PROTEIN AND GLYCOPROTEIN ELECTROPHORETIC PATTERNs OF ENRICHED FRACTIONS OF PRIMARY AND SECONDARY GRANULES FROM GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

The postnuclear supernatant fraction of sucrose homogenates of guinea pig polymorphonuclear leukocytes (PMNL) was subjected to differential centrifugation to obtain a total particulate fraction, a particle-free supernatant fraction, highly enriched fractions of primary and secondary granules, and a membrane-rich fraction. The various fractions were solubilized in buffer containing sodium dodecyl sulfate (SDS) and analyzed for protein and glycoprotein components by SDS-polyacrylamide gel electrophoresis.

The major glycoprotein components of the postnuclear supernatant fraction were found mainly associated with the enriched fraction of secondary granules and, to a lesser extent, with the membrane-rich fraction. No major glycoprotein components were visible in the polypeptide electrophoretic patterns of the primary granule fraction or of the particle-free cell supernate.

Attempts at separation of guinea pig granules by zonal sucrose density gradient centrifugation were only partially successful. Data supporting a species difference in this regard between rabbit and guinea pig PMNL granules are presented.

Recent studies have demonstrated a striking increase in the glycoproteins of granulocytes during maturation in the bone marrow (19, 20). Since the sequential formation of primary (or azurophilic) and secondary (or specific) granules is a major morphologic feature of this maturation process (1, 5, 6), we wondered whether the differences between glycoprotein patterns of mature and immature granulocytes were associated with the changing granular components of these cells. Although much progress has been made in elucidating the morphology and enzymatic composition of the granules from granulocytes (3, 4, 9, 24), little is currently known about the subcellular localization of glycoproteins in these cells.

In the present study, we have attempted to characterize the glycoproteins of various subcellular components of guinea pig exudate polymorphonuclear leukocytes (PMNL) after isolation of such components from sucrose homogenates by the technique of differential centrifugation. We present data that strongly suggest that the major glycoproteins in the postnuclear supernatant fraction of mature granulocytes are associated chiefly with the
granules or in the soluble fraction of PMNL. In zonal density gradient centrifugation, between the pattern of distribution of guinea pig PMNL and that of rabbit PMNL granules after addition, we have found a species difference in glycoproteins could be detected in primary membrane fraction of the cell. No major secondary granules and to a lesser extent with the membrane fraction of the cell. No major glycoproteins could be detected in primary granules or in the soluble fraction of PMNL. In addition, we have found a species difference between the pattern of distribution of guinea pig PMNL and that of rabbit PMNL granules after zonal density gradient centrifugation.

MATERIALS AND METHODS

Guinea Pig and Rabbit PMNL

Suspended PMNL were prepared from peritoneal exudates of male, strain 13 guinea pigs (400-500 g) and male, New Zealand white rabbits (4.0-4.5 kg) elicited by injecting 50 ml/kg animal weight of a sterile solution of sodium caseinate (Difco Laboratories, Detroit, Mich. 0.5% wt/vol in 0.9% sodium chloride). The animals were sacrificed 10 h postinjection by CO₂ narcosis and, after opening the peritoneal cavities, the exudate cells were collected by washing the cavities with iced NaKP (0.15 M NaCl, 0.005 M KCl, 0.01 M sodium phosphate buffer, pH 7.4). The cells were collected and washed twice with 50-ml volumes of iced NaKP by gentle suspension and low-speed (500 g) centrifugation. Contaminating erythrocytes were eliminated by hypotonic lysis (14). The granulocyte suspension was filtered through two layers of silk, washed twice with iced NaKP, and resuspended in the same medium. Cell counts of stained smears were performed as reported elsewhere (12). Such preparations routinely consisted of 92-95% PMNL. The balance of the cells were mononuclear (tissue macrophages and lymphocytes) with less than 1% eosinophils.

