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# Cationic Proteins of Polymorphonuclear Leukocyte Lysosomes

## I. Resolution of Antibacterial and Enzymatic Activities

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ABSTRACT

ZEYA, H. I. (University of North Carolina, Chapel Hill), AND J. K. SPITZNAGEL. Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. J. Bacteriol. 91:750–754. 1966.—A lysosomal fraction from polymorphonuclear (PMN) leukocytes of guinea pig peritoneal exudate was subjected directly to electrophoresis on cellulose acetate paper treated with cetyltrimethyl ammonium bromide. The lysosomal components resolved into seven bands moving towards the cathode. Assay of the eluted bands showed that the antibacterial activity was distinct from lysosomal enzymes and was associated with three cationic components (bands I, II, and III) which migrated most rapidly towards the cathode, ahead of lysozyme ribonuclease and deoxyribonuclease. Qualitatively, the antibacterial components appeared to be rich in arginine. The antibacterial components were absent in the pherograms of nuclear fractions of PMN leukocytes and in supernatant fractions that remained after lysosomes were removed from cell homogenates by centrifugation at 8,000 × *g*.

Leukocytic degranulation and participation of polymorphonuclear (PMN) granules in the bactericidal activity of leukocytes were described as early as 1894 by Kanthak and Hardy (6). Over the past decades, a number of partially characterized antibacterial substances in extracts of whole PMN leukocytes have been reported by various workers (10). As nuclei of PMN leukocytes, like nuclei of all mammalian cells, contain histones and protamines that in themselves possess antibacterial activity (10), the antibacterial property of whole leukocyte extracts could not definitely be attributed to extranuclear components. Utilizing cell fractionation methods, Cohn and Hirsch (2) demonstrated a number of acid hydrolases, as well as the bulk of the leukocytic antibacterial activity, in association with specific granules of PMN leukocytes. As these granules contained acid-soluble enzymes, such as lysozyme, ribonuclease, and deoxyribonuclease, the nature and the existence of the antibacterial substances independent of the hydrolytic enzymes could not be defined. The indication that the substances involved may be polycationic in nature came with the histochemical studies of Spitznagel and Chi (11).

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Using a method by which specific granules of PMN leukocytes can be subjected directly to electrophoresis, we have separated the contents of granules into several components which include, apart from known enzymes, three unique basic protein components possessing antibacterial activity. A preliminary report on some aspects of these studies has been published elsewhere (13).

This paper and the one following it describe the separation and the biochemical and biological characterization of cationic proteins isolated from specific granules of PMN leukocytes.

### MATERIALS AND METHODS

*PMN leukocytes.* The leukocytes were obtained from guinea pig peritoneal exudate induced by injection of 0.5% glycogen in 0.85% saline. White male guinea pigs weighing 400 to 600 g were used. The average peritoneal yield (80 to 100 ml) contained approximately 5.0 × $10^6$ cells per milliliter. Of these cells, 95% were PMN leukocytes.

*Preparation of granule fraction.* The granules from PMN leukocytes were prepared by homogenization and differential centrifugation of disrupted cells in 0.35 M sucrose. The granular fraction (8,000 × *g*) was suspended in 0.25 M sucrose and stored at 4 C. The nuclear fraction (twice washed with 0.25 M sucrose to remove contamination with large granules) and the post granular supernatant fraction were saved for electrophoretic comparison.

*Leukocytes from peripheral blood.* The leukocytes from peripheral blood of the guinea pig were separated according to the technique of Athens et al. (1). The red blood cells were removed by dextran agglutination and hemolysis by 5% dextrose.

*Paper electrophoresis of granules.* Oxoid paper (20 by 5 cm; Colab Laboratories, Inc., Chicago Heights, Ill.) was soaked in 0.5% cetyltrimethyl ammonium bromide (CTAB) in water for 3 min and was rinsed with acetate buffer (*p*H 4) for 5 min. Samples of granule suspension in 0.25 M sucrose were applied to strips from drawn tips of Pasteur pipettes. Electrophoresis was carried out for 1 hr (200 v; 0.002 amp) at room temperature in acetate buffer (*p*H 4; ionic strength, 0.05). The electrophoretic strips were stained with 0.5% Buffalo Black (Allied Chemical Corp., Morristown, N.J.) in methanol with 10% (v/v) glacial acetic, and were rinsed with a 10% solution of glacial acetic acid in methanol.

