DEMONSTRATION OF RICIN-BINDING SITES ON THE OUTER FACE OF AZUROPHIL AND SPECIFIC GRANULES OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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The polymorphonuclear leukocytes (PMNs) of the rabbit contain two types of granules, the azurophils and the specifics, which can be easily differentiated by electron microscopy because of differences in size and electron density. The two types of granules arise from opposite faces of the Golgi apparatus (1) during successive stages of PMN maturation (1, 2). The azurophils contain myeloperoxidase, lysozyme, and a wide variety of lysosomal hydrolases, while the specifics contain alkaline phosphatase, lysozyme, and lactoferrin, but are devoid of lysosomal hydrolases (3-5).

Membranes derived from both kinds of granules differ in their protein and lipid composition (6, 7). As part of a continuing investigation of the properties of granule and plasma membranes of these cells, we are attempting to localize the saccharide residues of membrane glycoproteins and glycolipids by using ferritin conjugates of plant agglutinins whose binding can be monitored by electron microscopy (8). In this paper, we report the binding of ferritin-linked ricin to the outer face of the membrane of isolated azurophil and specific granules of rabbit PMNs. This suggests, unexpectedly, that in the granule membrane carbohydrate residues face the cytoplasm.

MATERIALS AND METHODS

Cell Fractionation

Rabbit PMNs were obtained from glycogen-induced peritoneal exudates. The cells were homogenized, and a postnuclear (4,000 g-min) supernate was prepared as described previously (3). Granules were obtained by fractionating 2.5-ml aliquots of the postnuclear supernates through discontinuous sucrose gradients consisting of three layers (8.0, 5.5, and 4.0 ml) with densities 1.08, 1.16, and 1.32 g/ml, respectively, in 25-ml tubes. Centrifugation was performed at 29,000 rpm for 2 h at 8°C in a swing-out rotor (MSE no. 59590) operated by an MSE SS-65 ultracentrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex, England). The granules, which accumulated at the interface of the lower two layers, were collected by aspiration in about 1.0 ml, and diluted with 0.25 M sucrose to a protein concentration of 1 mg/ml.

Incubation of Granules with Ricin-Ferritin

Ricin-ferritin conjugates (RF) were prepared, within 10 days of use, according to a modification (9) of the method of de Petris and Raff (10), and stored in 0.1 M sodium cacodylate buffer (pH 7.4). Immediately before use, RF clumps were eliminated by centrifuging the preparation at 12,000 rpm for 5 min in a Beckman microfuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The incubation (see details in Results) was started by adding 20, 50, or 100 #l of the RF solution (0.5 mg/ml; molar ratio, ricin to ferritin, 4:6) to 250 #l of granule suspension in approximately 0.25 M sucrose, and terminated by fixation with 1.5 ml of an isotonic (340 mosM) solution of 1.5% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C. The fixed suspension was processed for microscopy according to the filtration procedure of Baudhuin et al. (11) which assures random sampling. Refixation in osmium tetroxide, en bloc staining with uranyl acetate, dehydration, and embedding were carried out according to standard techniques (12, 13).

Electron Microscopy

Thin silver sections were stained in the grid with lead citrate for 20-40 min and viewed in a Zeiss EM 9-S electron microscope.
**Morphometrical Analysis**

Volume and surface densities were determined according to the lattice method of Weibel et al. (14). Photomontages of strips of the entire depth of the pellicles, at a magnification of 48,000, were used in order to assess specimen composition and lectin binding on the same micrographs. Ricin binding was quantified by counting ferritin dots within a 20-nm wide band around each membrane profile by means of a ×10 loupe with an internal scale. The 20-nm limit was chosen because of the size of apoferritin, which has been estimated as 18.4 nm (15), and is in agreement with previous binding criteria (8, 9, 16).

**RESULTS AND DISCUSSION**

In preliminary experiments, the optimal ratio of RF to total granule protein was found to be approximately 1:10. Four separate binding experiments were performed under these conditions. Controls were done in the presence of 0.1 M α-lactose, a competitive inhibitor of ricin binding (17). As mentioned in Materials and Methods, sucrose, which does not interfere with ricin binding, was present in every instance. Figs. 1 and 2 show the whole depth of two of the particle pellicles obtained, and details of three different pellicle levels (circles) in which single ferritin molecules can be recognized. In the absence of lactose (Fig. 1 b–d) nearly all profiles of azurophil and specific granules are surrounded by arrays or little clusters of ferritin molecules. Some clusters are not connected with visible particle profiles, but very little RF remains unbound, as indicated by the lack of accumulation at the filter face of the pellicle (bottom of Fig. 1 a). In contrast to the granules, the profiles of the few mitochondria present show little, if any, apposed RF (Fig. 3). In micrographs of this type, ferritin dots were never seen inside any structure including disrupted granules.

In control experiments where granules and RF are exposed to 0.1 M α-lactose immediately before incubation, nearly all granule profiles remain free of attached RF as seen in Fig. 2 b–d, although occasional RF clumps are found in the vicinity of the granule membranes. Here, too, RF clusters which are not in apparent connection with particles are seen. The amount of RF concentrated at the bottom of the pellicle in Fig. 2 a is much more prominent than in Fig. 1 a, thus reflecting a much larger amount of unbound marker. High magnification micrographs of single granules with bound RF are shown in Figs. 3–4.

