LYSOSOMAL PHOSPHOLIPASES A_1 AND A_2 OF NORMAL AND BACILLUS CALMETTE GUERIN-INDUCED ALVEOLAR MACROPHAGES

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ABSTRACT

A single intravenous injection of 0.1 mg of heat-killed Bacillus Calmette Guérin (BCG) in 0.1 ml of Bayol F produced an accumulation of activated alveolar macrophages (BCG induced). Cells were collected 3.5–4.0 wk after injection. Phospholipases A and three lysosomal marker enzymes (acid phosphatase, β-glucuronidase, and lysozyme) were measured in homogenates, and the distribution of the phospholipases A and lysosomal, mitochondrial, and microsomal marker enzymes were examined after sucrose gradient centrifugation of a postnuclear (1,000 *g*) supernatant. Homogenates of normal and BCG-induced macrophages contained phospholipases A_1 and A_2 which had optimal activity at pH 4.0 in the presence of 2.0 mM ethylenediaminetetraacetate (EDTA). These activities were inhibited 50–70% by 2.0 mM $CaCl_2$. Homogenates of BCG-induced macrophages had specific activities of β-glucuronidase, acid phosphatase, and lysozyme, which were increased 1.5- to 3.0-fold over the controls, whether expressed as activity per mg protein or activity per 10^7 cells. The specific activities of the phospholipases A, on the other hand, were consistently lower than those of the control. Distribution of the phospholipases A and the lysosomal marker enzymes after sucrose gradient centrifugation suggested that the phospholipases A active at pH 4.0 in the presence of EDTA are of lysosomal origin since: (*a*) BCG treatment caused a selective increase in the density of particles which contained both the phospholipases A and three lysosomal marker enzymes; and (*b*) since the density of mitochondria and microsomes were not affected by BCG treatment. The increase in the density of lysosomes seen here may be related to previously described morphologic changes of BCG-induced alveolar macrophages.

INTRODUCTION

Recently, Franson et al. (1972) reported the presence of lysosomal phospholipases A_1 and A_2 in rat myocardial preparations. These enzymes were active at pH 4.0 in the presence of ethylenediaminetetraacetate (EDTA) and their activities were similar to phospholipase activities found in rat liver lysosomes with regard to pH optima, positional specificity, and inhibition by Ca^{2+} (Franson et al., 1971). Since lysosomes from both rat heart and rat liver which contained these phospholipases A shifted in distribution on sucrose gradients as a result of treatment with Triton WR-1339, as did those particles which contained acid phosphatase and β-glucuronidase, it was suggested that the phospholipases A active in heart preparations were of

macrophage origin. Indeed, phospholipases A with similar characteristics were found in normal rabbit alveolar macrophages, and these activities were presumed to be localized in macrophage lysosomes.

In this work, alveolar macrophages from normal and Bacillus Calmette Guérin- (BCG) induced rabbits were used to determine the subcellular localization of the phospholipases A. Phospholipases A activity and three lysosomal marker enzymes (acid phosphatase, β-glucuronidase, and lysozyme) were measured in homogenates of normal and BCG-induced alveolar macrophages. The distribution of these enzymes and marker enzymes for mitochondria and microsomes were determined after sucrose gradient centrifugation of a postnuclear supernatant. In homogenates of BCG-induced macrophages, the specific activities of the lysosomal marker enzymes were 1.5- to 3.0-fold greater than those of the control, while the specific activities of the phospholipases A_1 and A_2 were less than the control values. This suggests that BCG induction caused accumulation of some, but not all, of the lysosomal proteins. Distribution of the phospholipases A and the lysosomal marker enzymes after sucrose gradient centrifugation demonstrated that the phospholipases A_1 and A_2 are of lysosomal origin since BCG treatment caused a selective increase in the density of lysosomes which contain both the phospholipases A and the three lysosomal marker enzymes. Marker enzymes for mitochondria and microsomes indicated that the densities of these organelles were not affected by BCG treatment. The increase in lysosomal density of BCG-induced macrophages may be related to morphologic changes previously described (Leake and Myrvik, 1968).

