afforded by our present demonstration that ethacrynate-treated phagocytes attached appropriately to target cells and failed to degranulate on contact with zymosan particles. Nor did the drugs inhibit activation of target cells by ionophore A23187, further evidence that the site of action of the drugs was on the secreting phagocyte and not on the eggs.

The mechanism(s) underlying the action of the inhibitory agents is as yet unclear. On first inspection, these experiments would appear to provide confirmation of the hypothesis of Pollard's group (19) that "anion-transport-dependent osmotic lysis" of secretory granules is a common mechanism for the exocytosis of stored granule contents. Enhanced transport of anions (and counterions) into secretory granules after their fusion with the plasma membrane leads to osmotic swelling and "fission" of the granules; their contents are released extracellularly through the region of contact with the plasma membrane. Fusion, fission, and release can be signalled by such varied stimuli as thrombin or ionophore A23187 in the platelet (19), by ATP in isolated chromaffin granules (17), and by low Ca^{2+}, β-agonists, and dopamine which stimulate parathyroid hormone release from parathyroid cells (18).

SITS and pyridoxal are perhaps best characterized as inhibitors of anion transport (22, 23); ethacrynate (24), furosemide (25), and phloretin (26) are also potent inhibitors of anion transport in one or another cell type. Moreover, ethacrynate inhibits exocytosis of mast cell contents (27), and furosemide blocks the platelet response to collagen or ADP (28)—each at concentrations (0.1–1 mM) that inhibit anion transport nearly 800-fold (29). Further support for the anion-transport hypothesis is that, whereas variations in ambient [Ca] and [Na] failed to inhibit lysosomal enzyme secretion from dogfish phagocytes, replacement of permeant anions by impermeant $MeSO_4$ did decrease enzyme secretion. Only ouabain, which was minimally inhibitory for enzyme secretion, has no known effects on anion transport.

However, ethacrynate, furosemide, phloretin, and ouabain also have profound effects on cation transport across various membranes (30). Showell *et al.* (10) and Korchak and Hoffstein (11) have shown that replacement of external Na by choline decreases enzyme secretion from human PMNs. Moreover, the inhibitors could have other effects upon phagocytes, independent of these known actions on transport processes. Whatever the detailed mode of action of this group of inhibitors proves to be, we have found that agents previously known chiefly for their effects on membrane transport can inhibit not only lysosomal enzyme secretion but also the consequent cytolysis of resting cells. This class of agents may therefore prove to be of use in modifying those aspects of tissue injury and inflammation mediated by release of lysosomal constituents from phagocytes (14).

We thank Ms. Ann Scarborough for assistance in obtaining the light micrographs. The work was supported by grants from the National Institutes of Health (CA-19064 to P.D., RCDA, HL-00356 to S.H.; AM-11949, HL-19072, HL-19721, GM-23211 to G.W.) and from the National Foundation–March of Dimes (to G.W.).

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