Analytical Subcellular Fractionation of Alveolar Macrophages from Normal and BCG-Vaccinated Rabbits with Particular Reference to Heterogeneity of Hydrolase-Containing Granules

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Macrophages were obtained by pulmonary lavage from normal rabbits or rabbits that had developed pulmonary granulomas after receiving intravenous BCG vaccine 2–3 weeks earlier. The cells were disrupted in iso-osmotic sucrose and a low-speed supernatant was fractionated by isopycnic centrifugation on a linear sucrose density gradient. Three populations of hydrolase-containing granules (putative lysosomes) were found in both normal and BCG-induced macrophages. They were distinguished by their different distributions in the gradient and different sensitivities to disruption by digitonin and were termed: type A, containing lysozyme; type B, containing *N*-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase and possibly some lysozyme; type C, containing cathepsin D. Acid phosphatase appeared to be about equally distributed between type B and C granules. Type A and B granules from BCG-induced macrophages showed markedly greater equilibrium density than did those from normal macrophages. β -Glucuronidase and acid phosphatase had greater specific activity in the induced cells.

Macrophages collected from chronic granulomas induced in rabbit lungs by BCG (anti-tuberculosis) vaccine have a greater bactericidal power than macrophages from normal lungs (Myrvik, 1972). The nature of the bactericidal mechanism potentiated by vaccination is unknown, but macrophage granule (lysosome) hydrolases can be bactericidal *in vitro* (reviewed by Ginsburg & Sela, 1976), and increased killing power might be related to altered granule properties.

Comparisons between normal and BCG-induced rabbit alveolar macrophages show greater abundance of only certain granule hydrolases in the induced cell (Cohn & Wiener, 1963*a*; Franson & Waite, 1973). The induced cell also differs from the normal cell in containing a population of morphologically distinct hydrolase-containing granules, which sediment further when centrifuged into a linear sucrose density gradient (Cohn & Wiener, 1963*a*; Franson & Waite, 1973; Sorber *et al.*, 1973).

However, a great morphological diversity of granules is evident in electron micrographs of thin sections of rabbit alveolar macrophages (Cohn & Wiener, 1963*a*; Leake & Myrvik, 1970; Biggar & Sturgess, 1976; Nichols, 1976), which suggests the possible presence of distinct populations of granules even within the normal or BCG-induced macrophage. Such granule populations might differ in both enzyme content and function; some might be lysosomes,

capable of discharging hydrolases into phagocytic vacuoles, and others secretory granules. Secretion of lysozyme independently from other hydrolases has been reported for mouse peritoneal macrophages (Gordon *et al.*, 1974). These possible populations of granules have not been resolved in previous subcellular-fractionation studies.

In the present study, increased specific activity was again found in only certain hydrolases of the BCG-induced rabbit alveolar macrophage compared with the normal cell. An analytical density-gradient subcellular-fractionation technique was used to examine in detail the possible heterogeneity of the hydrolase-containing granules. Fractionation was by isopycnic centrifugation in a linear sucrose density gradient, and the distributions of six granule hydrolases were determined. Digitonin, a membrane-perturbing agent, selectively modified the distribution of the hydrolases. The results indicated the existence of three populations of hydrolase-containing granules in both normal and BCG-induced macrophages.

Experimental

Macrophages

Alveolar cells were collected from female New Zealand White rabbits, weighing 2–3 kg, by the lung-lavage technique of Myrvik *et al.* (1961), except that

the lavage solution was ice-cold 0.25M-sucrose containing 1 mM- Na_2EDTA , pH 7.4. Chronic granuloma (BCG-induced) alveolar cells were obtained from vaccinated rabbits 2–3 weeks after injection of 100 μg of BCG vaccine (Glaxo, Greenford, Middx., U.K.) in 0.25 ml of Freund's incomplete adjuvant (Difco, East Molesey, Surrey, U.K.) into a marginal ear vein (Sorber *et al.*, 1973). The cells were pelleted by centrifugation for 15 min at 4°C and 240g and then resuspended in 10–20 ml of the lavage medium by using a Pasteur pipette. Differential cell counts were performed on Giemsa-stained smears of this suspension. Most of the cells were again centrifuged under the same conditions and resuspended in 10–20 ml of ice-cold lavage medium containing 20 mM-ethanol and 5 units of heparin/ml. The cells were finally centrifuged and resuspended in 5 ml of this medium.