MATERIALS AND METHODS

Vaccination and Collection

New Zealand white rabbits (1.5–2.0 kg) were given a single injection of 0.1 mg heat-killed BCG in Bayol F (0.1 ml) into the marginal ear vein. Animals were killed by air embolism at 3.5–4.0 wk postvaccination. Alveolar macrophages were collected from the lung by lavage with sterile isotonic saline (Myrvik et al., 1961). Cultures were done on nasal swabs before vaccination and directly before sacrifice, and cultures were taken of the cell wash after sacrifice to determine the presence or absence and degree of bacterial infection due to *Bordetella bronchiseptica*. Grossly infected animals, or lungs which yielded abnormal numbers of cells, were not used in the experiments described. A yield of 0.1–0.3 ml of packed cells was obtained from each normal lung (8–10 g). The packed cells from ten normal lungs were pooled before homogenization (control group). 1–2 ml of packed cells per lung (15–25 g) was obtained as a result of BCG treatment. Wright smears indicated that 80–90% of the cells from BCG-treated lungs were macrophages. Viability studies based on the exclusion of trypan blue showed that greater than 85% of the cells were viable.

Cells were washed from the lung with 60 ml of sterile isotonic saline and were centrifuged at 800 rpm (100 *g*) for 5 min. Packed cells were twice resuspended and washed with isotonic saline and centrifuged at 800 rpm (100 *g*) for 5 min. The packed cells were then resuspended in 8.0 ml of 0.25 M sucrose and homogenized with a Potter-Elvehjem homogenizer. Cell disruption was monitored by phase microscopy. After cell collection, all steps were performed at 0°–4°C. Nuclei, cell debris, and the remaining intact cells were sedimented by centrifuging the homogenate at 1,000 *g* for 10 min. 4 ml of the 1,000 *g* supernatant was layered above 24 ml of a linear sucrose gradient from 0.73 to 1.61 M sucrose. 6 ml of 1.93 M sucrose were used as a cushion at the bottom of the tube. The samples were centrifuged at 24,000 rpm (64,000 *g*) for 2 h in a Beckman SW 25.1 swinging-bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions were collected from the top of the centrifuge tube by puncturing the bottom of the tube and pumping 1.93 M sucrose from below. The first 4.0 ml pumped from the gradient, fraction S, represents the amount of sample applied to the gradient. Thereafter, eight fractions of 3.0 ml each were collected. Enzyme assays were performed with aliquots of the homogenate and gradient samples which were dialyzed for 12 h against 6 liters of 1 mM sodium acetate buffer at pH 5.0 which ruptured lysosomes and removed sucrose.

The 1,000 *g* supernatant fluid contained 40–60% of the enzymatic activities of the homogenate, and this fraction exhibited typical enzymatic latency when lysosomes were ruptured. A 2- to 3-fold increase in enzymatic activities was observed in the soluble fraction when lysosomes were frozen and thawed (eight times), sonicated, or dialyzed. Dialysis was the method of choice in this study since this treatment resulted in the maximal recovery of phospholipase A activity. 85–98% of the five enzymatic activities applied to the sucrose gradient was recovered in the gradient fractions.

Enzyme Assays

Phospholipase A was assayed as described by Waite and van Deenen (1967). Phospholipases A_1 and A_2 specifically hydrolyze the fatty acid ester linkages in the C-1 and C-2 positions of phospholipids, respectively, to yield free fatty acid (FFA) and their mono-

acyl analogs. Reaction mixtures in a total volume of 1.0 ml contained (unless otherwise stated) 75 nmol of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (12,000 cpm added as an aqueous ultrasonic suspension), 2 μmol of EDTA or 2 μmol of $CaCl_2$, 100 μmol of sodium acetate buffer pH 4.0, and 25–100 μg of protein. Reaction mixtures were incubated for 20 min at 37°C. Preliminary studies showed that the reaction was linear for 45 min. Reactions were stopped by adding 2 vol of methanol, and the products were extracted by the method of Bligh and Dyer (1959). Radioactive lipids were separated by thin-layer chromatography on silica gel G plates which were first developed in chloroform: petroleum ether: (bp 63°–75°C) acetic acid (70:30:2 vol/vol) and then in chloroform:methanol:H_2O (70:30:4 vol/vol). Chromatograms were stained with I_2 vapor and those portions of the silicic acid gel that contained the radioactive compounds were each placed in scintillation vials containing Omnifluor (Pilot Chemicals Inc., Div., Watertown, Mass.) and counted.

