Neutral Proteases Confined to One Class of Lysosomes of Human Polymorphonuclear Leukocytes[1] (36164)

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Proteases of polymorphonuclear leukocytes (PMN) are presumed to play an important role in inflammatory reactions caused by immune complex diseases, microcrystalline and rheumatoid arthritis (1, 2). The acid cathepsins of rabbit PMN have been implicated in vascular injury such as arteritis or serum sickness and damage to the basement membranes of capillaries including those in glomeruli (3). Because PMN of several animals are known to possess acid proteases (4–7), it has been tacitly assumed that similar enzymes occur in human PMN. Human PMN appear to possess proteases active primarily at neutral pH (8–12). This indicates a fundamental difference between human and rabbit PMN and suggests that certain types of tissue injury occurring in rabbits differ from those occurring in man.

In order to augment the knowledge currently available on the proteases of human PMN, we have extended the investigations of others and attempted: to ascertain how many proteases are in human PMN, to characterize their pH optima, and to learn their subcellular location. We have found that the principal protease activities are associated with, and latent in, certain classes of membrane-bound granules of human PMN. Protease activity and benzyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (NBA esterase) activity, both optimally active at pH 7.2, sediment together through a sucrose density gradient in one class of granules. Aminopeptidase activity, also possessing a pH optimum of 7.2, sediments separately in another class of granules.

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Materials and Methods. Human blood, 500 ml from normal donors, was collected by venipuncture into a Fenwall Double Blood Pack ID12 (Travenol Laboratories, Morton Grove, IL) which contained 2250 units of heparin. Erythrocytes were removed by dextran sedimentation and the supernatant centrifuged at 126*g* to deposit PMN. Erythrocytes were removed by hypotonic lysis (13). The leukocytes were washed once in 0.34 *M* sucrose and resuspended in 0.34 *M* sucrose to make a 20% suspension. A smear of the resuspended material stained with Wright's stain showed less than 10% lymphocytes. This leukocyte suspension was then homogenized in a Potter-Elvejhem homogenizer for 225 sec using 5 × 45 sec bursts of homogenization with a 30 sec pause between each burst. After homogenization, the homogenate was centrifuged at 126*g* for 15 min to remove nuclei; and the organelles, in 2.7 ml of the homogenate, were separated on a 50 ml linear sucrose gradient. The gradient extended from a density of 1.129 to a density of 1.248. The gradient was centrifuged in a Beckman Model L-2-65B ultracentrifuge with a SW25.2 rotor at 55,000*g* for 60 min. Fractions (2.7 ml) were collected by upward displacement with 60% sucrose (density = 1.288).

Samples of each fraction provided material for determination of optical densities at 450 nm, protein assays by Lowry and co-workers' method (14), and enzyme assays. Neutral protease assay was carried out by the method of Press *et al.* (15), using denatured hemoglobin (Sigma Chemical Co., St. Louis, MO) at pH 7.2. The aminopeptidase assay was done at pH 7.2, using L-leucine-β-naphthylamide (Nutritional Biochemicals

