FATE OF SURFACE PROTEINS OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES DURING PHAGOCYTOSIS

I. Identification of Surface Proteins

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ABSTRACT

To study the fate of external membrane proteins during phagocytosis, rabbit peritoneal neutrophils were labeled by enzymatic iodination. Iodine was incorporated into at least 13 proteins ranging in size from ~250,000 to 18,000 daltons as judged from autoradiography of gels after SDS-polyacrylamide gel electrophoresis of labeled cells. The major contractile proteins of neutrophils, actin and myosin, were not labeled when intact cells were iodinated but were labeled when homogenates of these cells were iodinated. Nine of the iodinated proteins were released by mild protease treatment of intact cells. A plasma membrane-rich fraction was isolated by density centrifugation. This fraction was enriched at least 10-fold for lactoperoxidase-labeled acid-insoluble proteins. It was enriched to the same extent for the presence of iodinated wheat germ agglutinin that had been bound to the intact cells at 4°C before homogenization. Analysis by SDS-polyacrylamide gel electrophoresis revealed that the proteins of this fraction were predominantly of high molecular weight. However, only 8 of the 13 proteins iodinated on intact cells were found in this fraction. The remaining five were enriched in a dense fraction containing nuclei, intact cells, and membranous vesicles, and may represent a specialized segment of the neutrophil cell surface.

KEY WORDS phagocytosis - polymorphonuclear leukocyte - surface proteins - lactoperoxidase-catalyzed iodination - plasma membrane - cell fractionation - wheat germ agglutinin

Information concerning cell surface architecture and behavior is necessary to understand the role of the cell surface in recognition and interactions between cells. At present, the cell surface is seen as a fluid lipid bilayer interspersed with proteins that either span the bilayer or are located predominantly on one face or the other. Networks composed of spectrin and actin are present on the cytoplasmic face of erythrocyte membranes and may anchor and restrict the lateral mobility of proteins in these membranes (11, 34, 35). In nucleated cells, it has been proposed that microtubules and actin-containing microfilaments control the mobility of intrinsic membrane proteins, be-
cause agents that prevent their polymerization affect the redistribution of membrane-bound lectins and antibodies (7, 10, 33). We have chosen phagocytosis as a model system to study cell-surface structure and modulation during particle-cell interactions. These interactions are more amenable to analysis than cell-cell interactions because the target particle has a defined composition and can be essentially inert. At the same time, we hope to gain insight into a biological process which is the primary defense mechanism against infection and may have more biological relevance than studies on lectin-induced redistribution of membrane components.

Binding of ingestible particles to the surface of phagocytic cells triggers pseudopod formation and subsequent interiorization of 30–40% of the plasma membrane (18, 21, 22, 28, 42). A key question is whether the plasma membrane forming the phagocytic vesicle has the same properties as the remaining cell surface. Tsan and Berlin (38) presented convincing evidence that this may not be the case. They found that the number of carriers for lysine, adenine, and adenosine did not change after extensive phagocytosis and suggested that transport sites are excluded from sites of membrane interiorization. Ukena and Berlin (39) found that this postulated mosaicism could be destroyed by pretreating the cells with colchicine. On the other hand, Werb and Cohn (42) assaying 5'-nucleotidase and Charlampous et al. (5) assaying three plasma membrane marker enzymes were not able to detect differences in the specific activities of phagosomes and plasma membrane.

We have studied the fate upon phagocytosis of external membrane proteins of rabbit peritoneal polymorphonuclear neutrophils specifically labeled by lactoperoxidase-catalyzed iodination. In this paper, we describe the analysis of these labeled peptides and the isolation and characterization of a subcellular fraction enriched for plasma membrane. The companion paper (44) is concerned with the distribution of these proteins after labeled cells have ingested particles. A brief report of this work has appeared elsewhere (43).

