Biochemical differences in the mechanism of macrophage lysosomal exocytosis initiated by zymosan particles and weak bases

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(Received 14 January 1983/Accepted 17 March 1983)

1. By utilizing compounds with different inhibitory properties, discrete biochemical differences were found in the mechanism of selective lysosomal enzyme secretion by macrophages in response to stimulation with zymosan particles and methylamine. 2. Pretreatment of macrophages with trypsin markedly impaired the capacity of the cells to respond to stimulation with zymosan particles, but had no effect on methylamine-stimulated lysosomal enzyme secretion. 3. Similarly, the addition of phenylmethanesulphonyl fluoride or EDTA to the incubation medium substantially inhibited zymosan-induced lysosomal enzyme secretion, whereas the methylamine-stimulated response was unaffected by these agents. 4. The addition of 2-deoxyglucose to incubation media, however, strongly inhibited both zymosan- and methylamine-stimulated β -galactosidase secretion. 5. These findings are consistent with a mechanism for lysosomal enzyme secretion by macrophages, based on a receptor-dependent uptake of zymosan particles and a receptor-independent uptake of methylamine.

The selective secretion of lysosomal enzymes by mononuclear phagocytes can be effected by two distinct pathways of cell activation.

The major, pathologically important, pathway of triggering lysosomal secretion occurs in response to exposure of mononuclear phagocytes to sterile inflammatory agents such as zymosan particles (Schlorlemmer *et al.*, 1977a), group-A streptococcal cell walls (Davies *et al.*, 1974) and insoluble antigen-antibody complexes (Cardella *et al.*, 1974). The biochemical basis of this response is largely unknown, although it has been proposed by Schlorlemmer *et al.* (1977b) that stimulating agents such as those mentioned above activate the alternative pathway of complement by utilizing endogenous macrophage-derived complement components. This results in the generation of subcomponent C3b, which in other studies has also proved to be an effective, if somewhat slow, stimulant of lysosomal secretion (Schlorlemmer & Allison, 1976).

Lysosomal secretion can also be triggered in macrophage cultures after their exposure to weak bases such as methylamine (Riches & Stanworth,

1980) and chloroquine (Riches *et al.*, 1981). Here, too, the biochemical events underlying the secretory process remain largely unresolved, although it has been established that the mechanism of secretion by these agents is functionally distinguishable from that of zymosan-induced secretion, and, moreover, occurs independently of alternative-pathway complement activation (Riches & Stanworth, 1982).

In the present paper, we have attempted to define more precisely some of the biochemical processes that are involved in the initiation of lysosomal secretion by macrophages by both pathways of stimulation of secretion. Our findings extend our earlier observation of the functional separability of the pathways leading to lysosomal exocytosis by showing discrete biochemical differences in the responses of macrophages to zymosan particles and methylamine.

Materials and methods

Animals

Adult male BALB/C mice, weighing approx. 15–25 g, were obtained from the animal house of the Department of Immunology, University of Birmingham, U.K.

Tissue-culture materials

Medium 199, streptomycin and penicillin were obtained from Gibco Bio-Cult Ltd., Paisley, Scot-

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land, U.K. Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was purchased from Sigma Chemical Co., Poole, Dorset, U.K. Heat-inactivated foetal-calf serum was obtained from the Immunodiagnosics Research Laboratory, Department of Immunology, University of Birmingham, U.K. Falcon 3047 24-well tissue-culture plates were purchased from Becton Dickinson (U.K.) Ltd., Wembley, Middx., U.K.

Biochemical reagents

Lactic acid, NAD⁺ (grade AA-1), NADH (grade III), 4-methylumbelliferyl β -D-galactopyranoside, zymosan A, methylamine hydrochloride, EDTA, EGTA, phenylmethanesulphonyl fluoride, 2-deoxyglucose, soya-bean trypsin inhibitor (type 1-S), trypsin (type IX) and deoxyribonuclease I (DN-25) were purchased from Sigma.