*Elution of biological activity from pherograms.* The long edge of an unstained pherogram was cut away, stained with Amido Black, and used as a marker for elution of individual bands from the remainder. For antibacterial assay, protein was eluted from individual bands with 0.5 ml of 0.01 N HCl. Elution was carried out directly in 1-cm dialysis tubing for 30 min. Thereafter, the paper strip was removed from the bag to minimize loss of material by adsorption, and the eluate was dialyzed against 0.01 N HCl (2 liters) for 6 hr, under cold temperature, to remove the residual CTAB.

*Antibacterial assay.* The assay was carried out according to the method of Hirsch (4) in 2-ml plastic cups (Linbro Chemical Co., New Haven, Conn.). A citrate phosphate buffer of *p*H 5.6 was used as a medium for assay. Throughout the experiments, 18-hr cultures of *Escherichia coli* strain O117:H27 were employed. Fifty per cent inhibition of bacterial growth as estimated by comparison with control cups was taken as the end point.

*Lysozyme assay.* Lysozyme activity in each band was determined by the procedure of Shugar (9). Lysozyme substrate consisting of ultraviolet-irradiated *Micrococcus lysodeikticus* was obtained from Difco. Tests were standardized with crystalline egg white lysozyme. Individual bands from electrophoretic strips were eluted in 1.5 ml of 0.1 M phosphate buffer (*p*H 6.2) for 30 min, and eluates were assayed for lysis of *M. lysodeikticus*.

*Ribonuclease assay.* Ribonuclease was assayed according to the turbidimetric method of Houck (5). Commercial yeast ribonucleic acid (RNA) was used as the substrate. Elution of individual bands was carried out in 1 ml of 0.1 M acetate buffer (*p*H 5) for 30 min. The turbidity was measured at 400 mμ in a Beckman DB spectrophotometer.

*Deoxyribonuclease assay.* Deoxyribonuclease activity was determined according to the method of Schneider and Hogeboom (8). Highly polymerized DNA (Calbiochem) was used as substrate. Individual bands from electrophoretic strips were eluted in 1 ml of 0.1 M acetate buffer (*p*H 5) for 30 min. The eluates were incubated with the substrate for 2 hr at 37 C. The amount of deoxyribonuclease activity in each band was determined by comparison with the standard curve.

## RESULTS

*Resolution of PMN granule proteins* Attempts to resolve the constituents of PMN granules by zone electrophoresis on untreated acetate paper in a *p*H range from 2 to 9 were unsuccessful. The substances remained at the line of application, and even at very acidic *p*H failed to separate into bands. It was also difficult to elute the material from the paper. To avoid electrostatic bond formation between cationic groups in the proteins and the carboxyl groups of the cellulose acetate, the paper was treated with CTAB, a cationic detergent. The presence of residual CTAB on the paper and the acid *p*H of the suspending buffer seemed to cause the immediate lysis of the affixed granule suspension. Subsequent electrophoresis on the neutralized paper resolved granule components into seven or more bands, which migrated toward the cathode as shown in Fig. 1. The pattern was found to be reproducible in experiments from day to day.

For the purpose of description, the bands were designated by Roman numerals according to their mobilities towards the cathode, bands I and II being the fastest-moving components. The percentage of various electrophoretically separated components of PMN granules was estimated by determination of bound dye, on the assumption that bound dye would be proportional to the quantities of protein in the stained bands and that intrinsic dye-binding capacity of the components would be equal. The results are summarized in Table 1.

Bands I and II possessed the fastest mobilities and were found to be variable. At times, they separated into more than two bands or a single trailing fast-moving band. Band III was stained more heavily than the other bands, with a characteristic deep-blue color, providing an easily recognizable and very reproducible reference point in these patterns.

*Biological activities associated with protein*

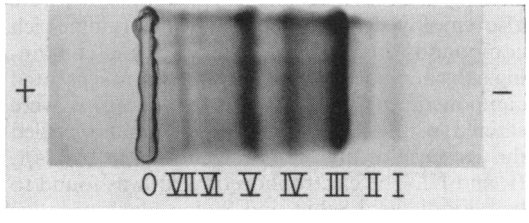


FIG. 1. *Zone electrophoresis of PMN lysosomes. Antibacterial bands: I, II, and III. Lysozyme: IV. Ribonuclease: V. Deoxyribonuclease: VI.*

TABLE 1. *Estimation of relative protein content and biological activities of various components obtained by electrophoresis of PMN lysosomes*

| Band | Mobility[a] | Relative protein content[b] | Antibacterial activity[c] | Lysozyme[d] | Ribonuclease[d] | Deoxyribonuclease[d] |
|---|---|---|---|---|---|---|
| I + II | 0.65 | 10 | 16 | 0 | 0 | 0 |
| III | 0.53 | 24 | 32 | 0 | 0 | 0 |
| IV[e] | 0.40 | 18 | 0 | 0.63 | 0 | 0 |
| V[e] | 0.29 | 20 | 0 | 0 | 0.5 | 0 |
| VI[e] | 0.14 | 3 | 0 | 0 | 0 | 0.2 |
| VII | 0.10 | 4 | 0 | 0 | 0 | 0 |
| Origin | 0 | 21 | 0 | 0 | 0 | 0 |

