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Distribution of Lysosomal Enzymes, Cationic Proteins, and Bactericidal Substances in Subcellular Fractions of Human Polymorphonuclear Leukocytes

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Separation of homogenates of human polymorphonuclear leukocytes (PMN) into different fractions by sedimentation in centrifugal fields that ranged from 126 × *g* to 50,000 × *g* resulted in a differential distribution of the lysosomal enzymes. Peroxidase, lysozyme, beta-glucuronidase, and acid phosphatase activity were separated from each other. This demonstrates that the lysosomes of human PMN comprise at least three and possibly four physically and chemically different cytoplasmic particles. Proteins which are more cationic than lysozyme and which may be analogous to cationic lysosomal protein of rabbit PMN were associated with lysozyme and beta-glucuronidase rich granules. Antibacterial activity was present in four of the five cell fractions which this work produced. These results are significant because they differ from those obtained with rabbits and because they directly influence future experimental design and interpretation, in attempts to analyze antibacterial, scavenging, and inflammatory capacities of human PMN. Since lysosomes differ physically, biochemically, and morphologically, they may well differ with respect to their function in the PMN.

The lysosomes of polymorphonuclear leukocytes (PMN) are closely linked with the antimicrobial and scavenging mechanisms of the PMN; knowledge of this lysosome system is essential to the understanding of these PMN functions. We describe below experimental results which suggest that the lysosomes of human peripheral PMN differ physically and chemically from the lysosomes of rabbit PMN and are heterogeneous.

There is already morphological and histochemical evidence (4, 9, 13) that lysosomes from rabbit PMN are heterogeneous. Centrifuged through sucrose density gradients, these particles separate into populations that differ in size, enzyme content, and molecular composition (3, 25). The lysosomes from guinea pig peritoneal exudate PMN have also been separated (17) into unlike populations by differential sedimentation. Evidence for morphological heterogeneity among PMN lysosomes of human peripheral blood (6, 22) makes it seem entirely reasonable that an hypothesis of physical and chemical heterogeneity would be valid for human PMN lysosomes.

To test this hypothesis, we compared the distribution of four lysosomal enzymes among subcellular fractions obtained by differential centrifugation of PMN separated from human peripheral blood and homogenized in 0.34 M sucrose. We examined each fraction for cationic proteins and antibacterial activity, which in the rabbit appear to coincide in one class of granules. We also measured the influence of homogenization time on the release and distribution of lysosomal enzymes because homogenization can rupture lysosomes and so influence distribution on cell fractions. If lysosomes are heterogeneous in composition and form, they may differ with respect to other properties which might correlate with resistance to mechanical breakage.

MATERIALS AND METHODS

Human blood, 500 ml, was collected by venipuncture into a Fenwal Double Blood-Pack JD-12 which contains 2,250 U.S.P. units of heparin (Travenol Laboratories, Morton Grove, Ill.). Erythrocytes were removed by dextran sedimentation, and the supernatant was centrifuged at 126 × *g* to deposit leukocytes. Contaminating erythrocytes were removed by hypotonic lysis (8). All stages in the preparation of the

fractions were performed at 4 C by using precooled equipment and reagents. The leukocytes were washed once with 0.34 M sucrose and divided into portions of 2.5 ml each. A smear of the resuspended material stained with Wright's stain showed less than 10% lymphocytes.

The portions were then homogenized for 90 sec, 180 sec, and 270 sec, respectively, the same glass mortar and Teflon pestle being used for each homogenization. After homogenization, each portion was centrifuged at 126 × *g* for 15 min; the deposit was designated fraction I. (Examination of a smear from this fraction showed that it contained unbroken cells, nuclei, and some granules adhering to the cells.) The supernatant fluid was recentrifuged at 800 × *g* for 20 min; this deposit was designated fraction II; the supernatant from this was recentrifuged at 10,000 × *g* for 30 min to give a deposit, designated fraction III; the supernatant fluid from this was centrifuged at 50,000 × *g* for 60 min to give a deposit, designated fraction IV; the supernatant fluid from this was designated fraction V. All deposited fractions were then resuspended in 4.0 ml of 0.34 M sucrose.

