Cationic Protein-Bearing Granules of Polymorphonuclear Leukocytes: Separation from Enzyme-Rich Granules

Abstract. *The cationic, antibacterial proteins of polymorphonuclear leukocytes are associated with a unique subcellular particle that is separable through zonal density gradient centrifugation from acid phosphatase–containing particles as well as from particles that contain alkaline phosphatase and lysozyme. Normal macrophages, macrophages stimulated by bacillus Calmette-Guérin, and liver cells lack this particle and the associated group of cationic proteins. Particles physically and biochemically similar to slower sedimenting enzyme-rich particles of polymorphonuclear leukocytes are shared by all the three cell types.*

The cytoplasmic granules of polymorphonuclear leukocytes are membrane-bound structures whose ultrastructural heterogeneity has been amply demonstrated by Florey and Grant (*1*). In polymorphonuclear leukocytes the cytoplasmic organelles, which Ehrlich called "specific granules," stain intensely with acidic dyes such as eosin and Fast Green at *p*H 8.1 (*2*), an indication that they contain strongly basic compounds. The subcellular particulate fraction of polymorphonuclear leukocytes has been shown by Cohn and Hirsch (*3*) to contain a number of hydrolytic enzymes, thus bringing the particles into the category of lysosomes (*4*). In addition to the enzymes, we have isolated from the polymorphonuclear granules a unique group of highly basic, apparently nonenzymatic proteins (*5*) that possess a number of biological activities (*6*). The cationic proteins of granular fractions constitute almost 50 percent of the soluble lysosomal proteins and have recently been separated into five to six components that individually show specific antibacterial action against various microorganisms (*7*). The protein components are highly arginine-rich, contain a high content of cystine, and differ from one another in their amino acid composition. Electrophoretically the six cationic components migrate toward the cathode ahead of lysozyme and ribonuclease.

The question that may be asked now is whether the group of antibacterial, arginine-rich basic proteins and the battery of lysosomal enzymes are packaged together in polymorphonuclear granules or whether they are associated with different subcellular particles. Cytochemical evidence for the existence of enzyme heterogeneity, in addition to the morphological heterogeneity among polymorphonuclear granules, has been presented by Wetzel *et al.* and by Bainton and Farquhar (*8*). This evidence has been further confirmed and extended in a very recent ultrastructural and histochemical study carried out by Bainton and Farquhar (*9*). However, the problem of localization of highly basic, apparently nonenzymatic, antibacterial proteins among polymorphonuclear granules remained to be resolved (*8, 9*). Recently Rahman *et al.* (*10*) have demonstrated the existence of biochemical heterogeneity among subcellular particles of rat liver cells, by cell fractionation methods. By a similar method, we have examined the distribution of the cationic proteins in the polymorphonuclear subcellular particles to see if these proteins are segregated in particles other than those containing enzymes, and if so, whether comparable particles exist in another phagocytic cell system (macrophages) and a nonphagocytic cell system (liver cells). This knowledge is of crucial importance in understanding the functional role of subcellular particles in determining the biological activity of the cell type.

We have subjected the subcellular particles of polymorphonuclear leukocytes to zonal sucrose gradient centrifugation and have compared under identical conditions the distribution of subcellular particles from lung macrophages, both unstimulated and stimulated by bacillus Calmette-Guérin (BCG), as well as liver cells.

This report presents evidence that the six cationic protein components of polymorphonuclear granules are exclusively associated with a particle that is absent from macrophages and liver cells. Most of the lysosomal enzymes of polymorphonuclear leukocytes are associated with a less rapidly sedimentable set of particles that are common to all the three cell types.

Polymorphonuclear leukocytes were obtained from rabbit peritoneal exudates induced by 0.5 percent glycogen in saline. Rabbit lung macrophages, both normal unstimulated and stimulated (by 4 weeks prior intravenous injection of BCG), were obtained according to the methods of Myrvik (*11*). A cellular suspension of rabbit liver was obtained by light homogenization of small pieces of liver in 0.34*M* sucrose. After removal of the connective tissue the cell suspension was washed twice with 0.34*M* sucrose before final disruption.