Subcellular fractionation by isopycnic centrifugation

The cells were disrupted with 15 strokes of a tight-fitting (B) pestle in a small Dounce homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.). The homogenate was centrifuged for 15 min at 4°C at 240g to remove nuclei and unbroken cells, and 4 ml of the supernatant (3600g-min supernatant) was layered on a 28 ml sucrose gradient extending linearly with respect to volume, from a density of 1.05 g/cm³ to 1.28 g/cm³ and resting on a 6 ml cushion, density 1.32 g/cm³, in a Beaufay small-volume automatic zonal rotor (Beaufay, 1966). All solutions contained 1 mM- Na_2EDTA , pH 7.4, 20 mM-ethanol and 5 units of heparin/ml. After centrifugation at 35000 rev./min at 4°C for 35 min (integrated angular velocity $3.3 \times 10^{10} \text{ rad}^2/\text{s}$), some 15 fractions were collected. Fractions were weighed and their densities determined. The centrifugation technique and associated equipment have been described previously (Leighton *et al.*, 1968). Results from isopycnic-

centrifugation experiments were expressed as frequency-density histograms as described by Peters (1976).

Analytical techniques

Enzymes and protein were determined by the micro methods described previously (Peters, 1976). Cathepsin D was assayed as described by Peters *et al.* (1972), except that the final haemoglobin concentration was 1 mg/ml. Enzymes were assayed at their pH optima determined on macrophage homogenates. DNA was assayed with calf thymus DNA (BDH, Poole, Dorset, U.K.) as standard (Giles & Myers, 1965). Latent *N*-acetyl- β -glucosaminidase was measured with 0.1% (v/v) Triton X-100 as the granule-disrupting agent (Peters *et al.*, 1972).

Results

Composition of cell suspensions

Over 95% of the cells from the lungs of normal rabbits were macrophages and the remainder were predominantly epithelial cells and small lymphocytes. Cells from chronic granulomatous lungs were 80–90% macrophages and up to 10% polymorphonuclear cells.

Enzyme activities

Table 1 shows the enzyme activities in 3600g-min supernatants from normal and BCG-induced alveolar macrophages. Although total protein was 5–6-fold greater in preparations from vaccinated rabbits the ratio of protein to DNA was unchanged. Peroxidase activity was not detected. The specific activities of two putative lysosomal enzymes, β -glucuronidase and acid phosphatase, were increased about 3-fold in the BCG-induced cells, but no

Table 1. Enzyme activities in 3600g-min supernatants from normal and BCG-induced pulmonary macrophage homogenates. The results are mean values $\pm$ S.D., expressed as munits/mg of protein. The numbers of animals tested are shown in parentheses. Significant differences (*t* test) between values for normal and BCG-induced cells are shown by: **P* < 0.001.

Enzyme	EC no.	Normal	BCG-induced
β -Glucuronidase	3.2.1.31	5.4 $\pm$ 0.9 (6)	17.5 $\pm$ 2.5 (4)*
Acid phosphatase	3.1.3.2	47.0 $\pm$ 14.5 (5)	158.7 $\pm$ 16.2 (4)*
β -Galactosidase	3.2.1.23	47.0 $\pm$ 8.5 (5)	48.6 $\pm$ 2.3 (4)
Lysozyme (muramidase)	3.2.1.17	59.1 $\pm$ 7.2 (7)	35.7 $\pm$ 8.6 (4)
<i>N</i> -Acetyl- β -glucosaminidase	3.2.1.30	97.6 $\pm$ 12.2 (10)	54.5 $\pm$ 6.5 (4)
Cathepsin D	3.4.23.5	34.9 (1)	41.7 (2)
Peroxidase	1.11.1.7	Not detectable	Not detectable
DNA (mg)		0.6 $\pm$ 0.2 (6)	3.9 $\pm$ 0.6 (4)*
Protein (mg)		5.7 $\pm$ 0.2 (6)	31.9 $\pm$ 6.6 (4)*
Protein (mg/mg of DNA)		9.1 $\pm$ 1.3 (6)	8.2 $\pm$ 1.0 (4)