Acid phosphatase (E.C.3.1.3.2) was determined by the method of Gianetto and de Duve (1955), using β-glycerophosphate as substrate. Inorganic phosphate was measured by the turbidimetric procedure of Eibl and Lands (1969). β-glucuronidase (E.C.3.2.1.3) was determined by a modification of the method of Talalay et al. (1946), as described by Canonico and Bird (1969). Lysozyme (E.C.3.2.1.17) was measured by the decrease in optical density (OD) at 450 nm, using *Micrococcus lysodeikticus* as substrate (Shugar, 1952). Nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome *c* reductase (E.C.1.7.99.1) and cytochrome oxidase (E.C.1.9.3.1) were measured by the method of Sottocasa et al. (1967). Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Materials

We synthesized 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine and 1-[^{3}H]palmitoyl-2-acyl-3-glycerophosphorylethanolamine by the procedure of Waite and van Deenen (1967). The 2-acyl-3-glycerophosphorylethanolamine which was used as the substrate for the preparation of the tritium-labeled phosphatidylethanolamine was prepared by hydrolysis of rat liver phosphatidylethanolamine with phospholipase A_1 from rat liver lysosomes (Franson et al., 1971). Heat-killed BCG was the generous gift of Dr. Quentin Myrvik. All substrates for the marker enzymes were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Fig. 1 shows the pH for optimal phospholipase A activity in homogenates of normal and BCG-

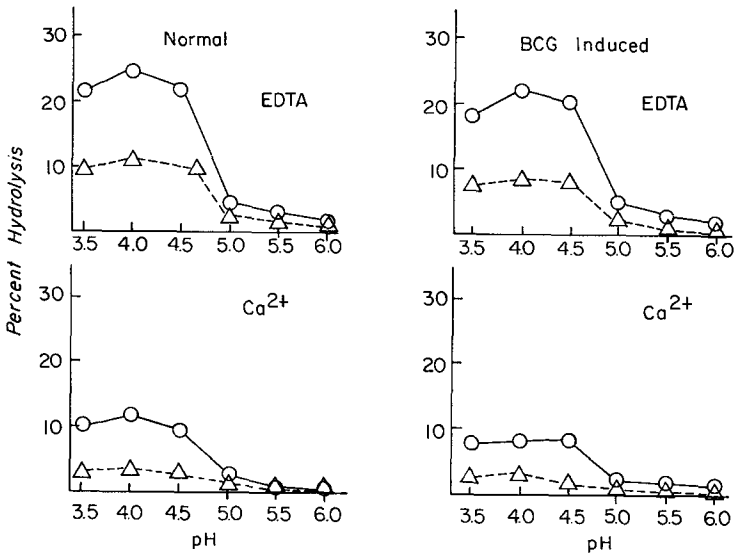

FIGURE 1 Optimal pH for phospholipase A activity of homogenates of normal and BCG-induced alveolar macrophages. Incubation mixtures contained 50–75 μg of protein, 50 nmol of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (12,000 cpm), 100 μmol of sodium acetate buffer, and 2 μmol of EDTA or $CaCl_2$ in a total volume of 1.0 ml. Reaction mixtures were incubated at 37°C for 20 min. The designations are 2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine (△) and [^{14}C]linoleic acid (○). All values have been corrected for nonenzymatic hydrolysis of substrate.