Protein (14), beta-glucuronidase (E.C. 3.2.1.31., reference 10), acid phosphatase (E.C. 3.1.3.2.; reference 1), lysozyme (E.C. 3.2.1.17., reference 20), and peroxidase (E.C. 1.11.1.7., reference 15) estimations were done on all fractions. Each assay was done in triplicate to allow calculation of a mean and standard deviation. All enzyme assays were done with the addition of 0.1% Triton T-X-100 (Rohm and Haas Co., Philadelphia, Pa.) to release activity latent within lysosomal membranes.

After biochemical assay, the corresponding fractions from each homogenization were pooled. Fractions II, III, and IV were concentrated by centrifugation and, after the addition of 0.1% Triton T-X-100, were subjected to cellulose acetate electrophoresis by the technique of Zeya and Spitznagel (23). Polyacrylamide gel electrophoresis was also done by using a *p*H 4.5 buffer and 17.5% polyacrylamide gel; a stacking gel was not used.

For antibacterial tests, fractions were prepared in the same way from two lots of 100 ml of human blood each but with the use of an homogenization time of 180 sec. The antibacterial tests were done with a modification of Muschel's technique (24).

RESULTS

The greatest percentage of acid phosphatase was in fraction V under all homogenization regimens (Table 1). Homogenization for more than 180 sec resulted in a decrease in the percentage of acid phosphatase present in fraction I and a corresponding increase in acid phosphatase in fraction V. Evidently, either acid phosphatase was associated with a very small particle or it was liberated from the lysosomes in molecular form by homogenization. When the acid phosphatase content was expressed in terms of relative specific activity (RSA, Table 2), fraction IV, regardless of the length of time during which the PMN had been homogenized, possessed acid phosphatase-containing granules in purer suspension than any other fraction. Thus, a particle rich in acid phosphatase was being sedimented selectively at 50,000 × *g* for 60 min. Homogenization for more than 180 sec caused the RSA of acid phosphatase in fraction IV to decrease from 4.1 to 2.5, whereas that in fraction V increased. Perhaps prolonged trauma liberated acid phosphatase in molecular form.

Lysozyme, under all conditions, was most concentrated in fraction III whether its presence was expressed as a percentage (Table 1) or as RSA (Table 2). Thus, the lysozyme-containing granules were either larger or of greater density than the granules containing acid phosphatase. Homogenization affected the association of lysozyme and granules relatively little, for after homogenization for 270 sec the lysozyme released from fraction I was no greater than that released after 90 sec. Although there was some decrease in the lysozyme activity in fraction II after 270 sec of homogenization, this may have represented disaggregation of granules in cytoplasmic fragments, an idea supported by the lysozyme activity which increased in fraction III as it decreased in fraction II.

Beta-glucuronidase was most concentrated in the same fraction as lysozyme, i.e., fraction III. There were, however, significant differences in the way the distribution of these enzymes was affected by the homogenization time. As the homogenization time was prolonged, the percentage and RSA of beta-glucuronidase in fraction III decreased; at the same time, they increased in fraction V. Under these conditions, the lysozyme percentage and RSA increased in fraction III but remained the same in fraction V. This suggested that these enzymes were contained in two different particles having similar physical characteristics but different degrees of fragility, or that they were contained in the same particle, the beta-glucuronidase being attached to the particle in such a manner that it was more readily released by prolonged homogenization.

The greatest percentage of peroxidase was in fraction I; however, the RSA of peroxidase was greater in fraction II than in any other fraction. The granules which contained peroxidase thus appeared to be larger or denser than those containing the other enzymes. Prolonged homogenization increased slightly the amount of peroxidase in fractions IV and V; this might be explained by postulating that the shearing forces broke a few of the granules into smaller fragments and perhaps released free peroxidase from them.