MATERIALS AND METHODS

Rabbit Peritoneal Polymorphonuclear Neutrophils (PMN)

New Zealand red female rabbits weighing 6–7 kg were primed three times, once every 10–15 d, by infusing 100–200 ml of 0.1% shellfish glycogen (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) into their peritoneal cavity. After this initial priming, the rabbits were used as cell donors as follows. Rabbits were infused with 200 ml of 0.1% glycogen in saline intraperitoneally. 4 h later, the fluid was recovered by placing a 16-gauge needle, perforated along each side with four small holes, into the peritoneal cavity and collecting the exudate by gravity into an ice-cold flask containing 500 U of heparin. The exudates were filtered through four layers of cheesecloth, centrifuged at 800 rpm for 8 min in a refrigerated International PRJ centrifuge (Damon/IEC Div., Damon Corp., Needham, Mass.), washed twice with ice-cold saline buffered with 0.02 M Na-phosphate pH 7.2 (PBS), and resuspended in Hank’s balanced salt solution (HBSS) buffered with 0.01 M N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES), (Sigma Chemical Co., St. Louis, Mo.), pH 7.3. The exudate cells were >98% PMN and were 100% viable as determined by trypan blue dye exclusion. Exudates which contained >1% erythrocytes were discarded. Cells stored in HBSS overnight at 4°C retained viability but had reduced phagocytic activity. Cells stored in exudate fluid overnight at 4°C and then prepared as above retained viability and phagocytic activity, as well as similar cell surface labeling patterns. All cell surface labeling experiments described below were performed immediately after harvesting the cells, however, to insure maximal cellular integrity.

Lactoperoxidase-Catalyzed Iodination

Rabbit peritoneal PMNs were labeled, with modifications, according to the method of Hubbard and Cohn (16). 5 x 10⁷ cells were incubated with 50 µg lactoperoxidase (Sigma Chemical Co.), 20 mU glucose oxidase (Aspergillus niger Type V, Sigma Chemical Co.), and 125 µCi carrier-free 125I-Na (Amersham Corp., Arlington Heights, Ill.) in 1 ml PBS. The reaction mixture was rolled at 4°C for 20 min and the reaction was terminated by addition of cold 5 x 10⁻⁴ M NaI. The cells were centrifuged at 800 rpm for 8 min at 4°C in the International PRJ and washed three times with ice-cold PBS. Labeled cells used for further experimentation were suspended in ice-cold HBSS at 5 x 10⁶ cells/ml. Samples to be analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were stored as cell pellets containing 5 x 10⁶ cells at −70°C for no more than 2 d. Samples to be assayed for acid-precipitable counts were either stored frozen as pellets, or 0.5–2.0 x 10⁶ cells were spotted on Whatman 3MM filter disks (2.5 cm) (Arthur H. Thomas Co., Philadelphia, Pa.). The filter disks could be stored at −20°C overnight. TCA-insoluble counts were determined on Whatman 3MM filter disks or in test tubes. Samples spotted on filter disks were incubated in 0.6 M TCA, 50 mM NaI for 2 h at 4°C. Approx. 30 disks were washed twice in 500 ml 0.6 M TCA, once in 500 ml acetone (−20°C), dried, and counted. Alternatively, 500 µg of bovine serum albumin (BSA) (crystalline, fraction V, Miles Laboratories Inc., Miles Research Products,
Kontes Co., Vineland, N.J.) for 2,000 rpm. Homogenization consisted of 3–5 1-min sequences of eight up-and-down strokes at 2,400 rpm for 30 min. Radioactivity was measured in a well-type gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with 75% efficiency.

Iodinated Wheat Germ Agglutinin

(WGA) Binding

Wheat germ agglutinin (WGA) was isolated from wheat germ lipase (Sigma Chemical Co.) according to the method of Marchesi (26) by affinity chromatography on ovomucoid-sepharose. The lectin was iodinated according to the procedure of Cuatrecasas (6) using chloramine T. BSA at a final concentration of 500 μg/ml was added to protect the agglutinin. Aliquots were kept frozen at −70°C. The labeled lectin migrated as one species on SDS-PAGE and had a spec act of 5–7 × 10⁶ cpm/μg.

To measure lectin binding, 10⁶ cells were incubated for 30 min at 4°C in a final volume of 0.2 ml PBS containing 200 μg BSA and various concentrations of lectin with or without competitor. At the end of the incubation, the cells were diluted with PBS/BSA, washed with 10 vol of PBS, and the pellets were counted. Alternatively, the cells were collected and washed free of unbound lectin by filtration through 1-μm nylon filters (Millipore Corp., Bedford, Mass.) according to the method of Cuatrecasas (6).

Intact cells were labeled with trace amounts of lectins to tag the cell surface receptors under conditions which Chang et al. (4) showed do not induce receptor redistribution. Over 90% failed to bind to intact cells in the presence of competing molecules (see Results). 10⁶ cells were incubated with 0.3 μg/ml ¹²⁵I-WGA in 10 ml PBS containing 100 μg/ml BSA, at 0°C for 20 min. Agglutination was not observed. The cells were washed twice with PBS/0.1% BSA and processed for membrane isolation. Under these conditions, 75% of the added lectin was bound to the cells.