[a] Microns (at *p*H 4) $cm^2hr^{-1}v^{-1}$.
[b] Relative percentage of Amido Black per band.
[c] Reciprocal of highest dilution showing 50% growth inhibition.
[d] Micrograms of crystalline egg white lysozyme, pancreatic ribonuclease, pancreatic deoxyribonuclease equivalent.
[e] Samples of purified commercial lysozyme, ribonuclease, and deoxyribonuclease displayed mobilities in this system identical to bands IV, V, and VI, respectively.

*bands*. For various assays, materials from individual bands were easily eluted. The antibacterial assay of bands eluted from electrophoretic strips revealed that the activity against *E. coli* was confined to bands I, II, and III, which migrated most rapidly towards the cathode (Fig. 1). Because of their low protein content, bands I and II were assayed together. The antibacterial activity is shown in Table 1 as the reciprocal of highest dilution showing 50% growth inhibition as compared with the controls. Usually, complete inhibition was found in cups preceding the 50% end point.

Assay for lysozyme showed the activity exclusively in band IV (Fig. 1; Table 1). Ribonuclease was detectable only in band V, whereas band VI showed deoxyribonuclease activity. Under these conditions, the arrangement of enzymatic bands seemed to follow closely their isoelectric points.

Samples of purified commercial lysozyme, ribonuclease, and deoxyribonuclease displayed mobilities identical to bands IV, V, and VI, respectively (Fig. 2).

A qualitatively identical pattern was obtained with granules from peripherally circulating PMN.

*Histochemical properties of bands resolved from PMN granules*. Histochemical techniques (7) were also employed for the detection of arginine-rich compounds and carbohydrate- and lipid-containing substances in the electrophoretically separated components of PMN granules. The strips were stained with a Sakaguchi method that revealed the presence of high arginine content in bands I, II, and III. The electrophoretic strip was found to be periodic acid-Schiff and Sudan black negative except at the point of application, indicating that under these conditions the compounds containing carbohydrate and lipid remained at the origin.

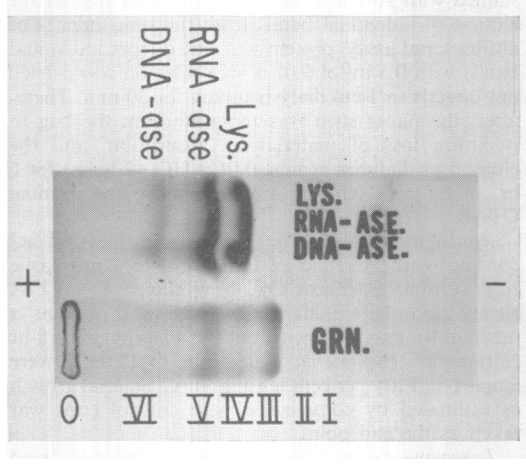


FIG. 2. *Comparative pherogram of samples of purified commercial lysozyme (Lys.), ribonuclease (RNA-ase), deoxyribonuclease (DNA-ase), and PMN lysosomes (GRN.). Antibacterial bands: I, II, and III. Lysozyme: IV. RNA-ase: V. DNA-ase:VI.*

*Comparison of pherograms of granular, nuclear and post granular supernatant fractions*. Samples from the supernatant fraction and the nuclear fraction of PMN leukocytes were compared electrophoretically with granule fractions. The results (Fig. 3a, 3b) revealed marked differences in the electrophoretic patterns of the different fractions. The nuclear fraction did not seem to resolve into definite bands, and the bulk of the material stayed at the point of application. In this preparation, care was taken to remove the contaminating large granules by repeated washings of the fraction with 0.25 M sucrose.