Macrophage collection, purification and culture

Unstimulated mouse peritoneal macrophages were isolated and purified as previously described (Riches & Stanworth, 1980). After being cultured overnight in medium 199 buffered with 20 mM-Hepes, pH 7.3, and supplemented with 10% (v/v) heat-inactivated foetal-calf serum, 100 μ g of streptomycin/ml and 100 units of penicillin/ml, the purified

optimal conditions that gave a linear release of reaction product with time. The general range of absolute enzyme activity recorded for β -galactosidase was 100–200 nmol of 4-methylumbelliferone liberated/min per culture, and that of lactate dehydrogenase was 6–12 μ mol of NAD⁺ reduced/min per culture.

Trypsin treatment of macrophages

Macrophages were pretreated with trypsin by the method of Halbwachs & Lachmann (1976). Cell monolayers prepared in multiwell plates were initially treated with deoxyribonuclease I (0.01% w/v) at 37°C for 10 min to prevent the formation of mucus-like material (presumably DNA released from senescent cells) during the subsequent trypsin-treatment stage. The macrophages were then rinsed with medium 199 and exposed to increasing concentrations of trypsin (0.5–10.0 mg/ml) for 30 min at 37°C. The cells were then rinsed once in 1% (w/v) soya-bean trypsin inhibitor to inactivate residual trypsin, and twice in medium 199, before being exposed to stimulating agents.

Data presentation

Values of relative β -galactosidase secretion were calculated as follows:

$$\text{Relative } \beta\text{-glucosidase secretion (\%)} = \frac{\% \text{ secretion in the presence of inhibitors}}{\% \text{ secretion in the absence of inhibitors}} \times 100$$

macrophage monolayers were rinsed three times with 0.01 M-phosphate-buffered saline (0.14 M-NaCl), and once with serum-free medium 199 before being challenged with 1 ml of serum-free medium 199 containing either methylamine (25 mM) for 4 h or zymosan particles (100 μ g/ml) for 6 h. At the end of the incubation period, the supernatants were removed and any detached cells were pelleted, and the cells attached to the culture wells were lysed in 1 ml of 0.1% (v/v) Triton X-100 in 0.9% NaCl. Detached cells were combined with the Triton lysate. Under these conditions of stimulation, approx. 50–55% of the total available β -galactosidase was secreted in the presence of zymosan particles, whereas methylamine released approx. 60–65% of the cellular β -galactosidase into the incubation medium. Lactate dehydrogenase release in these experiments was always less than 7% of the total available activity.

Enzyme assays

Both supernatants and cell lysates were analysed for activities of the lysosomal enzyme β -galactosidase and the cytoplasmic enzyme lactate dehydrogenase by automated continuous-flow methods that have been described in detail elsewhere (Riches & Stanworth, 1981). All assays were conducted under

Comparisons of means were made by Student's *t* test. Each point represents the mean $\pm$ s.d. for at least four experiments.

Results

Effect of trypsin

The involvement of a trypsin-sensitive cell surface component(s) [presumably a glycoprotein(s)] in the initiation of lysosomal enzyme secretion was investigated by exposing macrophages to increasing concentrations of trypsin (0.5–10.0 mg/ml) for 30 min before challenge with a fixed concentration of either zymosan (100 μ g/ml) or methylamine (25 mM) for 4 h at 37°C. As illustrated in Fig. 1, the ability of macrophages to respond to methylamine stimulation was unaffected by pretreatment of the cells with trypsin, indicating possibly the inaccessibility of a hypothetical weak-base carrier to trypsin, or, more probably, the lack of involvement of trypsin-sensitive membrane glycoproteins in this type of cell activation.

By contrast, β -galactosidase secretion in response to zymosan ingestion was markedly impaired by pretreatment of macrophages with trypsin (Fig. 1). A significant ($P < 0.01$) inhibition of secretion was detected with a trypsin concentration of 0.5 mg/ml;

Fig. 1. Concentration-dependent effects of pretreatment of macrophage monolayers with trypsin on the subsequent secretion of β -galactosidase induced by exposure to methylamine (25 mM, ○) and zymosan (100 μ g/ml, □) for 4 h at 37°C

For details, see the text. Bars represent $\pm$ S.D.