Cells were homogenized in 5 ml of 0.34*M* sucrose in a glass tube with a Teflon pestle. To prevent aggregation

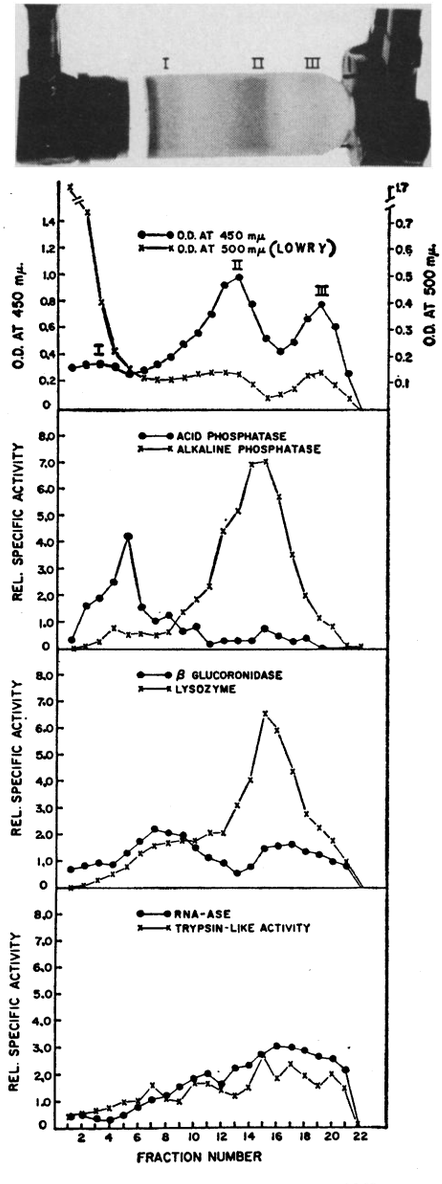

Fig. 1. (Top right) Sucrose density gradient centrifugation of post-400*g* supernatant of polymorphonuclear leukocytes. The gradient was centrifuged at 13,000 rev/min for 30 minutes. (Bottom right) Distribution of enzyme activities in relation to the gradient bands. In the top graph, "O.D. at 450 mμ" refers to polymorphonuclear lysosomal bands.

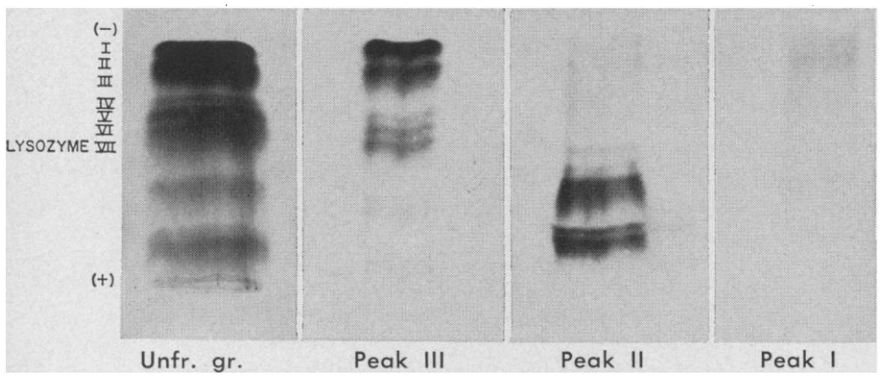

Fig. 2. Cellulose acetate electrophoresis of fractions from peaks I, II, and III of density gradient tube as shown in Fig. 1. *Unfr. gr.*, unfractionated granules obtained by differential centrifugation. Cathode at top. The six most cationic components are principally associated with peak III particles.

of the subcellular particles, homogenization was carried out for 1 to 1½ minutes and interrupted every 30 seconds without an attempt to disrupt 100 percent of the cells. All of the operations were carried out at ice-cold temperatures.

Homogenates were centrifuged at 400*g* in an H.S. head in a Servall centrifuge for 30 minutes to remove the nuclei and cell debris. At the end of centrifugation the supernate was carefully removed, and a 2-ml sample was immediately layered over the sucrose gradient. The sucrose density gradient was prepared just prior to use.

A 50-ml volume of linear density gradient of 20 to 50 percent sucrose (by weight) was prepared through a mixing chamber (triple outlet gradient mixer, Buchler Instruments, New Jersey) with the help of a polystaltic pump (Buchler Instruments). Two milliliters of the lightly centrifuged homogenate (post-400*g* supernatant) was layered over the gradient. Centrifugation was carried out in an S25.2 rotor in a Spinco model L 2-65B refrigerated centrifuge, fitted with rotor stabilizer, at 13,000 rev/min for 30 minutes, allowing an extra 10 minutes for acceleration and deceleration. After centrifugation, 2.5-ml fractions were collected by upward displacement of the gradient with 65 percent sucrose.