TABLE I

Specific Activities of Acid Phosphatase, β-Glucuronidase, Lysozyme, and Phospholipases A_1 and A_2 in Homogenates of Normal and BCG-Induced Alveolar Macrophages

	Specific activities			
	Activity/mg protein		Activity/10^7 cells	
	Normal	BCG	Normal	BCG
Acid phosphatase	48	93	56	116
β-glucuronidase	68	111	83	176
Lysozyme	3.2	8.2	6.8	20.1
Phospholipase A_1	60	40	110	98
Phospholipase A_2	82	50	140	105

Assay procedures were described in Materials and Methods. Enzymatic activity is defined as follows: acid phosphatase, nmol of product formed per min; β-glucuronidase, nmol of product formed per h; lysozyme, Δ OD at 450 nm/min × 100; and phospholipases A, nmol of product formed per min. Specific activity is expressed as activity per mg of homogenate protein or activity per 10^7 cells homogenized.

induced alveolar macrophages. In these preparations two phospholipases, A_1 and A_2, have optimal activity at pH 4.0 in the presence of 2 mM EDTA. These activities are inhibited 50–70% by 2 mM $CaCl_2$. The amount of [^{14}C]linoleic acid released is generally 2- to 3-fold greater than the [^{14}C]monoacylglycerophosphorylethanolamine released. This difference could be the result of either a phospholipase A_2 which is more active than a phospholipase A_1, or could be due to the concerted action of a phospholipase A_1 and a lysophospholipase. To determine the specificity of the enzymes in these preparations, we incubated both control and BCG-induced macrophage homogenates with a mixed-labeled substrate (1-[^{3}H]palmitoyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine). 30% of the mixed-labeled substrate was degraded and 70–90% of the products formed was FFA. These incubations gave rise to both [^{3}H]- and [^{14}C]monoacylglycerophosphorylethanolamines, which confirms the presence of both phospholipases A_1 and A_2 in these preparations. However, most (70–90%) of both monoacylglycerophosphorylethanolamines were further hydrolyzed, which indicates lysophospholipase activity in macrophages which has been previously described (Elsbach et al., 1966; Franson et al., 1972).

The specific activities of acid phosphatase, β-glucuronidase, lysozyme, and the phospholipases A_1 and A_2 in homogenates of normal and

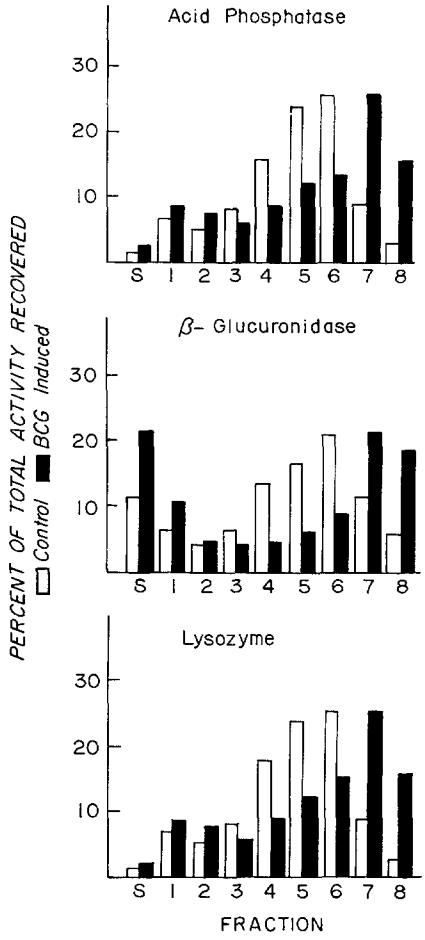

FIGURE 2 Distribution of acid phosphatase, β-glucuronidase, and lysozyme after sucrose gradient centrifugation. Homogenates of normal (□) and BCG-induced (■) alveolar macrophages were centrifuged at 1,000 *g* for 10 min and 4.0 ml of the 1,000 *g* supernatant fluid was layered above a continuous sucrose gradient. Fraction S represents the volume of 1,000 *g* supernate applied to the gradient, and fractions 1–8 are the gradient fractions (3.0 ml each) from the top to the bottom of the gradient. Enzymatic activity is expressed on the ordinate as the total activity of each fraction divided by the total activity recovered in the entire gradient times 100.