Cellulose acetate paper electrophoresis of

TABLE 1. *Distribution of enzymes in subcellular fractions after homogenization of human leukocytes*

Enzyme	Time of homogenization (sec)	Per cent of total activity in fraction[a]				
		I	II	III	IV	V
Acid phosphatase	90	27.1 ± 4.9[b]	4.5 ± 0.8	16.3 ± 2.8	13.1 ± 0.4	38.8 ± 1.0
	180	29.7 ± 2.6	3.8 ± 0.3	14.2 ± 1.1	17.3 ± 1.5	34.8 ± 1.2
	270	17.6 ± 2.3	3.9 ± 0.1	13.1 ± 1.5	15.6 ± 1.2	49.6 ± 2.5
Lysozyme	90	13.9 ± 1.0	15.6 ± 0.7	61.8 ± 2.0	6.4 ± 1.1	2.1 ± 0.5
	180	11.3 ± 1.0	16.9 ± 1.8	62.9 ± 2.0	6.6 ± 0.5	2.0 ± 0.5
	270	13.1 ± 1.0	9.4 ± 1.0	69.1 ± 1.9	6.1 ± 0.5	2.1 ± 0.5
Beta-glucuronidase	90	25.5 ± 0.7	11.8 ± 0.2	51.5 ± 1.5	5.6 ± 0.9	5.3 ± 0.4
	180	29.4 ± 0.9	11.2 ± 0.7	45.2 ± 1.7	7.0 ± 0.2	6.8 ± 0.2
	270	29.6 ± 1.0	10.1 ± 0.1	44.9 ± 2.6	7.6 ± 0.6	7.6 ± 0.6
Peroxidase	90	47.5 ± 5.5	27.6 ± 2.8	18.2 ± 1.0	3.4 ± 0.2	3.0 ± 0.2
	180	59.9 ± 3.1	15.3 ± 1.3	14.3 ± 0.9	5.7 ± 0.5	4.6 ± 0.5
	270	43.1 ± 4.6	20.3 ± 1.2	21.9 ± 1.4	8.9 ± 0.3	5.6 ± 0.4

[a] Fractions were prepared by resuspension of pellets which were consecutively sedimented as follows: fraction I, 126 × *g*; fraction II, 800 × *g*; fraction III, 10,000 × *g*; fraction IV, 50,000 × *g*; and fraction V, supernatant fluid from this last sedimentation.

[b] Mean ± standard deviation, n = 3.

TABLE 2. *Distribution of enzymes in subcellular fractions after homogenization of human leukocytes*

Enzyme	Time of homogenization (sec)	Relative specific activity in fraction[a]				
		I	II	III	IV	V
Acid phosphatase	90	0.69 ± 0.13[b]	0.63 ± 0.01	0.99 ± 0.06	2.80 ± 0.20	1.20 ± 0.04
	180	0.68 ± 0.07	0.59 ± 0.05	0.89 ± 0.09	4.10 ± 0.61	1.19 ± 0.06
	270	0.46 ± 0.07	0.42 ± 0.04	0.81 ± 0.13	2.50 ± 0.28	1.67 ± 0.09
Lysozyme	90	0.35 ± 0.03	2.20 ± 0.10	3.75 ± 0.20	1.39 ± 0.25	0.07 ± 0.01
	180	0.26 ± 0.03	2.64 ± 0.28	3.93 ± 0.27	1.57 ± 0.19	0.07 ± 0.01
	270	0.34 ± 0.03	1.02 ± 0.13	4.29 ± 0.49	0.98 ± 0.11	0.07 ± 0.01
Beta-glucuronidase	90	1.21 ± 0.14	3.89 ± 0.04	3.12 ± 0.16	1.22 ± 0.21	0.16 ± 0.01
	180	0.67 ± 0.04	1.75 ± 0.11	2.83 ± 0.21	1.67 ± 0.20	0.23 ± 0.01
	270	0.77 ± 0.06	1.10 ± 0.08	2.79 ± 0.34	1.23 ± 0.14	0.26 ± 0.01
Peroxidase	90	1.21 ± 0.14	3.89 ± 0.40	1.10 ± 0.08	0.74 ± 0.07	0.09 ± 0.01
	180	1.36 ± 0.10	2.39 ± 0.20	0.89 ± 0.08	1.36 ± 0.20	0.16 ± 0.01
	270	1.13 ± 0.14	2.21 ± 0.21	1.36 ± 0.18	1.44 ± 0.12	0.19 ± 0.01

[a] Fractions were prepared by resuspension of pellets which were consecutively sedimented at fraction I, 126 × *g*; fraction II, 800 × *g*; fraction III, 10,000 × *g*; fraction IV, 50,000 × *g*, and fraction V, supernatant fluid from this last sedimentation.