Fig. 1. Isopycnic fractionation of hydrolase-containing granules in 3600g-min supernatants from normal and BCG-induced rabbit alveolar macrophage homogenates

Graphs show frequency-density distributions for marker enzymes of hydrolase-containing granules from normal (—) and BCG-induced (····) macrophages. The results are averages ($\pm$ S.D.) for the following components, the numbers of experiments with normal and BCG-induced macrophages respectively being given in parentheses: (a) lysozyme (8, 6); (b) *N*-acetyl- β -glucosaminidase (9, 6); (c) β -glucuronidase (5, 4); (d) cathepsin D (1, 3); (e) β -galactosidase (5, 3); (f) acid phosphatase (5, 2). Frequency is defined as the fraction of total recovered activity present in the subcellular fraction divided by the density span covered. The cross-hatched area represents, over an arbitrary abscissa interval, the activity remaining in the sample layer. Recoveries of enzyme activity averaged 71–86%.

significant change in the specific activities of β -galactosidase, lysozyme, *N*-acetyl- β -glucosaminidase and cathepsin D was shown. *N*-Acetyl- β -glucosaminidase was typically found to be over 85% latent, indicating that the enzyme was sequestered within granules.

Subcellular fractionation

The distributions of most of the enzymes measured in density gradients are shown as frequency-density histograms in Figs. 1–3. In each instance the cross-hatched area represents components that had not sedimented into the gradient.

Fig. 1 shows the distribution of the six putative lysosomal enzymes: lysozyme, cathepsin D, *N*-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase and acid phosphatase. The great majority of the activity of each of these enzymes was found within the gradient. Differences were apparent between the distribution profiles of the individual

enzymes, both with normal and with BCG-induced cells. Additionally, all except cathepsin D were found in much denser regions of the gradient in experiments with the induced cells compared with normal cells.

Considering first the results with normal cells, all of the enzymes showed a single peak of activity with modal density of 1.16 g/cm³, but with differing distributions. Cathepsin D had a very broad distribution, the lysozyme peak was less broad and sharper still were the almost identical peaks of *N*-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase and acid phosphatase. With BCG-induced cells, activities of lysozyme, *N*-acetyl- β -glucosaminidase, β -galactosidase and β -glucuronidase all showed a peak at density 1.22 g/cm³. The profiles were skewed towards the less-dense regions of the gradient, particularly with β -glucuronidase. Acid phosphatase distribution was bimodal, with peaks at 1.16 and 1.22 g/cm³. The peak of the broad cathepsin D

profile was intermediate between these at about 1.17 g/cm^3 .

Cathepsin D was thus notable in that it was distributed among particles differently from the other hydrolases and in that this distribution was only slightly different in BCG-induced than in normal cells. Among the other five enzymes, the differences in distribution profiles between normal and induced cells showed the presence in the induced cells of distinctly denser particles carrying about half of the total acid phosphatase and the majority of each of the other enzymes. However, minor differences were apparent between the distribution profiles of these enzymes with either cell type, e.g. the broader profile of lysozyme. This indicated possible heterogeneity of enzyme content among these particles. Clarification of the localization of these five enzymes was obtained in experiments by using the membrane-perturbing agent digitonin to modify the distribution of the enzymes in the gradient.

Fig. 2 shows that after homogenization of the BCG-induced cells in the presence of 0.17 mM -digitonin the distribution of lysozyme in the gradient was profoundly altered. The major peak of activity

Fig. 2. Effect of cell homogenization in the presence of 0.17 mM -digitonin on granule hydrolase distribution in isopycnic density-gradient fractions from BCG-induced macrophages

Frequency-density distributions from experiments in the presence (—) or absence (···) of digitonin are shown. The enzymes are, with the number of experiments without and with digitonin respectively given in parentheses: (a) lysozyme (6, 1); (b) N-acetyl- β -glucosaminidase (6, 1). Further details are as given in Fig. 1.

in the gradient was found at about 1.12 g/cm^3 , and a residual peak was present at density 1.20 g/cm^3 . In contrast, the distribution of the other hydrolases was essentially unchanged, as illustrated with N-acetyl- β -glucosaminidase. Evidently lysozyme was carried by particles having higher sensitivity to digitonin than the particles carrying the other enzymes.

