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Activation of Lysosomal Enzymes in Polymorphonuclear Leukocytic Granules: The Role of Phospholipid–Protein Interaction*

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The concept of lysosomes as formulated in 1959 by DeDuve and his group(1) indicates that the activation and release of lysosomal enzymes depends on the membrane surrounding lysosomes. If the lysosomal membrane is intact, lysosomal enzymes are bound and inactive. If the membrane becomes labilized or ruptured, lysosomal enzymes are free and active. It has been concluded that the stability of the lysosomal membrane depends on protein as well as lipid portions of the membrane. Lysosomal enzymes become active during phagocytosis and participate in the intracellular digestion of engulfed particles(2). Such activation and release of lysosomal enzymes in phagocytic cells is due to a fusion of the lysosomal membrane with the membrane of a phagocytic vacuole(3–5).

In an attempt to elucidate a mechanism underlying this process, we have previously demonstrated that model membranes (liposomes) composed of negatively charged phospholipids induce a similar effect upon leukocytic granules in a cell-free system(6,7). It has been suggested that the activating effect of phospholipid model membranes on granulocytic lysosomes may be mediated by a phospholipid–protein interaction. This possibility prompted us to define the component of granules which interacts with phospholipids. Leukocytic granules possess a class of arginine-rich cationic proteins which do not exhibit activity of lysosomal marker enzymes and which may be obtained from granules by acid extrac-

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tion(8,9). In the present study, cationic proteins prepared from PMN leukocytic lysosomes are shown to complex with liposomes and thus prevent them from activating lysosomal enzymes in leukocytic granules. These results indicate that phospholipid–protein interactions play a role in the activation of latent lysosomal enzymes in isolated leukocytic granules and suggest that this mechanism may also be operative during phagocytosis.

MATERIALS AND METHODS

Leukocytic granules isolated from rabbit peritoneal PMN leukocytes and liposomes were prepared according to the method previously described(7).

The cationic protein fraction was prepared from isolated rabbit PMN granules by treatment with 0.05 *N* HCL for 30 min at 0°. The supernatant fluid obtained after centrifugation at 20,000*g* for 30 min was adjusted to pH 5.0 with 0.25 *M* acetate buffer and designated as the "cationic protein fraction." The protein content of the obtained material averaged 0.5 mg/cc and enzymatic measurements failed to show any activity of the PMN lysosomal marker enzymes: beta glucuronidase, beta *N*-acetyl glucosaminidase, acid phosphatase, myeloperoxidase, and lysozyme.

Liposomes were incubated with and without the cationic protein fraction at pH 5.0 (acetate buffer, 0.05 *M*) for 30 min. To these incubation mixtures isolated PMN granules were added, then the whole system was incubated for 20 min at 37° and free activity of beta glucuronidase and beta *N*-acetyl glucosaminidase was determined as described previously(7). Electrophoresis in polyacrylamide gel was performed using a cationic system of buffers at pH 4.3 as described by Reisfeld *et al.*(10) with omission of the sample gel. Protein was determined by the method of Lowry *et al.*(11) with crystalline bovine albumin (Armour Pharmaceutical Co.) as a standard. Phosphorus was determined by the method of Berenblum and Chain(12). The acid phosphatase activity with *p*-nitrophenyl phosphate as a substrate was determined according to Baggiolini *et al.*(13) and peroxidase activity was measured by the pyrogallin method according to Woodin *et al.*(14). Lysozyme was determined according to Smolelis and Hartsell(15).

RESULTS

The effect of cationic proteins derived from granules on activation of lysosomal enzymes by liposomes. Negatively charged liposomes possess the ability to activate lysosomal enzymes in PMN leukocytic granules(7). If liposomes are able to interact with proteins derived from granules, then such proteins should block the activating effect of liposomes. Indeed, the addition of the cationic protein fraction (CPF) significantly reduced the activating effect of liposomes. As shown in Fig. 1 (top), the free activity of the lysosomal marker enzyme, beta glucuronidase, which was 14% of total enzyme content present in preparations containing