All fractions were assayed for acid phosphatase, alkaline phosphatase, ribonuclease, β-glucuronidase, and lysozyme activities according to the methods described elsewhere (*5*). Trypsin-like activity was measured according to a modified method of Nelson *et al.* (*12*) by using 5 mg of Congocoll (Calbiochem) as substrate and employing an incubation time of 3 hours. Fifty percent of enzyme activity was found to be latent in the granule fractions when detergent was used to completely break down the granule membrane. The optical density of the gradient bands was measured turbidimetrically at 450 mμ. Protein was estimated by the Lowry method (*13*). Paper electrophoresis of intact lysosomal particles was performed according to the technique described elsewhere (*5*).

The results of sucrose density gradient centrifugation are shown in Fig. 1. The polymorphonuclear granules in the post-400*g* supernatant separated into three dense bands. The pattern was consistently reproducible. Peak I was the lightest band, whereas peak III constituted the heaviest band. The soluble protein and microsomes stayed in tube 1, whose pink color was a good marker for soluble hemoglobin and and other proteins. As shown in Fig. 1, the distribution of various enzyme peaks showed important differences. Acid phosphatase activity was mostly associated with lightest particles (peak I), with little activity at the level of peak II and no activity at peak III. On the other hand, alkaline phosphatase was mostly associated with peak II. Similarly, lysozyme activity was mainly attributable to peak II. However, the pattern of β-glucuronidase activity was different from the other enzymes in that it showed two peaks, one at the level of the ascending limb of peak III and another associated with particles in the ascending limb of peak II. Ribonuclease and trypsin-like activity were associated with peak II and peak III.

The specific cytoplasmic granules of rabbit polymorphonuclear leukocytes stain intensely eosinophilic with Wright stain. When samples from peaks I, II, and III were stained with Wright stain, peak III contained the typically spherical, intensely eosinophilic granules. The particles from peak I were stained as azurophilic amorphous material. The particles from peak II were stained light pink and were discrete, round, and flat in shape. The granular nature of material in peaks I, II, and III and variation in their sizes were further confirmed by electron microscopy.

Electrophoretic analysis on cellulose acetate of samples from peaks I, II, and III showed (Fig. 2) that the six most cationic components were exclusively associated with peak III particles. The absence from peak III pherogram of band VII, previously identified as lysozyme, correlated with the absence of that enzyme from peak III. Peak II contained the electrophoretically slower enzymic components. These components appeared only as traces in peak III. The presence of cationic components in peak III correlated with the intense eosinophilic staining of the particles.

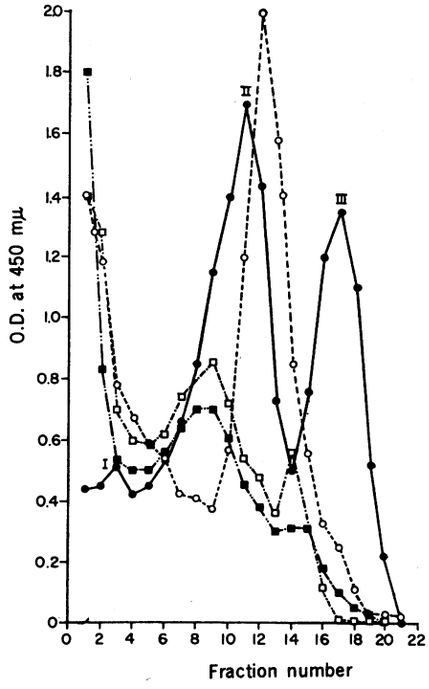

Fig. 3. Sucrose density gradient centrifugation of lysosomes; comparative distribution patterns of lysosomal particles of polymorphonuclear leukocytes, normal lung macrophages, BCG-stimulated lung macrophages, and liver cells. Particles comparable to peak III of polymorphonuclear leukocytes are absent from macrophages and liver cells. Peaks physically and biochemically similar to peak II of polymorphonuclear leukocytes exist in the other three cell types. ●——●, polymorphonuclear leukocytes; □-•-•-□, unstimulated lung macrophages; ■-••-■, BCG-stimulated macrophages; ○- - -○, liver.