Fig. 3 shows that when the cells were homogenized in the presence of 0.42 mM -digitonin the subsequent distribution of all five enzymes was changed. Essentially identical effects of digitonin were observed with either normal or BCG-induced macrophages, and only the results with the induced cells are presented. No peaks were seen at the usual position, density 1.22 g/cm^3 . Most of the lysozyme was either in the sample layer or in the immediately adjacent region of the gradient. Most of the N-acetyl- β -glucosaminidase, β -galactosidase and β -glucuronidase and about half of the acid phosphatase were also in the sample layer. A small peak of β -glucuronidase was evident with a modal density of about 1.14 g/cm^3 . The acid phosphatase that had entered the gradient was distributed as a broad peak with modal density of about 1.17 g/cm^3 . Digitonin at concentrations up to 0.42 mM had no effect on the specific activity or recovery of any of the hydrolases and did not alter the distribution of cathepsin D (results not shown). Evidently N-acetyl- β -glucosaminidase and β -galactosidase, most of the β -glucuronidase and about half of the acid phosphatase in both normal and BCG-induced macrophages were carried within digitonin-sensitive, and thus membrane-bound, granules.

Discussion

A novel discovery is the finding of three different types of hydrolase-containing granules in both the normal and the BCG-induced macrophages. The association of the six hydrolases, lysozyme, cathepsin D, N-acetyl- β -glucosaminidase, β -glucuronidase, β -galactosidase and acid phosphatase, with sedimentable components and the liberation of these enzymes by the detergent action of Triton X-100 and digitonin indicate their constraint within membrane-bound granules. They were distinguished by differences in their enzymic contents, and distribution profiles in the density gradient and their differential lability with digitonin. For convenience they are here referred to as: type A, disrupted by a low concentration of digitonin and containing lysozyme; type B, disrupted by a high concentration of digitonin and containing N-acetyl- β -glucosaminidase, β -galactosidase, β -glucuronidase and acid phosphatase and perhaps some lysozyme; type C, resistant to a high concentration of digitonin and having a broad profile of density distribution and containing cathepsin D and possibly some acid phosphatase.

Fig. 3. Effects of homogenization in the presence of 0.42 mM-digitonin on granule hydrolase distribution in isopycnic density-gradient fractions from BCG-induced macrophages

Frequency-density distributions from experiments in the presence (—) or absence (·····) of digitonin are shown. The enzymes are, with the numbers of experiments without and with digitonin respectively given in parentheses: (a) lysozyme (6, 3); (b) *N*-acetyl- β -glucosaminidase (6, 3); (c) β -glucuronidase (4, 2); (d) β -galactosidase (3, 2); (e) acid phosphatase (2, 3). Further details are as given in Fig. 1.

The evidence for the existence of type-A granules as a distinct entity appears unequivocal; 0.17 mM-digitonin selectively decreased the equilibrium density of part of the lysozyme without altering the distribution of acid hydrolases from the BCG-induced macrophages. The remainder of the lysozyme still gave a peak in the dense region of the gradient at 1.20 g/cm³, suggesting that it was also present in a separate population of granules, type B.