Subcellular Fractionation of Polymorphonuclear Neutrophils

For homogenization, cells were washed in 0.34 M sucrose containing 500 μg/ml BSA, 10 mM HEPES, pH 7.2, and 2 mM phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co.), a serine esterase inhibitor. They were resuspended in the same medium at a concentration of 7 × 10⁸ cells/ml and disrupted with a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle (Kontes Co., Vineland, N.J.). Homogenization consisted of 3–5 1-min sequences of eight up-and-down strokes at 2,000 rpm.

Cell breakage was monitored by phase-contrast microscopy and was terminated when ~80% of the cells were broken and intact free nuclei were observed. The homogenate was centrifuged at 2,000 rpm for 30 s or 400 rpm for 10 min in an International PRJ centrifuge to remove nuclei and unbroken cells, which interfere with subsequent plasma-membrane isolation. Further homogenization and washing of the low speed pellet to enhance recoveries was omitted because the nuclei are easily broken and subsequently bind plasma membrane. In general, the postnuclear supernate was layered on a discontinuous gradient composed of 1 ml each of 35, 45, and 50% wt/vol of sucrose containing 50 μg/ml BSA and 10 mM HEPES and overlayed with 8.6% sucrose, 10 mM HEPES. The gradients were centrifuged at 100,000 g for 1 h in a SW 50L or 50.1 rotor (Beckman Instruments, Inc., Spino Div., Palo Alto, Calif.). The material at the 35%–45% interface, which was highly enriched for plasma membrane (see Results), was collected, diluted with 5 vol 10 mM HEPES, 2 mM PMSF, and sedimented at 27,000 g for 45 min at 4°C.

SDS-PAGE

The method used for discontinuous polyacrylamide gel electrophoresis was essentially that described by Laemmli (25) and modified for slabs by F. W. Studier, J. Maizel, and others (personal communication). The cell pellets or subcellular fractions were heated at 100°C for 2 min in sample buffer containing 0.0625 M Tris (pH 6.8), 2% SDS (sequanal grade, Pierce Chemical Co., Rockford, Ill.), 10% glycerol, 5% 2-mercaptoethanol, 0.01% phenol red, and 2 mM PMSF. In some cases samples were concentrated under N₂ in the presence of PMSF before solubilization. The composition of the stacking gels, separating gels, and running buffer were as described by Laemmli (25). The acrylamide (Eastman, X5521, Fischer Scientific Co., Pittsburgh, Pa.) concentration of the stacking gel was 4% and the separating gel in most cases was 10%. The gels were electrophoresed at a constant current of 12–14 mA until the phenol red ran off and the SDS front (visible as a schlieren boundary) was ¼ in. from the bottom.

To visualize protein, the gels were fixed and stained with 0.2% Coomasie Blue R250 (Inoalex Corp., Biomedical Div., Glenwood, Ill.) in 50% methanol, 7% acetic acid for 1 h at 37°C. The gels were destained in 5% ethanol for 10 min and then in methanol/water/acetic acid (8:10:1) for 10 min. They were oxidized and stained as described. Ovomucoid was run as a positive standard.

To visualize labeled proteins, gels were dried onto Whatman 3MM filter paper and then permitted to expose Kodak NS-2T x-ray film (Pennsylvania X-Ray, Glenside, Pa.) for 2–8 d.

The following proteins served as molecular weight standards: β-galactosidase (130,000 daltons), phosphorylase a (94,000 daltons) (both from Sigma Chemical Co., Elkhart, Ind.) in water was added as carrier to samples in test tubes, and the samples were precipitated and washed with 3 ml each of 0.6 M TCA. The precipitates were pelleted by centrifugation at 2,400 rpm for 30 min. Radioactivity was measured in a well-type gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with 75% efficiency.
Co.), chymotrypsinogen (23,000 daltons), myoglobin (17,000 daltons) (both from Schwarz/Mann), BSA (crystalline fraction V, 67,000 daltons) (Miles Laboratories), myosin (200,000 daltons), and actin (45,000 daltons). Rabbit muscle myosin and actin were generous gifts of Doctors E. Elgart and V. T. Nachmias.