BCG-induced macrophages are shown in Table I. There was considerable variation from animal to animal with BCG treatment, which is probably due to differences in the degree of response to BCG. With the control animals, variability was minimized since the alveolar macrophages from ten normal rabbits were pooled before homogenization. For this reason, macrophages from control and BCG-treated rabbits were always compared. In six different experiments a similar trend was noted, and we present data from one study which is representative of this trend. When expressed as either activity per mg protein or activity per 10^7 cells, the specific activities of acid phosphatase, β-glucuronidase, and lysozyme are 1.5- to 3.0-fold greater in homogenates or BCG-induced macrophages than in homogenates of normal macrophages. On the other hand, the specific activities of the phospholipases A in homogenates of BCG-induced macrophages were consistently lower than control values. These data suggest that specific lysosomal proteins of macrophages are synthesized in response to BCG stimulation.

Since the substrate used in most phospholipase A assays was 2-[^{14}C]acyl-phosphatidylethanolamine, the release of [^{14}C]FFA represents maximal phospholipase A_2 activity as well as lysophospholipase activity; and the release of [^{14}C]lysophosphatidylethanolamine represents minimal phospholipase A_1 activity. It was not possible to selectively inhibit lysophospholipase activity with deoxycholate (DOC) since DOC inhibited both phospholipases A activities also.

Fig. 2 shows the distribution of acid phosphatase, β-glucuronidase, and lysozyme after sucrose gradient centrifugation of control and BCG-induced preparations (1,000 *g* supernatant). In the control, fractions 4–6 (average sucrose density 1.18) contain the majority (52–67%) of the three lysosomal marker enzymes. As a result of BCG treatment, however, there is an increase in the density of lysosomes which contain acid phosphatase, β-glucuronidase, and lysozyme. The relative recovery of lysosomal marker enzymes is 2- to 4-fold increased in fractions 7 and 8 (average sucrose density 1.23) while a concomitant decrease in recovery is noted in fractions 4–6. The increased β-glucuronidase activity in the S fraction is probably from ruptured lysosomes.

The distribution of the phospholipases A_1 and A_2 after sucrose gradient centrifugation is shown in Fig. 3. Fractions 4–6 of the control contain most of the phospholipase A activity recovered (56%). As a result of BCG treatment, lysosomes which contain the phospholipases A are more dense and are found in fractions 7 and 8. There is a 2.5-fold increase in the phospholipase A activity recovered in fractions 7 and 8, while there is a concomitant decrease in the recovery in fractions 4–6. Since the particles which contain the phospholipases A shift in distribution as did the lysosomes which contain acid phosphatase, β-glucuronidase, and lysozyme (Fig. 2), these data are consistent with a lysosomal origin for the phospholipases. However, it is not possible to determine if the lysosomal enzymes are equally distributed in all BCG-induced granules since all the granules from the BCG-induced macrophages have sedimented to the 1.93 M sucrose cushion.

To demonstrate that the increase in lysosomal density which results from BCG-induction is not

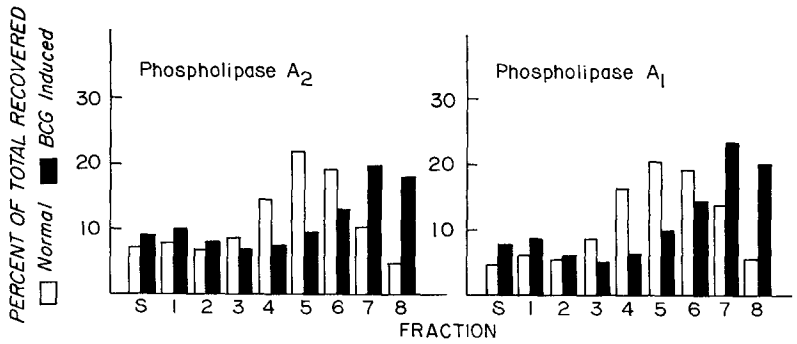

FIGURE 3 Distribution of phospholipases A_2 and A_1 after sucrose gradient centrifugation. Centrifugation of the homogenates of normal (□) and BCG-induced (■) alveolar macrophages and expression of enzyme activity are described in the legend of Fig. 2.