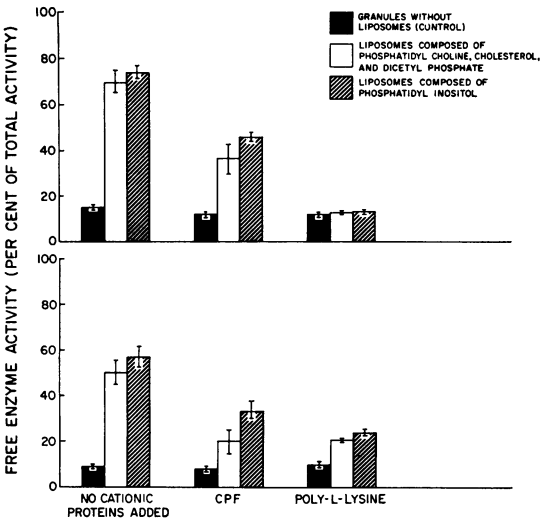

FIG. 1. Effect of cationic proteins on activation of lysosomal marker enzymes in PMN leukocytic granules by liposomes. CPF refers to the cationic protein fraction isolated from leukocytic granules. Each bar represents the mean of three experiments performed in duplicate. Brackets represent standard deviations. (Top) beta glucuronidase, (bottom) beta *N*-acetyl glucosaminidase.

granules alone, rose to 50 and 58% upon the addition of either type of liposomes. In the presence of the cationic protein fraction the activating effect of liposomes was reduced to 20 and 33%, respectively. For comparison a polycationic protein, poly-L-lysine (Sigma Chemical Co.) in a concentration of 1 mg/cc was utilized in the experimental system and demonstrated a similar but more marked effect. As we have previously shown(7), albumin in concentrations ranging from 0.5 to .5 mg/ml, the latter 10 times higher than the cationic protein fraction used, did not reduce the activating effect of liposomes.

Activation of another lysosomal marker enzyme, beta *N*-acetyl glucosaminidase by liposomes was also significantly reduced in the presence of the cationic protein fraction or poly-L-lysine (Fig. 1, bottom). Thus, the activating effect of liposomes upon PMN leukocytic granules was diminished by the cationic protein fraction derived from leukocytic granules. This indicates that proteins present in leukocytic granules interact with liposomes which activate lysosomal enzymes.

Formation of complexes between cationic protein fraction and membrane-forming phospholipids. The nature of the interaction between liposomes and the cationic protein fraction was examined by the following experiment. The cationic protein fraction was incubated at room temperature for 30 min with and without liposomes and then centrifuged for 20 min at 20,000*g*. The protein in the supernatant fluid after sedimentation was determined. The same material was analyzed by electrophoresis in polyacrylamide gel. As is seen in Fig. 2, the cationic protein fraction before the addition of liposomes was composed of one strongly cationic band and at least six less-defined components. After addition of liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phos-

phate the intensity of the most cationic band decreased. Indeed, the content of soluble protein in the supernatant fraction was only 42% of that observed before the addition of the liposomes. Liposomes composed of phosphatidyl inositol when mixed with the cationic protein fraction caused complete disappearance of protein from the soluble supernatant fluid as observed in electrophoresis and determined quantitatively.

The formation of complexes between lysosomal proteins and liposomes was dependent on the concentration of liposomes as shown in Fig. 3. As noted, cationic proteins forming complexes with liposomes were more readily sedimented as the concentration of liposomes increased. Liposomes composed of phosphatidyl inositol induced sedimentation at a lower concentration than did

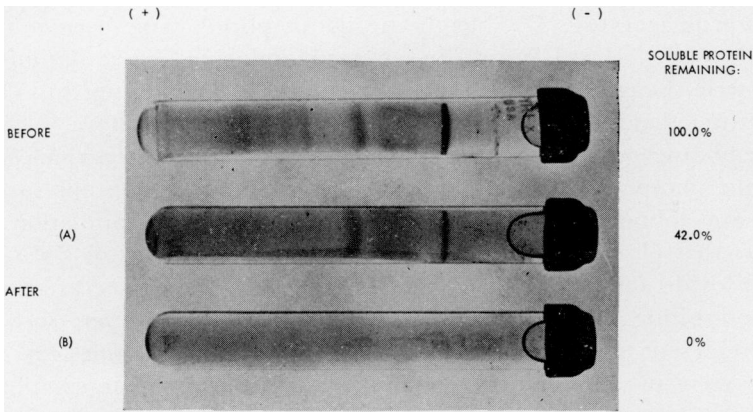

FIG. 2. Polyacrylamide gel electrophoresis of the cationic protein fraction before and after addition of liposomes. (A) Liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phosphate; (B) liposomes composed of phosphatidyl inositol.