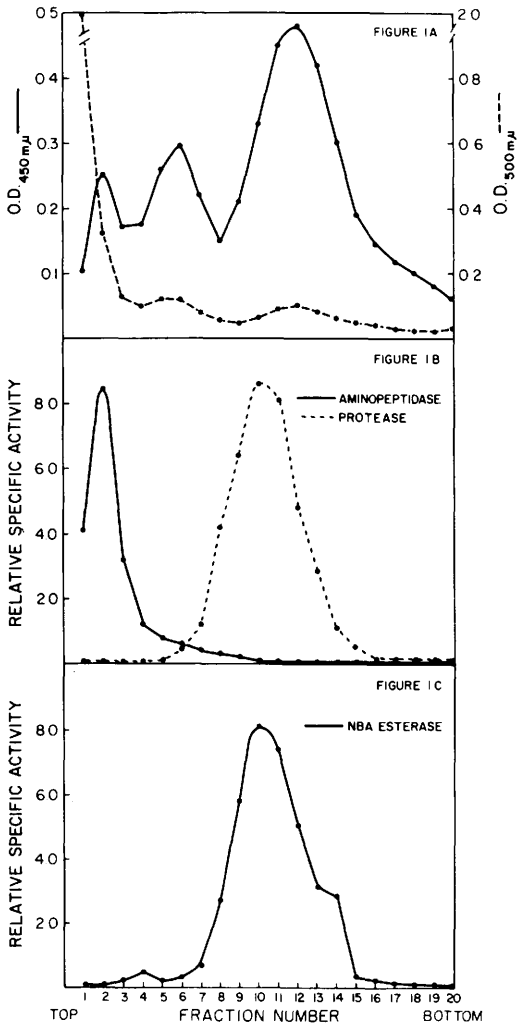

FIG. 1A. The densitometric profile of human PMN sucrose density gradient: The optical density at 450 nm shows three peaks. Protein by Lowry's method is shown as optical density at 500 nm. (B) Distribution of aminopeptidase and protease activities. Aminopeptidase was associated with the top band or band I. The protease was associated with the bottom band or band III. (C) The NBA esterase activity was associated with band III of the sucrose density gradient.

Co., Cleveland, OH) as substrate, according to the method of Behal and Folds (16). Janoff's (17) method, which employs benzyloxycarbonyl-L-alanine-*p*-nitrophenyl ester as substrate at pH 6.5 served to measure NBA esterase activity. Triton-X (0.1%), which was added to all fractions before enzyme assays were begun, released latent enzyme activity (usually 60–70% of total).

Results and Discussion. Three bands of increased optical density designated I, II, and III (Fig. 1A) appeared in the sucrose gradient, when human peripheral PMN were processed as described in the Materials and Methods. Band I appeared at the top of the gradient at a density of 1.128; band II appeared at a density of 1.156; band III appeared at a density of 1.195. Although most of the protein remained at the top of the gradient at the point of application, Fig. 1A indicates that small peaks of protein corresponded to bands II and III. The aminopeptidase sedimented in band I; and neutral protease sedimented in the ascending limb of band III (Fig. 1B). NBA esterase activity was also associated with band III (Fig. 1C).

Measurements of enzymes, commonly associated with cytoplasmic granules of PMN, provided additional markers for the protease-rich fractions. Band I contained acid phosphatase (active on *p*-nitrophenyl phosphate) activity, as well as the aminopeptidase activity. Band III contained, in addition to the neutral protease and NBA esterase, β-glucuronidase and β-glycerol phosphatase, enzymes which usually associated with lysosomes as well.

The proteolytic enzymes all displayed clear-cut pH optima. The aminopeptidase, the protease and the esterase were found to be most active at pH values near neutrality; all were inactive at pH values below pH 6.0. This is consistent with the results of others (9), which suggested that the acid proteases found in rabbits and other animals were not found in human PMN.

These observations extend those of Janoff and Zeligs (9), who reported the presence of a neutral protease in human PMN granules and showed that *in vitro* it hydrolyzes denatured hemoglobin and rabbit glomerular basement membranes. They also relate to observations of Davies *et al.* (18), who found that, in addition to acid protease, rabbit PMN granules possess a neutral protease and an aminopeptidase. These investigators found the protease was entirely associated with the PMN granules, but of the aminopeptidase (measured with L-leucine-β-naphthylamide), 80% failed to sediment in the granule fraction. Of the NBA esterase [Davies *et al.*

(18)] however, 60% sedimented with the granules and 35% remained in the supernatant fluid. The essential point is that, in these experiments, the investigators centrifuged granules out of homogenized cytoplasm from PMN according to now classical techniques, which were devised for use with rabbit PMN. Not only are these techniques probably not appropriate for use with PMN from species other than the rabbit (19), they are also sure to produce highly heterogeneous granule populations. Our contribution, which is based on the use of granules resolved into different classes with sucrose density gradient centrifugation, shows that the aminopeptidase of Davies *et al.* (18), exists in human PMN but that, together with acid *p*-nitrophenyl phosphatase, it is associated with a fraction of membrane-bound granules of a density 1.129, which may or may not be lysosomal in the strict sense [De Duve *et al.* (20)]. The neutral protease and esterase of human PMN, however, are associated, together with two lysosomal enzymes, in a granule fraction of density 1.190.