Type-B granules appeared to contain essentially all the *N*-acetyl- β -glucosaminidase, β -galactosidase and β -glucuronidase. The three enzymes showed modal densities identical with that of the dense peak of lysozyme after treatment with 0.17 mM-digitonin and can therefore be assigned to the type-B granule. Both type-A and type-B granules showed striking increases in modal density in induced cells. Similar observations of increased density of hydrolase-containing granules in induced cells, compared with normal rabbit alveolar macrophages, have been reported by others (Cohn & Wiener, 1963a; Franson

& Waite, 1973; Sorber *et al.*, 1973), but the granules in each cell type were not further resolved. Although Leake & Myrvik (1964) indicated, on the basis of differential latency, that lysozyme might be in a separate population of granules from those containing acid hydrolases in the BCG-induced macrophages, they later concluded (Sorber *et al.*, 1973) that this was an artifact of selective adsorption and inactivation of lysozyme on membrane fragments. Not all macrophage β -glucuronidase was in type-B granules. The small amount of β -glucuronidase that still migrated into the gradient to peak at density 1.14 g/cm³ after granule disruption with 0.42 mM-digitonin probably represented endoplasmic-reticulum enzyme. Dual localization of β -glucuronidase in lysosomes and endoplasmic reticulum has been reported for rat liver (Fishman *et al.*, 1967).

The third population of granules, type C, was evidenced by the different distribution of cathepsin D compared with the enzymes of the type-A and -B granules in both normal and induced cells. Not only

was the distribution profile markedly flatter, but also the equilibrium density was increased only slightly in the induced cells.

Acid phosphatase appeared to be present in two granule types. In BCG-induced cells the peak of activity in the dense region of the gradient was coincident with the peaks of the dense type-A and -B granules, but its precise association cannot, however, be ascertained from the present data. The second peak of activity, coincident with the peak of cathepsin D, suggested possible association with the type-C granule. This was further evidenced by the distribution of activity in the gradient after treatment with 0.42 mM-digitonin. A peak was no longer observed in the dense region of the gradient and there was a concomitant increase in activity in the sample layer, behaviour typical of enzymes of type-A and -B granules. The profile of the activity that did remain in the gradient closely resembled that of cathepsin D. The differences in distribution and modal density between acid phosphatase and cathepsin D in the normal cells could be accounted for by the presence of the two acid phosphatase-containing granule types that were unresolved in this situation.

The three granule types of the rabbit alveolar macrophage might be functionally as well as biochemically distinct. There is evidence that some granule hydrolases can be discharged faster than others into phagocytic vesicles or the extracellular environment (Cohn & Wiener, 1963*b*; Bainton, 1973; Ackerman & Beebe, 1974; Page *et al.*, 1974). Most strikingly, release of lysozyme from mouse peritoneal macrophages has been shown to be under separate physiological control from the release of other hydrolases. Lysozyme secretion from cells maintained *in vitro* was continuous, whereas no *N*-acetyl- β -glucosaminidase, β -glucuronidase, β -galactosidase or cathepsin D was secreted (Gordon *et al.*, 1974). Studies with human polymorphonuclear leucocytes have shown comparable selective secretion of lysozyme independently of other granule hydrolases under appropriate experimental conditions (Goldstein *et al.*, 1974; Wright *et al.*, 1977). It thus seems reasonable to expect that the three rabbit alveolar-macrophage granules may differ in function and control.

Whether all three granule types can function as lysosomes remains to be seen. Leake & Myrvik (1970), Sorber *et al.* (1974) and Leake *et al.* (1974) have argued from morphological studies that the dense granules characteristic of the induced cells might not fuse with phagocytic vacuoles, a necessary criterion for lysosomes. What alternative function the dense type-A and -B granules might have is not at present clear. However, Stossel *et al.* (1972) found that transfer of acid phosphatase and β -glucuronidase from granules to phagocytic vacuoles was more efficient in induced than in normal alveolar macro-

phages. Thus at least some of the dense type-B granules probably function as lysosomes. Histochemical evidence has confirmed the presence of acid phosphatase in digestive vacuoles in these cells (Cohn & Wiener, 1963*b*; Nichols, 1976). Lysozyme and cathepsin D have not to our knowledge been sought in macrophage phagocytic vacuoles. Type-A lysozyme-containing granules, in particular, might be specialized for a secretory function. Nevertheless, it would be remarkable if lysozyme were not transferred to phagocytic vacuoles; lysozyme is the only macrophage granule component having well-authenticated anti-microbial activity and would be expected to have a role in intracellular killing of ingested micro-organisms (Ginsburg & Sela, 1976; Klebanoff & Hamon, 1975).

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