Two binding experiments that were carried out both in the presence and in the absence of α-lactose were analyzed morphometrically. The results are summarized in Table 1. As expected, the two preparations, which differ only with respect to α-lactose, have very comparable relative volume and surface densities of profiles of the main particles present, and are therefore stereologically equivalent. The main components are the specific granules which account for approximately half of the total particle volume compared to about one-third for the azurophilis. In the absence of α-lactose, an average of 30 ferritin molecules were counted per micrometer of membrane for both types of granules. In the presence of α-lactose, these values were 3.8 and 6.1 for azurophil and specific granules, respectively. By visual comparison of RF binding in Figs. 1 and 2, one would have expected the latter figures to be even lower. Indeed, the morphometric data obtained for the α-lactose control may be largely overestimated due to the packing of considerable amounts of unbound RF around the particles situated at the bottom of the pellicle (Fig. 2 a).

When higher concentrations of RF were used (50 μg RF per 250 μg of total granule protein), the RF binding density of either granule type remained unchanged. Under such conditions, the morphometric analysis was complicated by the comparatively large amounts of free marker adjacent to the granules lying at the bottom of the pellicle. With a lower RF to granule protein ratio (10:250 μg) the binding density was lower, but there was no apparent difference in RF binding to either type of granule. In addition, sizable numbers of granule profiles were free of marker, indicating that the RF concentration was nonsaturating.

The α-lactose-sensitive binding of RF indicates that sugar residues are exposed at the outer surface of azurophil and specific granules of rabbit PMNs. This was an unexpected finding. Since granules fuse with endocytic vacuoles, one would expect the inner rather than the outer surface of the granule membrane to resemble the outer aspect of the PMN plasmalemma which, like other cell surfaces, avidly binds RF (unpublished results). On the other hand, the membrane recognition process, which must precede fusion, may be mediated by specific glycoproteins exposed at the granule surface and therefore available for lectin binding. Similar sites have not yet been demonstrated on the surface of the endocytic vacuoles, i.e. on the cytoplasmic face of the plasma membrane. Experi-
FIGURE 1 Granule preparation incubated with ricin-ferritin (RF). The incubation mixture contained portions of a granule fraction and RF suspension corresponding to 250 and 25 μg of protein, respectively, 0.017 M sodium cacodylate buffer, pH 7.4, and 0.25 M sucrose. Incubation was carried out for 30 min at 4°C and terminated by glutaraldehyde fixation as described. Fig. 1a represents a survey of the whole depth of one pellicle, x 13,100. Fig. 1b–d are higher magnifications (X 48,000) of three pellicle levels, indicated by circles in Fig. 1a, and show the bound RF on the surface of azurophil (A) and specific (S) granules. Note clusters of RF, apparently unrelated to any structure (Fig. 1b, double arrow). Scale bars, 0.5 μm.
FIGURE 2. Preparation identical to that in Fig. 1 except for the presence of 0.1 M α-lactose in the incubation mixture. The four micrographs (a–d) correspond to those in Fig. 1. Note the minimal RF binding to structures in Fig. 2 b–d, the accumulation of unbound RF (arrow) at the bottom of the pellicle in Fig. 2 a, and the presence of free clusters of RF (double arrow, Fig. 2 d). Scale bars, 0.5 μm.
Figure 3  High magnification of a specific granule encrusted with RF particles, and of a mitochondrion (M) without apposed RF. The poor degree of preservation of mitochondria is typical for subcellular preparations from rabbit PMN (see references 3 and 4), × 120,000. Scale bar, 0.1 μm.

Figure 4  High magnification micrograph of two azurophils and one specific granule (left-hand side) showing the details of bound RF. × 120,000. Scale bar, 0.1 μm.

Table 1  Morphometric Analysis

<table>
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<tr>
<th>Addition to granule preparations</th>
<th>Particles identified</th>
<th>Percent of total particles</th>
<th>Vv/Vvr</th>
<th>BAt/BAr</th>
<th>RF,</th>
<th>RF/Bi</th>
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<tr>
<td>RF</td>
<td>Azurophils</td>
<td>18.0</td>
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<td>0.22</td>
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<td></td>
<td>Specifics</td>
<td>69.0</td>
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<td>0.66</td>
<td>3241</td>
<td>31.0</td>
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<td>Mitochondria</td>
<td>2.0</td>
<td>0.04</td>
<td>0.04</td>
<td>—</td>
<td>—</td>
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<tr>
<td>RF + 0.1 M α-lactose</td>
<td>Azurophils</td>
<td>18.0</td>
<td>0.30</td>
<td>0.23</td>
<td>99</td>
<td>3.8</td>
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<tr>
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<tr>
<td></td>
<td>Mitochondria</td>
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<td>0.05</td>
<td>0.07</td>
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</tbody>
</table>

* The relative volume and surface densities (Vv and BAt) of each type of particle are given with respect to the densities of all particles present (Vv and BAt).
† RFn represents the total number of bound RF markers, and Bi is the total membrane length in micrometers determined for both azurophil and specific granules. RFn/Bi is the average RF binding density expressed as the number of bound RF markers per micrometer of membrane. Mitochondrial profiles were not included in the binding calculations because of the extremely small population present in each pellicle.
‡ Unidentifiable profiles accounted for an average of 12% (range 7–15) of the total particles present.
ments are in progress further to characterize membranes involved in the fusion process.

SUMMARY
The presence of carbohydrate residues on the outer surface of PMN granules has been demonstrated by the use of ricin-conjugated ferritin. The binding of the lectin was inhibited by α-lactose. No difference in the binding densities of azurophil or specific granules was observed.

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REFERENCES