Spontaneous lysosomal enzyme secretion by a murine macrophage-like cell line

Wendy JESSUP and Roger T. DEAN

Department of Applied Biology, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

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Lysosomal enzyme secretion by the murine macrophage-like cell line, P388D1, was compared with that of normal peritoneal macrophages. Unlike macrophages, lysosomal hydrolase secretion by P388D1 cells occurred spontaneously *in vitro* and was not further stimulated by the presentation of inflammatory agents such as zymosan and asbestos.

Macrophages can influence their environment by the secretion of a wide variety of products. For example, the macrophage is the predominant cell in chronic inflammatory lesions and a striking correlation has been demonstrated between the capacity of certain materials to induce inflammation *in vivo* and the release of macrophage lysosomal hydrolases *in vitro* (Schorlemmer *et al.*, 1977). It has been suggested, therefore, that secreted macrophage hydrolases may be important factors in the initiation and maintenance of chronic inflammatory lesions.

The cell line P388D1, originally derived from cultured murine lymphoblastoid cells, has been shown to possess several properties characteristic of macrophages, including adherence to glass and plastic, phagocytosis of latex, receptors for C3 and the Fc fragment of immunoglobulin, staining for non-specific esterase, antibody-dependent cytotoxicity and secretion of lysozyme (Koren *et al.*, 1975; Snyderman *et al.*, 1977; Ralph & Nakoinz, 1977). These cells may provide a convenient model for the study of macrophage function.

In the present paper we report the selective secretion of lysosomal hydrolases by P388D1 cells and compare this activity with that of normal murine peritoneal macrophages.

Experimental

Materials for tissue culture

Plastic multi-well dishes (35 mm diameter) were from Costar, Cambridge, MA, U.S.A. Tissue culture media, sera and antibiotics were obtained from Flow Laboratories, Irvine, Scotland, U.K. The sera were inactivated by heating at 56°C for 30 min.

Biochemical reagents

Zymosan from *Saccharomyces cerevisiae* and

p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside were from Sigma Chemical Co., Poole, Dorset, U.K.; polystyrene latex particles [0.81 μ m diameter, 5% (v/v)] were from Difco, Detroit, MI, U.S.A.; dextran sulphate (DS 500) was from Serva, Heidelberg, F.R.G.; heparin (preservative-free) was from Paines and Byrne, Greenford, Middx., U.K.; pyruvate and NADH were from Boehringer Mannheim GmbH, F.R.G.; Triton X-100 was from BDH, Poole, Dorset, U.K. Chrysotile asbestos was a UICC standard reference sample of South African chrysotile.

Macrophage collection and culture

Macrophages were obtained from normal Swiss mice (T.O. strain) by peritoneal lavage as described previously (Dean *et al.*, 1979a). The cells were cultivated in medium 199 containing 10% (v/v) heat-inactivated pig serum, 100 i.u./ml of penicillin and 100 μ g/ml of streptomycin at 37°C and gassed with 5% CO₂ in air. After establishment of cultures ($1.5\text{--}2.0 \times 10^6$ cells/3 ml of medium) overnight the medium was changed and the experimental treatment started. During experiments the medium was changed every 2 days; release of enzyme into the medium was calculated from the cumulative activities of all medium changes. At the end of the experiment medium was collected and the cells lysed in 1.5 ml of phosphate-buffered saline containing 0.1% (v/v) Triton X-100 and removed by scraping with a silicone rubber bung.

P388D1 culture

The continuous murine cell line P388D1 (kindly supplied by Dr. S. Foster, ICI Pharmaceuticals Division, Alderley Park, U.K.) was maintained by bi-weekly passage in minimum essential medium (Eagle's) containing 10% (v/v) heat-inactivated

foetal calf serum, 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml. Experimental cultures were established by transferring 3 ml portions ($1.5\text{--}2.0 \times 10^6$ cells) to plastic culture dishes (35 mm diameter). In experiments where stimuli were added the cells were allowed 1 h to adhere and spread before application of the agents. At the end of the experiment the medium was removed and non-adherent cells sedimented in a bench centrifuge. Cells remaining attached to the dish were lysed in 1.5 ml of phosphate-buffered saline containing 0.1% (v/v) Triton X-100, scraped off with a silicone rubber bung and the lysate combined with the pelleted cells.

Enzyme assays

Lactate dehydrogenase (EC 1.1.1.27) and β -N-acetyl-D-glucosaminidase (hexosaminidase; EC 3.2.1.30) were assayed as described by Dean *et al.* (1979b). Lysozyme (EC 3.2.1.17) was measured by the turbidimetric assay described by Barrett & Heath (1977).