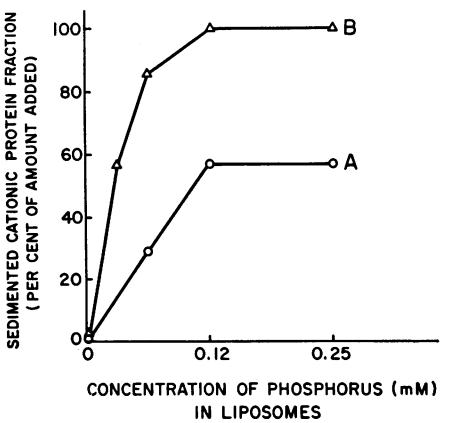

FIG. 3. Sedimentation of cationic protein fraction by liposomes. (A) Liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phosphate; (B) liposomes composed of phosphatidyl inositol.

liposomes composed of phosphatidyl choline, cholesterol, and dicetyl phosphate. It is probable that this effect is not only the function of phosphate groups present in both preparations of liposomes but it depends on the overall number of hydrophilic and hydrophobic groups of phospholipids interacting with cationic proteins.

DISCUSSION

Phospholipids which constitute about 50% of the cell membrane of PMN leukocytes(16) appear to participate in membrane changes leading to activation and release of lysosomal enzymes associated with degranulation. "A phospholipid effect," i.e., increased incorporation of phosphorus into acidic phospholipids, accompanies phagocytosis(17). Membrane phospholipids have been noted to interact with staphylococcal leucocidin, an agent inducing degranulation, possibly through steric changes in the cell membrane of PMN leukocytes(18). The recent demonstration that negatively charged phospholipids in the form of liposomal model membranes were able to elicit activation of lysosomal enzymes(7) suggests that specific phospholipid groups in the leukocytic cell membrane may have a role in the membrane fusion associated with phagocytic degranulation.

The fact that phospholipids complex with cationic proteins of PMN granules does not provide absolute evidence that these noncatalytic proteins are responsible for controlling the activity of lysosomal enzymes. However, such cationic proteins represent structural constituents of leukocytic granules(19). Whether they are present in the lysosomal membrane or whether they are complexed with acidic mucopolysaccharides found within PMN leukocytic granules(20) remains to be elucidated. Structural proteins that have been described in microsomes(21) and mitochondria(22,23) also exhibit ability to bind phospholipids. It is possible that during phagocytosis a cationic protein(s) contributing to the structural integrity of PMN leukocytic granules interacts with negatively charged phospholipids unmasked in a cell membrane after particle attachment (Fig. 4). This interaction may produce transitional changes labilizing the granule membrane and freeing lysosomal enzymes which become active and gain access to ingested

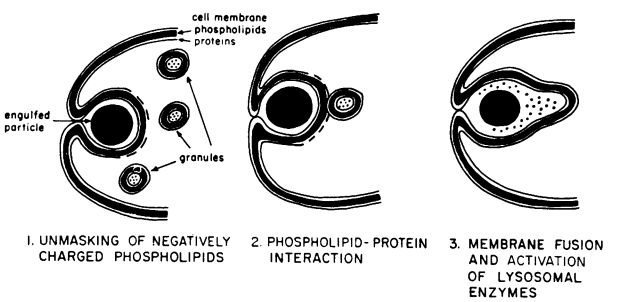

FIG. 4. A tentative mechanism of activation of lysosomal enzymes during phagocytosis in PMN leukocytes.

material. Therefore, it would appear that the molecular basis of activation of lysosomal enzymes during phagocytosis may be characterized by a phospholipid–protein interaction.

SUMMARY

A cationic protein fraction (CPF) prepared by acid extraction of isolated leukocytic granules was tested for its ability to interact with liposomal model membranes. CPF preincubated with liposomes significantly inhibited their ability to activate the lysosomal marker enzymes, beta glucuronidase, and beta *N*-acetylglucosaminidase in intact leukocytic granules. A similar inhibitory effect was observed with the polycationic protein, poly-L-lysine. CPF formed insoluble complexes with liposomes as revealed by sedimentation studies and electrophoresis in polyacrylamide gel. It is suggested that membrane-forming phospholipids in the form of liposomes interact with lysosomal protein(s) to labilize the lysosomal membrane and activate lysosomal enzymes.