Fig. 2. Concentration-dependent effects of phenylmethanesulphonyl fluoride (PMSF) on the secretion of β -galactosidase induced by exposure of macrophages to methylamine (25 mM) for 4 h (○) or zymosan (100 μ g/ml) for 6 h (□)

For details, see the text. Bars represent $\pm$ S.D.

and maximum inhibition, which reached approx. 50%, was obtained by pretreatment of macrophages with concentrations of trypsin greater than 5 mg/ml. Interestingly, the degree of inhibition of zymosan-stimulated lysosomal enzyme secretion appeared to be inversely related to the duration of zymosan exposure, i.e. greater overall inhibition was achieved with shorter zymosan challenge periods (D. W. H. Riches, unpublished work). It is presumed that this finding is related to the kinetics of re-expression of the zymosan-binding protein, after its removal by trypsin.

Effect of phenylmethanesulphonyl fluoride

Evidence of an involvement of a phenylmethanesulphonyl fluoride-sensitive site (possibly a serine esterase) in the initiation of lysosomal enzyme secretion from macrophages was sought by exposing cells to this inhibitor (0.05–1.0 mM) in the presence of a fixed concentration of zymosan (100 μ g/ml) or methylamine (25 mM) for 6 h or 4 h respectively. Exposure of macrophages to zymosan in the presence of increasing concentrations of phenylmethanesulphonyl fluoride strongly inhibited β -galactosidase secretion in a concentration-dependent fashion (Fig. 2), with maximum inhibition (81%) at 1 mM-inhibitor. Methylamine-induced β -galactosidase secretion, however, was totally refractory to inhibition by phenylmethanesulphonyl fluoride in the concentration range used in the present study (Fig. 2).

Effect of bivalent-cation chelators

The influence of Ca^{2+} and Mg^{2+} on zymosan- and methylamine-stimulated β -galactosidase secretion by

Fig. 3. Influence of EDTA on the secretion of β -galactosidase induced by exposure to methylamine (25 mM, ○) or zymosan (100 μ g/ml, □) for 4 h or 6 h respectively

For details, see the text. Bars represent $\pm$ S.D.

macrophages was investigated by adding either EDTA [to chelate Ca^{2+} (1.8 mM) and Mg^{2+} (0.8 mM) from the culture medium] or EGTA (to chelate Ca^{2+} alone) to macrophage cultures 5 min before adding stimulating agents. As shown in Fig. 3, the addition to the culture medium of EDTA at concentrations of 1–2 mM had no effect on zymosan-stimulated β -galactosidase secretion, but when it was added at 3–5 mM the secretory response was virtually abolished, indicating a definite requirement for Ca^{2+} and/or Mg^{2+} in the initiation of zymosan-stimulated lysosomal enzyme secretion. Identical concentrations of EDTA, however, had no effect on methylamine-stimulated β -galactosidase secretion (Fig. 3),

indicating the lack of dependence of this type of stimulus on extracellular bivalent cations.

The effect of chelating Ca^{2+} alone by adding EGTA (1–5 mM) to the culture medium is illustrated in Fig. 4, which shows that neither methylamine- nor zymosan-stimulated β -galactosidase secretion was affected by the addition of EGTA, thereby illustrating that lysosomal-enzyme secretion initiated by both types of stimulus is not dependent on extracellular Ca^{2+} . It is not clear, however, whether Ca^{2+} would substitute for Mg^{2+} in the absence of Mg^{2+} .

Effect of 2-deoxyglucose

The energy-dependence of lysosomal enzyme secretion was studied by exposing macrophage monolayers to zymosan (100 $\mu\text{g}/\text{ml}$) or methylamine (10 mM) for 6 h in the presence of 2-deoxyglucose, a competitive inhibitor of glucose transport

and of glycolysis. Glucose is present in medium 199 at a concentration of 5 mM; thus high concentrations of 2-deoxyglucose (2.5–50 mM) had to be added to the culture medium to overcome the effects of exogenous glucose. Contrary to the effects of the other agents used in the present study, the addition of 2-deoxyglucose to incubation media led to a concentration-dependent inhibition, of similar magnitude, of both zymosan- and methylamine-stimulated β -galactosidase secretion (Fig. 5). In both cases, significant inhibition was achieved with 2.5 mM-2-deoxyglucose, and maximum inhibition (approx. 80%) was attained with concentrations of 2-deoxyglucose greater than 25 mM.