Presentation of stimuli to cells

These were suspended directly in the appropriate culture medium at the following concentrations: zymosan, 50 µg/ml; chrysotile asbestos, 50 µg/ml; dextran sulphate, 50 µg/ml; latex, 2 µl of the 5% (v/v) stock/ml of culture medium.

Results are expressed as means $\pm$ s.d. of triplicate cultures (except where these do not exceed the span of the points in the Figure) unless otherwise stated. Results are from single experiments and are representative of several separate experiments.

Results and discussion

Hydrolase secretion during cultivation in vitro

When unstimulated macrophages were maintained in culture, there was a progressive accumulation in the culture medium of the lysosomal enzyme hexosaminidase (Fig. 1a). Since an exactly equivalent release of the cytosolic marker enzyme lactate dehydrogenase was also measured, we concluded that lysosomal hydrolase release by

Fig. 1. Release of enzymes by unstimulated cells during cultivation *in vitro*

(a) Peritoneal macrophages: $1.5\text{--}2.0 \times 10^6$ cells/culture were incubated in medium 199 containing 10% (v/v) heat-inactivated pig serum, 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml. (b) P388D1 cells: $1.5\text{--}2.0 \times 10^6$ cells/culture were incubated in minimum essential medium (Eagle's) containing 10% (v/v) heat-inactivated foetal calf serum, 100 i.u. of penicillin/ml and 100 µg of streptomycin/ml. All cultures were incubated at 37°C in 5% CO₂ in air. Δ, Lysozyme; ●, hexosaminidase; ○, lactate dehydrogenase.

macrophages under these culture conditions was entirely attributable to non-specific cell lysis rather than any selective secretory event. This is in accordance with other observations (Davies *et al.*, 1974; Mørland & Kaplan, 1978). We found no evidence of any spontaneous specific hydrolase release such as that previously reported in long-term cultures (Schnyder & Baggiolini, 1978), even when the incubation period was extended to 12 days. This discrepancy might be accounted for by differences between the strains of mice and culture conditions used in the parallel experiments. Alternatively, it is possible that an apparent 'basal' release may occur in circumstances where macrophages have been pre-exposed to certain kinds of stimulation *in vivo* (e.g. by infection, etc.). It is unlikely that failure to detect selective lysosomal hydrolase release by the unstimulated macrophages in the present work was due to a functionally impaired cell population, since total activities of both hexosaminidase and lactate dehydrogenase increased as cultivation proceeded (hexosaminidase and lactate dehydrogenase intracellular activities increased from 3.47 to 6.74 $\mu\text{mol h}^{-1}$ (10^6 cells) $^{-1}$ and 252 to 490 m-units/ 10^6 cells respectively between day 0 and day 8 in the experiment in Fig. 1(a) and the cells continuously synthesized and selectively secreted lysozyme (Fig. 1a), a characteristic and constitutive function of macrophages under all conditions studied (Gordon *et al.*, 1974).

In similar experiments, the time course of enzyme release by P388D1 cells was studied (Fig. 1b). The cells, after a lag period of 24 h, divided in culture with a doubling time of about 36 h until a final cell density of $3.0\text{--}3.5 \times 10^6$ cells/culture was reached. In the experiment shown in Fig. 1(b) the specific intracellular activities at 24 and 96 h respectively were 1.45 and 1.39 $\mu\text{mol h}^{-1}$ (10^6 cells) $^{-1}$ for hexosaminidase, and 374 and 232 m-units/ 10^6

cells for lactate dehydrogenase. In agreement with earlier work (Ralph & Nakoinz, 1977), it was found that these cultures secrete lysozyme continuously. However, P388D1 cells differed from unstimulated macrophages in that they secreted a large proportion of their lysosomal hydrolase (measured by release of hexosaminidase into the culture medium) independently of concomitant cell lysis and death (i.e. not accompanied by an equivalent release of lactate dehydrogenase). Thus unstimulated P388D1 cells apparently secrete lysosomal enzymes *in vitro* both selectively and spontaneously. Such spontaneous secretion continued even after confluence (72 h) for at least a further 24 h (Fig. 1b). It is interesting that similar behaviour has been suggested for J774, also a murine cell line with macrophage-like properties (Mørland & Kaplan, 1978).