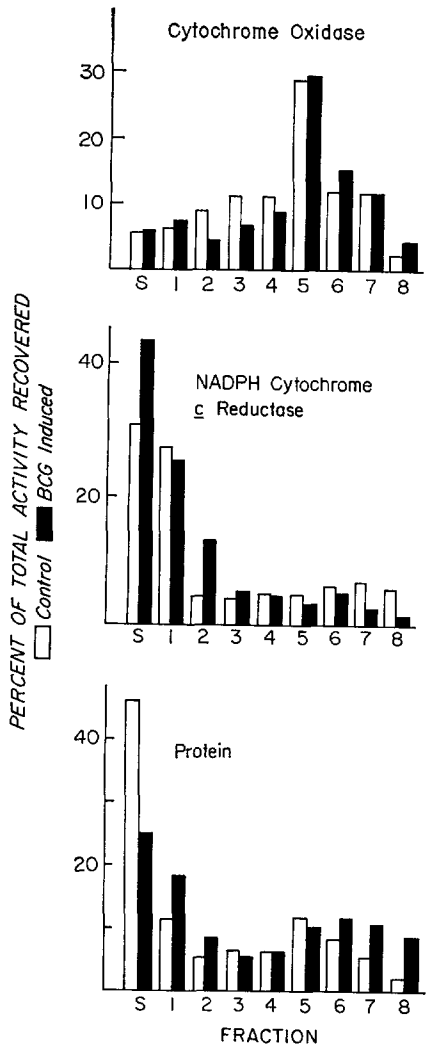

FIGURE 4 Distribution of cytochrome oxidase, NADPH cytochrome *c* reductase, and protein after sucrose gradient centrifugation. Centrifugation of the homogenates of normal (□) and BCG-induced (■) alveolar macrophages and expression of enzyme activity are described in the legend of Fig. 2.

due to a general increase in the density of all organelles, the isopycnic sedimentation of marker enzymes for mitochondria and microsomes was determined. Fig. 4 illustrates that there is no significant change in the distribution of microsomes (NADPH cytochrome *c* reductase) or mitochondria (cytochrome oxidase) in fractions 4–8. The recovery of protein in normal and BCG-induced preparations is compatible with the increase in lysosomal density and the distribution shift of lysosomal enzymes (Figs. 2, 3).

DISCUSSION

Lysosomal phospholipases A presumably function to degrade phospholipids contained within phagocytic and autophagic vacuoles. While homogenates of alveolar macrophages were reported to have phospholipase activity (Elsbach, 1966), the subcellular localization of this activity is unknown.

The data presented here are consistent with the hypothesis that the phospholipases A are of lysosomal origin. Phospholipases A with similar characteristics (positional specificity, pH optima, and inhibition by Ca^{2+}) have been demonstrated in rat liver and rat heart preparations (Franson et al., 1971, 1972). In both cases, the lysosomes which contained the phospholipases were isolated by decreasing their density by in vivo treatment with Triton WR-1339. It was suggested (Franson et al., 1971, 1972), that the cells which contained the Triton-filled lysosomes were phagocytes and had an origin common to that of the alveolar macrophages (Boak et al., 1968). Other workers have used dextran-500 which was phagocytized to increase lysosomal densities and thus facilitate the separation of lysosomes from mitochondria with which they normally sediment (Canonico and Bird, 1970). In this study, BCG treatment resulted in an increase in the density of macrophage granules which contained acid phosphatase, β-glucuronidase, lysozyme, and the phospholipases A_1 and A_2 (Figs. 2, 3). The increase in density in this case, however, is probably not due to the intracellular accumulation of BCG or its components since such small amounts of BCG in oil produce density changes in lysosomes which persist for at least 8 wk; and it has been suggested by Leake and Myrvik (1968) that a sensitivity mechanism is operative.