In the supernatant fraction, some pattern was discernible, although quite different from the

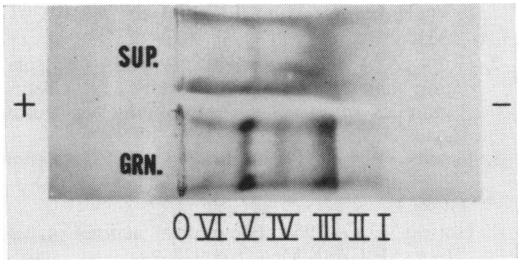


FIG. 3a. *Comparative pherogram of supernatant fraction after centrifugation at 8,000 g × (SUP.) and PMN lysosomes (GRN.). Antibacterial bands: I, II, and III. Lysozyme: IV. RNA-ase: V. DNA-ase: VI.*

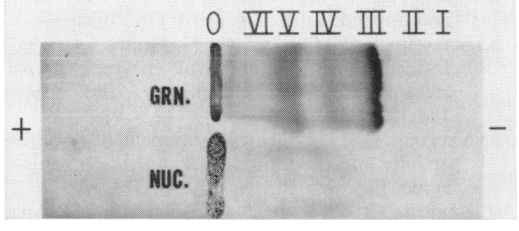


FIG 3b. *Comparative pherogram of nuclear fraction (NUC.) and PMN lysosomes (GRN.) Antibacterial bands: I, II, and III. Lsyozyme: IV. RNA-ase: V. DNA-ase: VI.*

granule fraction. Bands corresponding to I, II, and III of intact granules were absent in the pherogram of the supernatant fraction. However, some smudged, streaked material was visible at the level of bands I, II, and III of the granule fraction. Another band corresponding to the ribonuclease band of intact granules was visible in the supernatant fraction. These substances either could have been derived from some ruptured granules during the process of fractionation or they may have represented components originating from ribosomes of PMN cytoplasm. However, a consistently reproducible electrophoretic pattern of PMN granules quite different from that of nuclear and supernatant fractions showed that the components visible on the granule pherogram seemed to have their origin in PMN granules.

The fact that the granule fraction could be directly subjected to electrophoresis was a definite advantage over extraction procedures whereby some important components might escape analysis or be altered.

## DISCUSSION

The use of paper electrophoresis in the separation of cationic proteins has been severely limited due to the avidity with which cationic proteins are adsorbed to materials such as cellulose acetate. The paper acquires a negative charge when in contact with buffer solutions, thereby leading to adsorption of the positively charged proteins to the paper (12). Under these conditions severe streaking has occurred with the result that the resolution of such protein mixtures has been unsatisfactory. To minimize the electrostatic adsorption of cationic proteins to paper, attempts have been made to esterify the carboxyl groups of the paper with diazomethane (12), or to block them with detergents such as sodium cetyl-sulfonate and Tween 20 (3). By these methods, a positively charged paper is produced on which cationic proteins can move below their isoelectric points with little or no adsorption. Proteins bearing net negative charge are adsorbed on such modified paper.

In our studies, pretreatment of the paper with the cationic detergent CTAB made possible the determination of mobilities of lysosomal cationic proteins and the use of these mobilities as an analytical tool. The residual CTAB on the paper and the acidity of the buffer helped in immediate lysis of applied granules. This offered the advantage of direct electrophoretic analysis of the subcellular components of PMN. The pherograms were consistently reproducible. The detergent-treated paper did not seem to exert a detrimental effect on the biological activities of the protein components. In this respect, the short running time of 1 hr required for electrophoresis was undoubtedly advantageous.

The electrophoretic pattern showed that basic proteins possessing diverse biological activities resolved under these conditions. The granule components resolved into multiple bands of which I, II, and III were found to be antibacterial, and bands IV, V, and VI accounted for lysozyme, ribonuclease, and deoxyribonuclease activities, respectively. The mobilities toward the cathode seemed to be directly proportional to the isoelectric points of the known proteins.

The antibacterial activity of PMN granules was demonstrable only in the fastest-moving bands, I, II, and III. At *p*H 4, this fast mobility did not necessarily mean that proteins in these bands were basic, but the fact that they moved faster than lysozyme pointed to the possibility of their having isoelectric points above 11.0. The histochemical detection of high arginine content in these bands was yet another indication of the basicity. Further evidence of the strongly cationic nature of the proteins in bands I, II, and III of PMN lysosomes is discussed in the following paper.

Nuclear basic proteins like histones and protamines are known to have antibacterial effects,

and PMN leukocyte nuclei, like all mammalian cells, contain histones. The possibility of nuclear contamination of the granular fraction (8,000 $\times$ *g*) was minimized by careful homogenization of leukocytes in hypertonic sucrose solution in the cold, followed by repeated sucrose washing of the fraction. Consistently reproducible differences in pherograms of granules compared with those of nuclear fraction showed that the components essentially originated from PMN granules. A qualitatively identical electrophoretic pattern given by PMN lysosomes of peripheral blood would indicate that the antibacterial proteins do not arise from the inflammatory conditions produced during the induction of aseptic peritoneal exudation.

A comparative study, with the help of this technique, of basic protein composition of PMN lysosomes may provide a useful area for the investigation of species- and disease-associated variability of resistance against infection.

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