Finally, it seems that, unlike proteases of rabbit PMN, the proteases of human PMN are maximally active at pH values near or greater than 7.2. Stiles and Fraenkel-Conrat (21) have reported the presence of small amounts of acid proteases in human leukocytes. However, they also reported the presence of neutral proteases in the leukocytes. We have failed to demonstrate acid proteases in human PMN, although we used the same techniques that easily demonstrate acid protease in rabbit PMN. Failure to find acid proteases in human PMN is not necessarily final proof that they are absent, but these results and those of others are pointing in that direction.

With respect to their pH optima, at least, human PMN proteases appear to be unique. Hence, it is necessary to study proteases in the human PMN in their own right. Similarly, it is important to know where these enzymes are located in these cells and how they relate to each other and to other enzymes, for it is now clear that substantial differences exist between the physical and biochemical features of rabbit and human PMN lysosomes.

Summary. We find that protease and esterase activity of human PMN exist in a latent form in a class of membrane-bound particles which sediment at a distinctive density. Readily separated from these granules by sucrose density gradient centrifugation is a separate class of particles, which is associated with an aminopeptidase not previously described in human PMN lysosomal granules. Protease, esterase, and aminopeptidase act optimally at or near pH 7.2.

1. Janoff, A., Ser. Haematol. 3, 96 (1970).
2. Thomas, L., Proc. Soc. Exp. Biol. Med. 115, 235 (1964).
3. Cochrane, C., and Aikin, B., J. Exp. Med. 124 733 (1966).
4. Cohn, Z., and Hirsch, J., J. Exp. Med. 112, 983 (1960).
5. Lapresle, C., and Webb, T., Biochem. J. 84, 455 (1962).
6. Wasi, S., Murray, R., Macmorine, D., and Movat, H., Brit. J. Exp. Pathol. 47, 411 (1966).
7. Lebez, D., and Kopitar, M., Enzymologia 31, 271 (1970).
8. Moulter, L., and Atiyeh, W., Blood 15, 52 (1960).
9. Janoff, A., and Zel'gs, J., Science 161, 702 (1968).
10. Janoff, A., and Scherer, J., J. Exp. Med. 128, 1137 (1968).
11. Prokopowicz, J., Thromb. Diath. Haemorrh. 19, 84 (1968).
12. Lazarrus, G., Daniels, J., Brown, R., Bladen, H., and Fullmer, H., J. Clin. Invest. 47, 2622 (1968).
13. Fallon, H., Frei, E., Davidson, J., Trier, J., and Burk, D., J. Lab. Clin. Med. 59, 799 (1962).
14. Lowry, O., Rosenbrough, N., Farr, A., and Randall, R., J. Biol. Chem. 193, 265 (1951).
15. Press, E., Porter, R., and Cebra, J., Biochem. J. 74, 501 (1960).
16. Behal, F., and Folds, J., Arch. Biochem. Biophys. 121, 364 (1967).
17. Janoff, A., Biochem. J. 114, 157 (1969).
18. Davies, P., Krakaner, K., and Weissmann, G., Nature (London) 228, 761 (1970).
19. Welsh, I. R. H., and Spitznagel, J., Infec. Immunity 4, 97 (1971)
20. De Duve, C., Pressman, B., Gianetto, R., Watriaux, R., and Appelmans, F., Biochem. J. 60, 604 (1955).
21. Stiles, M., and Fraenkel-Conrat, J., Blood 32, 119 (1968).

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