To confirm that the inhibition of secretion obtained with 2-deoxyglucose was not due to an osmotic effect, macrophages were also exposed to zymosan or methylamine in the presence of identical concentrations of glucose. As illustrated in Fig. 5, zymosan-stimulated β -galactosidase secretion was unaffected by glucose at concentrations up to 25 mM, but a significant inhibition ($P < 0.02$) of approx. 17% was recorded at a glucose concentration of 50 mM. Methylamine-stimulated β -galactosidase secretion, however, was slightly enhanced by 50 mM-glucose (Fig. 5).

Discussion

The initiation of acid hydrolase secretion by macrophages exposed to zymosan particles and methylamine has been shown by ourselves (Riches & Stanworth, 1982) and by others (Jessup *et al.*, 1982) to be mediated by two functionally distinguishable mechanisms of macrophage stimulation. These observations have now been extended in the present study, in which we have demonstrated specific biochemical differences in the responses of macrophages towards these two types of secretory stimulus.

The secretion of lysosomal β -galactosidase in response to methylamine accumulation was found to be unaffected by three potential receptor-activity-modifying agents, i.e. pretreatment of the cells with trypsin, deprivation of the bivalent cations Mg^{2+} and Ca^{2+} , and exposure of the cells to phenylmethane-sulphonyl fluoride, an irreversible serine esterase inhibitor.

These findings are therefore consistent with the receptor-independent mechanism of weak-base uptake proposed initially by de Duve *et al.* (1974) and which has more recently been expanded by Ohkuma & Poole (1978, 1981) and by Poole & Ohkuma (1981). The cellular uptake and lysosomal accumulation of weak bases is viewed as being a function, firstly, of the large pH gradient which is maintained across the lysosomal membrane partly by a Donnan equilibrium (Reijngoud & Tager, 1977) and partly

Fig. 4. Influence of EGTA on the secretion of β -galactosidase induced by exposure to methylamine (25 mM, ○) or zymosan (100 $\mu\text{g}/\text{ml}$, □) for 4 h or 6 h respectively. For details, see the text. Bars represent $\pm$ S.D.

Fig. 5. Concentration-dependent effects of 2-deoxyglucose (○, □) or glucose (●, ■) on β -galactosidase secretion induced by exposure of macrophages to methylamine (10 mM, ○, ●) or zymosan (100 $\mu\text{g}/\text{ml}$, □, ■) for 6 h. For details, see the text. Bars represent $\pm$ S.D.

by an ATP-driven proton pump (Schneider, 1981), and, secondly, of the much greater permeability of the plasma and lysosomal membranes to the neutral free base $R-NH_2$ compared with the protonated conjugate acid $R-NH_3^+$. Thus, following the passive diffusion of the free base, CH_3NH_2 , through the lipid bilayer of the plasma membrane in a receptor-independent fashion, methylamine will diffuse into the lysosomes and become protonated ($CH_3NH_3^+$), thereby retarding its back-diffusion. Furthermore, continual acidification of the lysosomes by the membrane-located ATP-driven proton pump would result in further accumulation of methylamine, which, in turn, partially dissipates the pH gradient across the lysosomal membrane (Ohkuma & Poole, 1978). As the intralysosomal concentration of the weak base increases, the lysosomes can be seen to undergo marked osmotic expansion (Fedorko *et al.*, 1968; Seglen & Gordon, 1979) and, as revealed by electron-microscopic examination, to fuse with one another and, more importantly, with the plasma membrane, thereby releasing the lysosomal contents (acid hydrolases and accumulated weak base) into the extracellular environment (Riches *et al.*, 1981). However, it is not yet clear if the ability of weak bases to induce lysosomal enzyme secretion by macrophages can solely be ascribed to their effects on the lysosomes. Many weak bases, including methylamine, have been shown to inhibit transglutaminase, an enzyme implicated in cross-linking the sub-membrane microfilament mat in 3T3 cells (Davies *et al.*, 1980; Levitzki *et al.*, 1980), and which therefore may be important in the restriction of lysosome-plasma-membrane fusion.

In contrast with methylamine-stimulated lysosomal enzyme secretion, β -galactosidase secretion in response to zymosan stimulation was strongly inhibited by pretreating macrophages with trypsin, or by adding phenylmethanesulphonyl fluoride or EDTA to the incubation medium during exposure to zymosan. Moreover, in other studies (D. W. H. Riches, unpublished work), these potential receptor-activity-modifying agents have also been found to impair markedly the attachment of zymosan particles to macrophages, thereby indicating that they probably influence the early stages of secretion rather than influencing a metabolic process subsequent to ligand uptake. This conclusion is confirmed with the observation that, when added 2 h after the zymosan particles, the inhibitory activity of phenylmethanesulphonyl fluoride, EDTA and trypsin is considerably less (D. W. H. Riches, unpublished work).