[b] Mean ± standard deviation, n = 3.

fraction III (Fig. 1) showed basic proteins which were electrophoretically similar in their relation to lysozyme to those demonstrated in the rabbit (23). Electrophoresis on 17.5% acrylamide gels (Fig. 2), which under the conditions used acted as molecular sieves, failed to show protein bands closer to the cathode than lysozyme. Thus these basic proteins of human PMN probably have a greater molecular weight than lysozyme (molecular weight 15,000).

The bactericidal assay, a modification of Muschel's technique (24), showed that, although fraction I had no ability to inhibit growth, all other fractions had a LD_{50} between 10 and 25

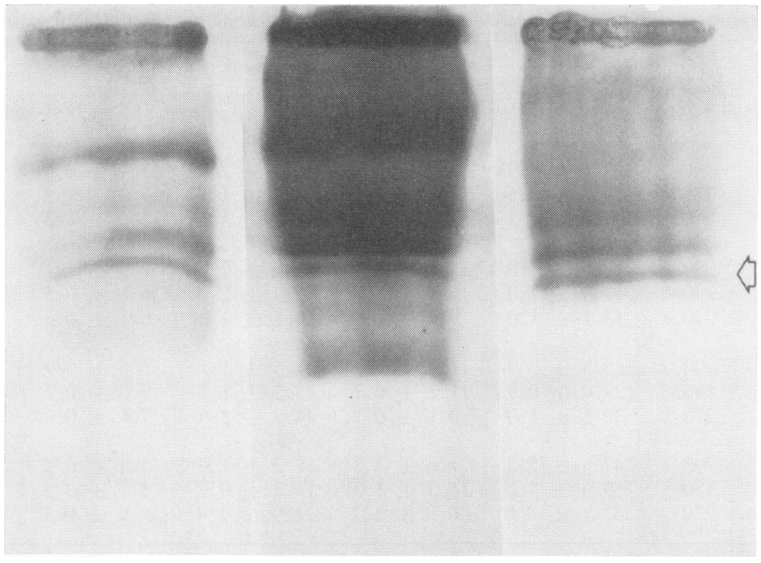

FIG. 1. *Cellulose acetate paper electrophoresis. Buffer pH 4.5, origin at top, cathode at bottom; from left to right fractions II, III, and IV. Arrow points to position of lysozyme activity.*

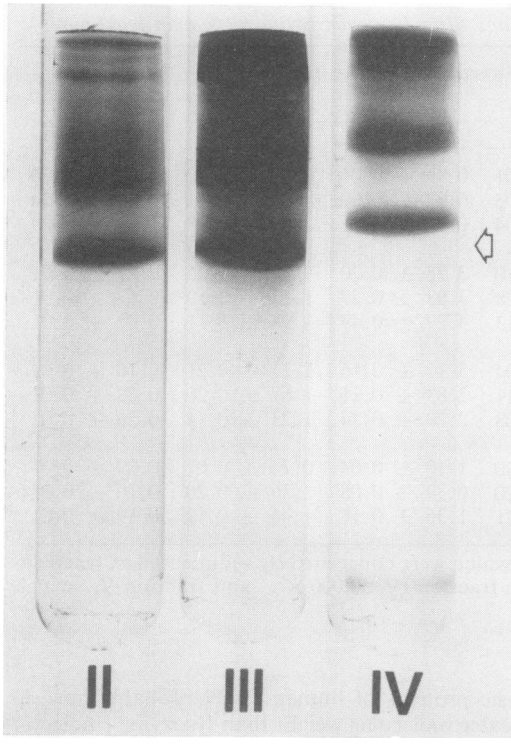

FIG. 2. *Acrylamide gel electrophoresis. Acrylamide (17.5%), buffer pH 4.5, origin at top, cathode at bottom. Numerals show fraction used and arrow points to position of lysozyme activity.*