In view of the physical as well as biochemical heterogeneity of the subcellular particles of polymorphonuclear leukocytes, we examined other cells under identical conditions to compare the distributions of particles and the associated enzyme activities. As shown in Fig. 3, the particles comparable to peak III particles of polymorphonuclear leukocytes were entirely absent from macrophages and liver cells. However, peaks physically comparable to peak II of the polymorphonuclear particles were found in both macrophage and liver cell homogenates. The unstimulated normal lung macrophages and BCG-stimulated macrophages showed practically similar distribution patterns. Unlike the polymorphonuclear leukocytes where acid phosphatase appeared exclusively in peak I, the acid phosphatase in macrophages and liver was equally associated with both of the lighter peaks, peaks I and II. Most of the lysosomal enzymes in macrophages and liver cells were associated with the major peak II. Nevertheless the distribution of enzymes in the major peak of these cells was not uniform and indicated the presence of further heterogeneity. In addition to the differences in the distribution of enzymes between subcellular particles of polymorphonuclear leukocytes and the other three cells there were also quantitative differences. Alkaline phosphatase activity of polymorphonuclear leukocytes was 20 times higher than that of macrophages and liver lysosomes. Peroxidase and trypsin-like activities were negligible in normal macrophages as well as BCG-stimulated macrophages, although trypsin-like activity was present in liver lysosomes. On the other hand, catheptic activity, compared to polymorphonuclear leukocyte, was higher in macrophage lysosomes.

It is concluded that the six cationic proteins of polymorphonuclear granular fractions are exclusively confined to a group of unique, heavy, intensely eosinophilic particles that are poor in enzymes, such as acid phosphatase, alkaline phosphatase, and lysozyme, and are evidently absent from normal macrophages, BCG-stimulated macrophages, and liver cells. Concomitant with the absence of these particles, the group of six cationic components is not found in the cytoplasmic organelles of these three cell types.

Note added in proof: Since this manuscript was submitted, we have had the privilege of reading the manuscript of M. Baggiolini, J. G. Hirsch, and C. de Duve (*J. Cell. Biol.*, in press). Their work on the distribution of enzymes in polymorphonuclear leukocyte granules was in part known to us during our investigation of the localization of cationic proteins in granules.

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Hemophilia A: Polymorphism Detectable by a Factor VIII Antibody

Abstract. *Plasma from 54 patients with hemophilia A was tested for neutralizing activity with a human antibody to factor VIII. The plasma from 52 patients had no demonstrable neutralizing activity. Two plasma samples had neutralizing activity equivalent to that of normal plasma despite the lack of factor VIII clotting activity. Apparently, most patients with hemophilia A do not synthesize factor VIII, whereas a few synthesize an inactive molecule with a presumed genetic structural mutation of the active site but with antigenic determinants in common with normal factor VIII. Thus, hemophilia A is a disease caused by more than a single genetic mechanism.*

Hemophilia A is considered a hereditary defect resulting in the decreased synthesis of factor VIII. If a molecule with similar antigenic determinants but without an active site were present in hemophilic plasma, an antibody to factor VIII might combine with a "defective molecule" and, therefore, antibody neutralization studies could be used to detect the presence or absence of such a molecule. A relatively small number of patients with hemophilia A have been so tested with variable results (*1*). We have studied a large population of hemophiliacs and report results indicating that a few patients with hemophilia A have antibody-neutralizing activity in their plasma.

Plasma from hemophiliacs was prepared by high-speed centifugation of nine volumes of venous blood added to one volume of a balanced citrate anticoagulant. Plastic syringes and tubes were used. Serum from a patient with a spontaneous factor VIII inhibitor, which had been characterized as an IgG immunoglobulin (*2*), was heated for 30 minutes at 56°C and then absorbed twice with a 25 percent $Al(OH)_3$ gel. The absorbed serum was then saturated with 40 percent $(NH_4)_2 SO_4$, and the precipitate was isolated by high-speed centrifugation and redissolved in normal saline. The inhibitor was further purified by zone electrophoresis on starch and stored at −20°C. The concentration of factor VIII in plasma of patients with hemophilia A was determined by the two-stage method of Pool and Robinson (*3*). Residual factor VIII in the second incubation mixture of the antibody-neutralization test was measured by a one-stage factor VIII assay based upon the partial thromboplastin time (*4*).

Samples were tested for material that neutralized factor VIII antibody as follows. The antibody was diluted in normal plasma to a point (1:7) where all of the antibody was neutralized when incubated at room temperature for 90 minutes. When the antibody was similarly diluted in buffer or in a single prototype hemophilia A plasma, there