Zonal Density Gradient Centrifugation

Since excellent separations of rabbit and human PMNL granules have been achieved using zonal sucrose density gradient centrifugation (3, 4, 24, 29) we thought that this technique might allow us to obtain relatively pure granular fractions from guinea pig PMNL. In an effort to conform to the methods used by Baggiolini et al. (3) for separating granules from rabbit PMNL, we prepared granular suspensions using the sucrose lysis technique of Cohn and Hirsch (9). We found, as have others (7, 26), that for guinea pig PMNL it was necessary to add a fixed concentration of sodium heparin (0.5 mg/ml) to the cellular suspensions (6-8 × 10⁶ exudate cells/ml). Essentially complete lysis of the cells (as observed by phase microscopy) and the formation of a highly viscous suspension was achieved in this way. The high viscosity was dramatically reduced by manually forcing the suspension through a column of glass beads (A. S. Aloe, Co., St. Louis, Mo., average diameter 0.2 mm) supported on a porous polyethylene disk in a 20-ml plastic syringe after prewetting the glass bead column with 0.34 M sucrose. A ratio of 1 ml of packed glass beads/ml of lysate was routinely used. After collecting the effluent, the column was washed with an aliquot of 0.34 M sucrose equal in volume to the starting lysate, and the effluent and wash were combined. This procedure resulted in removal of 95-99% of the starting lysate DNA (unpublished data) with recovery of 50-90% of the initial lysate granular enzyme activity.

All zonal sedimentations were carried out at 4°C, using the Sorvall SZ-14 Reorienting Density Gradient Zonal Rotor driven by the Sorvall RC2-B centrifuge fitted with the Sorvall Manual Rate Controller (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Gradients were prepared either according to the procedures of Cline and Ryel (8) or as described by Baggiolini et al (3).

Differential Centrifugation

For differential centrifugation, a postnuclear granular suspension (PNGS) was prepared from a homogenate of the PMNL suspension in 0.34 M sucrose. A 30-50% (vol/vol) suspension of the exudate cells was mixed with 0.5% wt/vol sodium heparin in 0.9% NaCl, 0.005 M KCl, 0.01 M sodium phosphate buffer, pH 7.4. The suspension was then centrifuged at 1,000 rpm for 10 min (International centrifuge model PR-2, Damon/IEC Div., Damon Corp., Needham Heights, Mass.) and the supernate carefully decanted and reserved. The pellet was then resuspended in the starting volume of 0.34 M sucrose with 0.045% (vol/vol) NaCl and rehomogenized as above. It was found that the additional NaCl was needed to prevent swelling of the nuclear pellet and to allow clean separation of the pellet from the supernate. A second 1,000-rpm supernate was collected and combined with the original. The pellet was then resuspended in 0.34 M sucrose by pipetting and was recentrifuged as above. This third supernate was combined with the first and second, diluted to 10 ml total volume with 0.34 M sucrose, and cleared of nuclear debris and chromatin strands by two sequential centrifugations as above. The small pellets yielded by these latter two steps were resuspended in 1.0 ml 0.34 M sucrose and centrifuged at 1,000 rpm for 5 min in order to wash free adherent granules. This final supernate was combined with the other, and the volume of the combined supernates was adjusted with 0.34 M sucrose to give a final concentration of granular material equivalent to 1 × 10⁶ exudate cells/ml.

Phase microscope observations of the supernates and pellets at each stage of the above sequence revealed the following: (a) two sequential 100-stroke homogenizations resulted in 95-98% cell breakage; (b) washing the nuclear pellet recovered significant numbers of granules; (c) sequential 1,000 rpm × 10 min centrifugations cleared the granular suspension of virtually all nuclear fragments and chromatin strands; and (d) the final granular
suspension was well dispersed and free of visible granular aggregation.

Five subcellular fractions were collected from the PNGS as outlined in Fig. 1.

Pellets for all analyses were routinely prepared simultaneously and were washed once in 0.34 M sucrose before extraction or fixation.

**Enzyme Assays**

All fractions were suspended in 0.5% cetyltrimethylammonium bromide (CETAB) in 0.01 M potassium phosphate buffer, pH 7.0, before enzyme assay. Myeloperoxidase (MYPO) was assayed by the guaiacol method (18), using O-methoxyphenol (MC & B Manufacturing Chemists, Norwood, Ohio) as substrate. Alkaline phosphatase (AP) assays were carried out using p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo., 104 phosphatase substrate) as described by Rosenblum et al. (25). Activities were expressed as enzyme units per milliliter of starting granular suspension. The extraction volume for each pellet was equal to the original volume of PNGS from which the fraction was derived.