Secretion induced by applied stimuli

The responses of P388D1 hydrolase secretion to the presence of various added stimuli were compared with the well-characterized effects of these agents on macrophage lysosomal enzyme release. The agents included materials which are known to induce inflammation when administered *in vivo* [both of particulate (zymosan and asbestos) and soluble (dextran sulphate) nature] and non-inflammatory polystyrene latex spheres (Schlorlemmer *et al.*, 1977). These were supplied continuously for 24 h, after which hexosaminidase accumulation in the medium was measured (Table 1). Both cell types are highly phagocytic and accumulated large amounts of zymosan and latex. The asbestos particles were too large to be internalized, but both macrophages and P388D1 cells aggregated along the surface of the fibres. In the presence of dextran sulphate all cells rounded up and lost their spread, stellate morphology.

Table 1. Secretion of hexosaminidase by peritoneal macrophages and a macrophage-like cell during exposure to various stimuli

Cultures of peritoneal macrophages or P388D1 cells ($1.5\text{--}2.0 \times 10^6$ cells/3 ml) were established as described in the Experimental section. Cells were exposed continuously to the stimuli for 24 h, then cells and media were separately harvested and assayed for hexosaminidase and lactate dehydrogenase (LDH) activities. Enzyme release is expressed as a percentage of the total (i.e. medium plus cell) activity in the cultures at the end of the experiment. Results are means $\pm$ S.D.; $n = 6$ for peritoneal macrophages and $n = 3$ for P388D1 cultures.

Stimulus	Release during 24 h continuous exposure to stimulus (%)			
	Macrophage		P388D1	
	Hexosaminidase	LDH	Hexosaminidase	LDH
None (medium only)	14.6 $\pm$ 2.4	13.1 $\pm$ 6.2	39.1 $\pm$ 1.4	3.7 $\pm$ 1.3
Zymosan (50 $\mu\text{g/ml}$)	61.2 $\pm$ 2.0	28.9 $\pm$ 9.6	39.8 $\pm$ 0.7	3.8 $\pm$ 0.7
Chrysotile asbestos (50 $\mu\text{g/ml}$)	69.3 $\pm$ 1.8	37.8 $\pm$ 2.2	45.2 $\pm$ 2.5	6.5 $\pm$ 2.8
Latex spheres [2 μl of 5% (v/v) stock/ml]	19.3 $\pm$ 0.9	18.0 $\pm$ 6.4	9.8 $\pm$ 0.7	3.6 $\pm$ 0.2
Dextran sulphate (50 $\mu\text{g/ml}$)	36.6 $\pm$ 3.3	29.2 $\pm$ 3.0	34.1 $\pm$ 1.5	5.8 $\pm$ 3.2

The specific, basal secretion of hexosaminidase by P388D1 cells, but not by macrophages, which was measured in control cultures (Table 1) corresponds with the results of the previous experiment (Fig. 1). Incubation of normal macrophages with the inflammatory agents zymosan and asbestos induced significant selective hydrolase secretion, as previously reported by several workers (see, e.g., Dean *et al.*, 1979a). Dextran sulphate (50 µg/ml) did not induce significant (i.e. $0.10 \leq P \leq 0.15$) selective hexosaminidase release from macrophages although it caused a slight decrease in basal secretion by P388D1 cells. Latex, although internalized, was ineffective as an inducer of macrophage hydrolase secretion.

In contrast, P388D1 cells did not respond to the particulate inflammatory stimuli; no release was measured beyond that basal release which occurred even in control cultures. Since this was not due to the failure of the particles to associate with the cells, any difference in the P388D1 cell response must presumably lie in the manner in which the stimuli interact with the lysosomal system to induce secretion. It is possible that P388D1 cultures secrete at their maximum rate during constitutive unstimulated hydrolase release and are therefore unable to demonstrate any increased secretion in response to externally applied stimuli. The basal secretion by P388D1 cells may be similar functionally to that by macrophages storing previously endocytosed materials (Dean *et al.*, 1979a), although in this case it is not known whether such cells are insensitive to further stimulation. Alternatively, the mechanism of lysosomal enzyme release by these cells may differ from that of normal macrophages, such that they are not subject to the same controls and stimuli. Clearly, if the former situation exists, P388D1 cells may

provide a useful model in investigation of the control of hydrolase secretion. An understanding of this process would be of importance in achieving regulation of lysosomal enzyme hypersecretion in conditions such as chronic inflammation, I-cell disease (Sly & Stahl, 1978) and, possibly, cystic fibrosis (Hösli & Vogt, 1977).

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