REFERENCES

1. DeDuve, C., *in: Subcellular Particles* (T. Hayashi, Ed.), p. 128. Ronald Press, New York, 1959.
2. Cohn, Z. A., and Hirsch, J. G., The influence of phagocytosis on the intracellular distribution of granule associated components of polymorphonuclear leukocytes. *J. Exp. Med.* **112,** 1015–1022 (1960).
3. Lockwood, W. R., and Allison, F., Electron micrographic studies of phagocytic cells. I. Morphological changes of the cytoplasm and granules of rabbit granulocytes associated with ingestion of rough pneumoncoccus. *Brit. J. Exp. Pathol.* **54,** 593–600 (1963).
4. Zucker-Franklin, D., and Hirsch, J. G., Electron microscope studies on the degranulation of rabbit leukocytes during phagocytosis. *J. Exp. Med.* **120,** 569–576 (1964).
5. Horn, R. G., Spicer, S. S., and Wetzel, B. H., Phagocytosis of bacteria by heterophil leukocytes. Acid and alkaline phosphatase cytochemistry. *Amer. J. Pathol.* **45,** 327–335 (1964).
6. Hawiger, J., Collins, R. D., Horn, R. G., and Koenig, M. G., Interaction of artificial phospholipid membranes with isolated polymorphonuclear leukocytic granules. *Nature* London **222,** 276–278 (1969).
7. Hawiger, J., Horn, R. G., Koenig, M. G., and Collins, R. D., Activation and release of lysosomal enzymes from isolated leukocytic granules by liposomes. A proposed model for degranulation in polymorphonuclear leukocytes. *Yale J. Biol. Med.* **42,** 57–70 (1969).
8. Zeya, H. I., and Spitznagel, J. K., Antibacterial and enzymic basic proteins from leukocyte lysosomes: Separation and identification. *Science* **142,** 1085–1087 (1903).
9. Zeya, H. I., and Spitznagel, J. K., Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. *J. Exp. Med.* **127,** 927–941 (1968).
10. Reisfeld, R. A., Lewis, V. J., and Williams, D. E., Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* London **195,** 281–283 (1962).
11. Lowry, O. H., Rosenbrough, N. J., Far, L., and Randall, R. J., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275 (1951).
12. Berenblum, I., and Chain, E., An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32,** 295–298 (1938).
13. Baggiolini, M., Hirsch, J. G., and DeDuve C., Resolution of granules from rabbit heterophil

leukocytes into distinct populations by zonal sedimentation. *J. Cell Biol.* **40,** 529–541 (1969).
14. Woodin, A. M., The extrusion of protein from rabbit polymorphonuclear leukocyte treated with staphylococcal leucocidin. *Biochem. J.* **82,** 9–15 (1962).
15. Smolelis, A. N., and Hartsell, S. E., The determination of lysozyme. *J. Bacteriol.* **70,** 731–736 (1949).
16. Woodin, A. M., and Wieneke, A. A., Composition and properties of a cell-membrane fraction from the polymorphonuclear leukocyte. *Biochem. J.* **99,** 493–500 (1966).
17. Karnovsky, M. L., Metabolic basis of phagocytic activity. *Physiol. Rev.* **42,** 143–168 (1962).
18. Woodin, A. M., and Wieneke, A. A., The participation of phospholipids in the interaction of leucocidin and the cell membrane of the polymorphonuclear leukocyte. *Biochem. J.* **105,** 1029–1038 (1967).
19. Zeya, H. I., Interactions among lysosomal constituents of polymorphonuclear leukocytes. *Fed. Proc.* **28,** 265 (1969).
20. Fedorko, M. E., and Morse, S. I., Isolation, characterization, and distribution of acid mucopolysaccharides in rabbit leukocytes. *J. Exp. Med.* **121,** 39–48 (1965).
21. Ward, K. A., and Pollak, J. K., The composition of rat liver microsomes. The structural proteins of rat liver microsomes. *Biochem. J.* **114,** 41–48 (1969).
22. Richardson, S. H., Hutlin, H. O., and Green, D. E., Structural proteins of membrane systems. *Proc. Nat. Acad. Sci. U.S.A.* **50,** 821–826 (1963)
23. Zahler, W. L., Saito, A., and Fleischer, S., Removal of structural protein from mitochondria. *Biochem. Biophys. Res. Commun.* **32,** 512–518 (1968).