In normal rabbits the intravenous injection of 0.1 mg of heat-killed BCG in oil results in the formation of granulomatous lesions in the lung and an accumulation of activated macrophages in the alveolar spaces (Myrvik et al., 1962). Activated macrophages have higher levels of acid hydrolase activities and greater antimicrobial activity than do immature and relatively undifferentiated macrophages. Leake and Myrvik (1968) reported a 2- to 4-fold increases in the lysozyme content of BCG-induced rabbit alveolar

macrophages at 3–4 wk postvaccination. Using the same conditions for macrophage activation, we found a comparable increase in homogenate specific activities of acid phosphatase and β-glucuronidase, as well as lysozyme (Table I). Yet, a decrease was noted in the specific activity of the phospholipases A. If phospholipases are not inactivated or secreted into the medium during macrophage activation, it would appear that some selective process occurs in the synthesis of lysosomal proteins during macrophage induction which does not involve the phospholipases. Also, dialysis as a means of rupturing lysosomes could alter the observed phospholipase activities by removal of an inhibitor or by inactivating less protein. Cohn and Weiner (1963) and Mizunoe and Dannenberg (1965) found variable response of enzyme content in BCG-induced macrophage lysosomes. In the latter case, both lysozyme and acid phosphatase activities were increased 20–50% over control values at 2–3 wk after an intravenous injection of 40 mg of BCG (2–20-mg doses). On the other hand, Mizunoe and Dannenberg (1965) found that the cellular content of a nonspecific lipase was decreased. It is possible that the phospholipases described here may have nonspecific lipase activity.

An increase in the number of electron-opaque granules at 3 wk postvaccination has been demonstrated by Leake and Myrvik (1968). This morphologic alteration, as suggested by Leake and Myrvik (1968), may be due to the storage of large amounts of newly synthesized proteins which could account for the increased lysosomal densities found here. In this regard, Sorber et al. (1972) have demonstrated that these electron-opaque granules are found in sucrose gradient fractions 7 and 8 in preparations from BCG-induced macrophages. It is not possible to determine whether the denser lysosome produced as a result of BCG treatment is due to (*a*) the conversion of primary lysosomes to denser, secondary lysosomes, or (*b*) the BCG-induced synthesis of primary lysosomes which are more dense than normal and which have increased content of certain enzymes (lysozyme, acid phosphatase, and β-glucuronidase), but not others (lipase and phospholipases).

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REFERENCES

BLIGH, E. G., and W. J. DYER. 1959. *Can. J. Biochem. Physiol.* **37**:911.

BOAK, J. L., G. H. CHRISTIE, W. C. FORD, and J. C. HOWARD. 1968. *Proc. R. Soc. Lond. B Biol. Sci.* **169**:307.

CANONICO, P. G., and J. W. C. BIRD. 1969. *Cytobios.* **14**:23.

CANONICO, P. G., and J. W. C. BIRD. 1970. *J. Cell Biol.* **45**:321.

COHN, Z. A., and F. WEINER. 1963. *J. Exp. Med.* **118**:1009.

EIBL, H., and W. E. M. LANDS. 1969. *Anal. Biochem.* **30**:51.

ELSBACH, P. 1966. *Biochim. Biophys. Acta.* **125**:510.

FRANSON, R., M. WAITE, and M. LAVIA. 1971. *Biochemistry.* **10**:1942.

FRANSON, R., M. WAITE, and W. WEGLICKI. 1972. *Biochemistry.* **11**:472.

GIANETTO, R., and C. DE DUVE. 1955. *Biochem. J.* **59**:433.

LEAKE, E. S., and Q. N. MYRVIK. 1968. *J. Reticuloendothel. Soc.* **5**:33.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. Randall. 1951. *J. Biol. Chem.* **193**:265.

MIZUNOE, K., and A. M. DANNENBERG. 1965. *Proc. Soc. Exp. Biol. Med.* **120**:284.

MYRVIK, Q. N., E. S. LEAKE, and B. FARISS. 1961. *J. Immunol.* **86**:128.

MYRVIK, Q. N., E. S. LEAKE, and S. OSHIMA. 1962. *J. Immunol.* **89**:745.

SHUGAR, D. 1952. *Biochim. Biophys. Acta.* **8**:302.

SORBER, W. A., E. S. LEAKE, and Q. N. MYRVIK. 1972. *Bacteriol. Proc.* 107.

SOTTOCASA, G. L., B. KUYLENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. *J. Cell Biol.* **32**:415.

TALALAY, P., W. H. FISHMAN, and C. HUGGINS. 1946. *J. Biol. Chem.* **166**:757.

WAITE, M., and L. L. M. VAN DEENEN. 1967. *Biochim. Biophys. Acta.* **137**:498.