**Polyacrylamide Gel Electrophoresis**

Pellets from the differential centrifugation protocol were prepared for polyacrylamide gel electrophoresis by extraction in 0.1 M sodium phosphate buffer, pH 7.1, containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 1% (vol/vol) 2-mercaptoethanol. Solubilization was achieved by heating at 100°C for 10 min in a boiling water bath with vigorous stirring. Centrifugation of the extracts at 1,000 g for 15 min sometimes yielded traces of undissolved material in the total granule fraction. The presence or absence of this material, however, had no significant effect on the gel electrophoretic patterns obtained with the SDS extract of this fraction. No insoluble material was found in any of the other subcellular fractions after SDS treatment.

These SDS extracts were electrophoresed and stained for protein and glycoprotein as previously described (20), with the following modifications: (a) all extracts were diluted with the extracting medium to make the protein concentration of all extracts identical, or nearly so, so that equal quantities of Lowry protein material could be placed on each gel in equal volumes; (b) electrophoresis was carried out for 4.5 h; and (c) gels stained for...
glycoproteins were not fixed in trichloroacetic acid before staining but were soaked for 48-72 h in two or three changes of 25% (vol/vol) isopropanol and 10% (vol/vol) acetic acid to remove SDS.

**Protein Determination**

The protein concentration of each SDS extract was measured by the technique of Lowry et al. (21). At the dilutions assayed (1:50-1:100 in 0.1 N NaOH), no interference from SDS or 2-mercaptoethanol could be detected.

**Electron Microscopy**

A portion of the peritoneal exudate containing the PMNL was diluted 1:2 in 1.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, and centrifuged at 500g for 10 min. The supernate was discarded and the pellet covered with fresh 1.5% glutaraldehyde in Sorensen's buffer. Cell fractions for electron microscope analysis were collected as shown in Fig. 1. All samples were fixed for 2-4 h in 1.5% glutaraldehyde in 0.1 M Sorensen's buffer. After removal of the glutaraldehyde, the pellets were washed through several changes of cold buffer and subsequently fixed in Dalton's chrome osmium (10) for 1.5 h. After osmication and en bloc staining for 1 h with 0.5% aqueous uranyl acetate, pH 4.9, the samples were dehydrated through an ascending series of ethanol solutions in water, treated with two changes of propylene oxide, infiltrated, and embedded in Epon-Araldite (23).

After polymerization, ultrathin sections were cut on an LKB Ultratome III. These were collected on Formvar-coated copper grids, stained with uranyl acetate in 50% ethanol and lead citrate, and examined in a Siemens Elmiskop 1A. Care was taken to analyze several blocks from the pellets of each of the cellular fractions to minimize the possibility of sample errors due to layering effects resulting from centrifugation.

**RESULTS**

**Comparison of Rabbit and Guinea Pig PMNL Granule Separations by Zonal Sedimentation**

Using zonal sucrose gradient density centrifugation, we were readily able to distinguish two discrete populations of rabbit granules but were never able to resolve the overlapping distributions

![Figure 2](https://example.com/figure2.png)

**Figure 2** Distribution of rabbit and guinea pig MYPO and AP by zonal sucrose density gradient centrifugation. Five-step gradient (15, 25, 35, 45, and 50% wt/vol sucrose, 200 ml/step, with 300 ml 60% wt/vol sucrose cushion) centrifuged at 15,000 rpm for 30 min. 40-ml sample of heparin sucrose lysate (3 x 10⁷ cells/ml) was used for each gradient. Ordinate is enzyme activity in fraction relative to activity corresponding to uniform distribution through the gradient. Radial distance increases from right to left. Enzyme recoveries: rabbit MYPO, 86%; rabbit AP, 107%; guinea pig MYPO, 75%; guinea pig AP, 95%.
TABLE I

Enzyme Ratios of Enriched Primary and Secondary Granular Pellets from Guinea Pig PMNL Homogenates

| Granular Fraction | MYPO EU† | % | AP EU† | % | Ratios* |
|-------------------|----------|---|--------|---|---------|
| Total             | 25.0 (±2.75) | 100 | 8.82 (±1.15) | 100 | 2.8 |
| Primary           | 6.53 (±1.00) | 26 | 0.49 (±0.03) | 5.5 | 13.3 |
| Secondary         | 1.17 (±0.15) | 4.7 | 1.34 (±0.03) | 15 | 0.87 |

*MYPO EU to AP EU.
†Total enzyme units (EU) in 1.0 ml of CETAB extract of a pellet derived from 1.0 ml of granular suspension (PNGS).
All measurements are means ± SEM from three experiments.

of guinea pig granules into two separate populations, despite the use of a wide variety of gradients and sedimentation conditions. Fig. 2 presents the optimal separations of MYPO (primary granule marker enzyme) and AP (secondary granule marker enzyme) which were achieved by zonal centrifugation of both rabbit (Fig. 2 a) and guinea pig (Fig. 2 b) granule suspensions.