The identity of the membrane components involved in zymosan attachment and uptake by macrophages have been the subject of much speculation in recent years. Schorlemmer *et al.* (1977b) have suggested that, on exposure of macro-

phages to alternative-complement-pathway activators, including zymosan particles, a C3-convertase is generated from endogenously derived complement components, which can then facilitate the attachment of activating ligands to the macrophage membrane via C3b receptors, whereas in human monocytes the zymosan binding site can be clearly distinguished from C3b receptors (and Fc receptors) on the basis of differential trypsin sensitivity (Czop *et al.*, 1978). However, in view of the abundance of mannan in the zymosan coat, coupled with the finding that the uptake of zymosan particles by macrophages can be inhibited by mannan from the cell walls of *Kloeckera brevis* and *Saccharomyces cerevisiae* (Sung *et al.*, 1981), a likely candidate to facilitate zymosan attachment would seem to be the mannosyl/fucosyl receptor (Stahl *et al.*, 1978), which promotes the uptake by macrophages of glycoconjugates terminated with mannose, L-fucose or *N*-acetylglucosamine. Indeed, the recognized functional characteristics of this receptor are compatible with those identified in the present study as being necessary for the induction of lysosomal enzyme secretion by zymosan, i.e. its activity is inhibited by trypsin treatment of macrophages (Stahl *et al.*, 1978), and also by removal of bivalent cations from the incubation medium (Stahl *et al.*, 1980). Its sensitivity to phenylmethanesulphonyl fluoride, however, remains to be ascertained.

Thus the secretion of lysosomal enzymes by macrophages after stimulation with zymosan can be viewed as a sequel to the initial interaction of the stimulus with a cation-dependent trypsin-sensitive component located on the macrophage plasma membrane. The strong inhibition of attachment and lysosomal enzyme secretion by phenylmethanesulphonyl fluoride also suggests that the zymosan-binding site is either closely coupled to, or dependent on, a serine esterase activity, possibly analogous to that implicated in stimulus-secretion coupling in mast cells (Becker & Austen, 1966), and in phagocytosis, superoxide anion production and lysosomal enzyme secretion by neutrophils (Musson & Becker, 1977; Kitagawa *et al.*, 1980). The molecular mechanism by which irreversible serine esterase inhibitors block stimulus-secretion coupling is at present unclear. Modification of the active site of the putative enzyme by phenylmethanesulphonylation, or (for di-isopropyl fluorophosphate) by phosphorylation, would seem to be the most attractive explanation. However, other studies have shown that certain non-phosphorylating analogues of di-isopropyl fluorophosphate, e.g. di-isopropyl methyl phosphate, can also block lysosomal enzyme secretion in neutrophils (Becker *et al.*, 1978), indicating that these agents may exert their effects at a number of levels.

The only agent found to inhibit both zymosan- and methylamine-stimulated lysosomal enzyme secretion was the glycolysis inhibitor, 2-deoxyglucose. The precise mode of inhibition, however, is not clear. Macrophages derive most of their energy by the anaerobic oxidation of glucose (Karnovsky *et al.*, 1970), and therefore the decrease in macrophage ATP content that accompanies 2-deoxyglucose metabolism (Michl *et al.*, 1976b) might well account for the inhibition of methylamine-stimulated lysosomal enzyme secretion, in view of the proposed involvement of an ATP-driven proton pump in the lysosomal accumulation of weak bases. Zymosan ingestion, however, can take place in spite of the large decrease in cellular ATP produced by 2-deoxyglucose metabolism (Michl *et al.*, 1976a). Therefore, this implies that either 2-deoxyglucose itself, or, more probably, the decrease in cellular ATP that accompanies its metabolism, inhibits a process distal to zymosan uptake, e.g. phagosome/lysosome and/or plasma-membrane/lysosome fusion.

We acknowledge generous financial support from the Arthritis and Rheumatism Council.

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