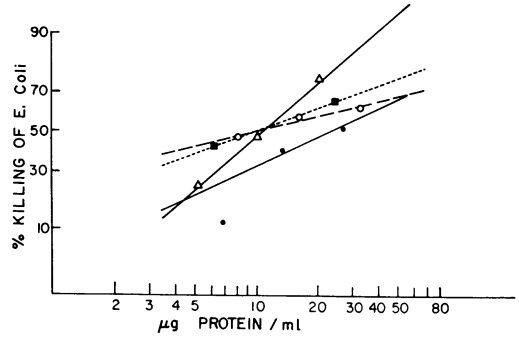

FIG. 3. *Results of bactericidal assay. Fraction II (●), fraction III (○), fraction IV (■), fraction V (△), fraction I not shown, failed to inhibit bacterial growth. Reaction of fractions with bacteria at room temperature in the presence of 0.01% Triton-X-100.*

μg of granule protein per ml for 10^6 bacteria (Fig. 3). (The assay for fraction I is not shown as it had no lethal action.)

DISCUSSION

The distribution of the enzymes as shown by differential centrifugation demonstrates that there is a physical and biochemical heterogeneity among the lysosomes of the human PMN and suggests that this heterogeneity goes beyond the concept of specific and azurophilic granules. We found at least three and possibly four types of lysosomes in human PMN: (i) small or low-density granules containing acid phosphatase, (ii) large or dense granules which

contain peroxidase, and (iii) one or two granule populations intermediate in size or density (or both) but which may differ in mechanical fragility. These intermediate granules contain lysozyme, beta-glucuronidase, or both. These results extend morphological observations by Watanabe et al. (22) who found three types of granules and of Daems (6) who demonstrated four types of granules in human PMN. The results are also consistent with those of Daems and van der Ploeg (7) who found that peroxidase was confined to the largest granules of human PMN.

An alternate explanation of our results would be that there is differential breakdown of a homogeneous granule into different fractions, depending on the duration of homogenization. We reject this explanation because of the morphological and histochemical findings cited above and because such an explanation would require a graded increase of all the enzymes in all of the lighter fractions as the time of homogenization increases; this is not shown in the distribution of lysozyme. The presence of maximum amounts of peroxidase in a heavier fraction than any other enzyme also mitigates against this explanation. More definitive proof must await analysis of homogenized human PMN on density gradients.

That the forces which bind beta-glucuronidase among the 10,000 × *g* particles are tenuous compared with those binding lysozyme suggests that the function as well as the composition of these granules may differ. The release of beta-glucuronidase from phagocytosing human PMN has been shown by Pruzansky and Patterson (19) and May et al. (16); this phenomenon may be due to physical stresses upon the beta-glucuronidase granule during phagocytosis or due to granule involvement in the formation of the phagosome.

A dissimilarity between species is suggested by comparison of human with rabbit PMN which have been shown (25) to contain, in association with the peroxidase-rich granules, proteins more basic and of lower molecular weight than lysozyme. Proteins which were similar in their electrophoretic migration on cellulose acetate to those found in rabbit PMN (23) are found in the human PMN, but when they are electrophoresed on polyacrylamide gels they seem to have a greater molecular weight than lysozyme and they appear to be associated with the intermediate size granule of fraction III rather than the peroxidase-rich fraction II.

The antibacterial activity of PMN has been associated with lysozyme (18), peroxidase (12), and basic proteins (23). The failure to find significant differences between the bactericidal activity of fractions II, III, IV, and V, despite the fact that these antibacterial substances are present in different proportions in each fraction, suggests that there may be more than one mechanism available to kill bacteria in the PMN.

Some earlier experiments of different investigators on the human PMN (21) were done with the same experimental conditions which Cohn and Hirsch (5) used for the isolation of granules from rabbit PMN. Our results suggest that these conditions are of restricted applicability to human PMN. Others (11) removed the nuclei and cell debris in a centrifugal field of 2,000 × *g* for 10 min, which our experiments show would also deposit the heavy granule class, or (2) used homogenization conditions more severe than those which in our experiments resulted in increased release of lysosomal enzymes into the supernatant fluid fraction. Thus our results suggest that there should be a revision in the interpretation of earlier experiments, which were designed on the assumption that there was only one class of granule, and that the wide differences in physical properties of human lysosomes should be taken into account in future experimental design.

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