Attempts at collecting particulate material from various peak regions of the guinea pig gradients for polyacrylamide gel electrophoresis were uniformly unsatisfactory because of our inability to recover and concentrate, even from pooled peak fractions, sufficient amounts of protein to be visualized after electrophoresis and staining.

Differential Centrifugation of Guinea Pig PMNL Granule Suspensions

Our failure to obtain clear separation of primary and secondary granules by zonal techniques prompted us to try differential centrifugation as a method for generating enriched granule fractions. One previous study indicated that some degree of separation of guinea pig granules could be achieved by this procedure (22). Measurements of MYPO and AP activities of CETAB extracts of the granular pellets obtained by the centrifugation scheme outlined in Fig. 1 showed that considerable relative enrichment of these two granular enzymes was achieved (see Table I). The primary granular pellet had a MYPO to AP ratio of 13.3 whereas that of the secondary granular pellet was less than 1.

It should be borne in mind that considerable sacrifice of recovery has been accepted in order to achieve the degree of relative enrichment reported as indicated by the percentage of total granular pellet enzymes recovered in each of the pellets.

Polyacrylamide Gel Electrophoretic Patterns of Guinea Pig Granular Pellet Extracts

The gel electrophoretic pattern of each granular pellet is shown in Fig. 3. For comparison and completeness we have included the electrophorograms derived from SDS extracts of the "membrane" pellet and the cytosol from the same cells. The pellets from which these extracts were derived were prepared simultaneously with those whose enzyme ratios were presented in the previous section.

The polypeptide electrophoretic patterns of the total particulate fraction (gel A-P) and the secondary granular pellet (gel C-P) are strikingly similar with regard to both the number and positions of bands as well as the intensity of the Coomassie blue staining. The electrophoretic pattern of protein components in primary granule gel (gel B-P), however, showed distinct differences from that in secondary granule gel. In particular, components migrating in region II of the primary granule gel are resolved into one medium and four lightly stained bands, in contrast to the heavily stained pair of bands in region II of the secondary granule gel. Most of the major components in regions III and IV of the primary granule gel differ either in position or in staining intensity from the major components in these regions of the secondary granule gel. In no way should the correspondence between band positions from one granular pellet electrophoretic pattern to another be interpreted at this juncture as identity between protein and/or polypeptide components.

For emphasis, it should be noted that the protein electrophoretic patterns of the membrane (gel D-P) and soluble (gel E-P) fractions are...

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considerably different from any of the granular pellet gel patterns.

Examination of the gels stained for glycoproteins reveals that in this regard more striking differences between the granular pellets exist. It is evident that the primary granule gel (gel B-G) is nearly devoid of periodic acid-Schiff (PAS)-positive bands. On the other hand, essentially all of the glycoprotein bands visible on the gel of the total particulate fraction (gel A-G) are clearly seen on the secondary granule gel (gel C-G), with no additional bands being noted.

The intensely PAS-positive band in region IV of both total and secondary granule gels (gel A-G and C-G, respectively) is absent in the primary granule gel (gel B-G) as well as in the soluble fraction (gel E-G). In addition, the membrane fraction (gel E-G) contains a PAS-positive band migrating in region IV, having an \( R_f \) which is less than that of the region IV band of the secondary granule gel. Together, these two bands may account for the more widely spread region IV band in the total granule gel. The nature of this rapidly migrating PAS-positive material is unknown at present, although similarly migrating material from erythrocytes has been identified as glycolipid (13, 17).

Although certain of the membrane glycoprotein bands, particularly those in regions I and II, appear to have a reasonably close correspondence to total and/or secondary granule gel glycoprotein bands, identity between these bands should not be assumed.

**Electron Microscope Observations**

Each of the pelleted cell fractions was extensively surveyed by electron microscopy to ascertain the relative amount and type of granule present in each pellet. Peritoneal exudate cells fixed by the same method were also examined, and one is shown in Fig. 4 for comparison. Electron micrographs in Figs. 5–8 show cell fractions A (total postnuclear particulate fraction), B (primary or azurophilic granule pellet), C (secondary or specific granule pellet), and D (membrane pellet). Fractions B (Fig. 6) and C (Fig. 7) were relatively homogeneous, consisting primarily of membrane-
enclosed granules. The major difference in appearance of these populations was in the average granule diameter. The range was 0.25–0.90 mm in B and 0.12–0.40 mm in C. In both fractions the granules exhibited variability in staining intensity. Elongate rather than circular forms were common in both populations; however, the elongate form was more often observed in the secondary granule pellet. Fractions A and D were heterogeneous, fraction A much more so than D. The granule morphology in fraction A is shown in Fig. 5. All of the components represented in fractions B, C, and D were present in this fraction which was examined in order to control any possible morphologic changes resulting from the manipulation of the samples during homogenization, sedimentation, and preservation. Fraction D (Fig. 8), the "membrane fraction," consisted almost wholly of membranous vesicles of varying diameter, and both free and membrane-associated ribosomes.

DISCUSSION

Using MYPO as a primary granule marker enzyme and AP as the corresponding secondary granule marker, we have evolved a method for generating, by differential centrifugation of guinea pig PMNL PNGSs, pellets of granules which are selectively enriched in one or the other marker enzymes. We have found that there are striking differences in the glycoprotein electrophoretic patterns of the two granule populations. Our data
suggest that most of the major glycoprotein components of the postnuclear supernatant fraction of PMNL are associated with secondary granules. No major glycoprotein components could be detected in the enriched primary granule fraction. In the light of previous findings reported from this laboratory documenting the presence of more glycoproteins (20) and greater glucosamine incorporation (19) in mature than in immature granulocytes from guinea pig bone marrow, we are tempted to interpret these glycoprotein differences from the viewpoint of PMNL maturation. It is well documented that primary granules appear in the granulocyte before secondary granules and, in fact, probably decrease in number relative to secondary granules during cell maturation (5). Our present data are, therefore, consistent with the view that the preponderance of the glycoprotein synthetic activity in the later stages of cell maturation is associated with the synthesis of secondary granules.

Results of our extensive, early attempts at zonal sucrose density gradient centrifugal separation of guinea pig PMNL granular suspensions corresponded poorly to the excellent separations which can be readily achieved from rabbit PMNL granular suspensions. It may be that this failure to resolve two distinct populations of guinea pig granules by this technique reflects an inherent species difference between rabbit and guinea pig. The morphologic similarities between the intact cells of the two species at both the light and electron microscope levels is well documented (27) but one previous study of the differential centrifugal distribution of guinea pig granular enzymes (22) strongly suggests a considerable degree of granular size heterogeneity in this species. Other investigators have suggested that a more complex array of granules may exist in the guinea pig, perhaps related to the degree of maturity of the cell (15, 28). It may well be that the overlap between MYPO and AP so consistently seen with our gradient experiments reflects a considerable similarity in sizes and/or densities of guinea pig granular particles with quite different enzymatic contents. Although it is becoming apparent that the rabbit PMNL contains certainly two, probably three, and perhaps four distinct granule types (4), the exploitable size-density differences between granules of the rabbit PMNL may be greater than those of the guinea pig.

In cells such as liver and kidney many of the hydrolases of lysosomes have been shown to be glycoproteins (16). The role that glycoproteins play in secondary granular function is still undefined, however. There is recent evidence that secondary granules within intact human PMNL may be subject to degranulating stimuli which have little or no effect on primary granules (11). On the other hand, Avila and Convit (2) have reported that secondary granules in cytoplasmic extracts from human PMNL fail to be activated by compounds which trigger release of the lysosomal enzymes of primary granules. Conceivably, the glycoprotein differences which we have demonstrated are involved in determining this type of selective granular function.

Whatever may be the role of these granular differences, we believe that this technique of primary and secondary granular fraction enrichment may provide a useful tool for further elucidating structural and functional differences.
between the granules of mature and maturing granulocytes.

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