**Enzyme Assays**

Alkaline phosphatase, acid-phosphatase, and β-D-glucuronidase were measured spectrophotometrically by the methods of Michell et al. (27). Substrates were obtained from Sigma Chemical Co.

**RESULTS**

**Lactoperoxidase-Catalyzed Iodination of Rabbit Peritoneal PMNs**

**THE LABELING TECHNIQUE:** Rabbit peritoneal PMNs were labeled at 4°C to minimize pinocytosis and reduce transport of 125I into the cell, using a modification of the procedure of Hubbard and Cohn (16). Viability of the cells was examined during each labeling experiment by trypan blue exclusion, and was always >95%. In addition, the phagocytic activity of labeled cells was measured in most experiments and found to be identical to that of unlabeled cells (44). The efficiency of incorporation of 125I into TCA-precipitated material was ~1% of the added label, and was proportional to input 125I in the range tested, between 50 and 350 μCi/ml. This is in the range in which incorporation is proportional to iodide concentration (16). Increasing the concentration of lactoperoxidase threefold and/or of glucose oxidase fivefold did not increase the total incorporation of 125I into acid-insoluble material.

Because PMNs contain numerous oxidases and myeloperoxidase (for review see reference 24), it was essential to demonstrate that the incorporation was dependent on added reagents and not a consequence of labeling by these endogenous enzymes and intracellular free 125I. As shown in Table I, when the reaction mixture did not contain lactoperoxidase, incorporation of 125I into acid-insoluble material was reduced to 4% of the complete reaction. In the absence of glucose oxidase and/or glucose, incorporation was <1% of the complete reaction. This strong dependence on external enzymes was observed in spite of the capacity of these cells to trap free iodine. In fact, in various experiments, between 50 and 90% of the cell-associated isotope was acid-soluble and could not be removed by washing the cells with excess cold water.

| Reaction Mixture       | TCA-precipitate (cpm/10^7 cells) | % of Control |
|------------------------|---------------------------------|--------------|
| Complete reaction      | 249,000*                        | 100.0        |
| Minus lactoperoxidase  | 10,800                          | 4.3          |
| Minus glucose oxidase  | 2,700                           | 1.1          |
| Minus both enzymes     | 1,600                           | 0.7          |

* This amount of radioactivity represents 2.5 \times 10^5 atoms/cell.

10^7 PMNs were incubated in 0.1 ml PBS containing 15 μCi 125I with or without enzymes under the conditions described in Materials and Methods. The reaction was stopped with 5 \times 10^{-4} M Na_2S_2O_3, the cells were washed three times, and the cell pellet was precipitated and counted.

**Analysis of Labeled Polypeptides by SDS-PAGE:** The labeled polypeptides of iodinated cells were analyzed by slab gel electrophoresis in the presence of SDS. Washed cell pellets were suspended in an SDS solution containing mercaptoethanol and PMSF, a protease inhibitor, and dissolved by heating to 100°C for 2 min. Fig. 1a shows the electrophoretic distribution of the 125I-labeled proteins of these cells, detected by autoradiography, and Fig. 1a' shows the distribution of total proteins detected by Coomassie blue staining. At least 13 labeled proteins ranging in size...
FIGURE 1 SDS-PAGE of rabbit polymorphonuclear neutrophils labeled by lactoperoxidase-catalyzed iodination. Whole cells were labeled, washed, and then solubilized in electrophoresis sample buffer and analyzed on 10% acrylamide slab gels, as described in Materials and Methods. (a) Autoradiograph. (a') Coomassie blue stain. Numbers at right indicate mol wt (× 10^6) of standards listed in Materials and Methods. Myosin (M) and actin (A) migrate where indicated.

from ~250,000 daltons to 18,000 daltons were detected. The 11 numbered bands in Fig. 1a showed the least variation in incorporation during these experiments. The apparent molecular weights and other characteristics of these proteins are listed in Table III.

When lactoperoxidase or glucose oxidase were run simultaneously with iodinated cells, they migrated between bands 4 and 5. Occasionally a small amount of labeled material was found in this region and may represent lactoperoxidase bound to the cells. Cells labeled in the absence of either enzyme did not contain any labeled peptides when analyzed by electrophoresis. CHCl₃:methanol (2:1) or n-butanol extraction of the cells did not change the labeled protein profile.

Two major cell components which co-migrate with myosin (M) and actin (A) were not labeled, suggesting that these contractile components were not exposed at the external surface of PMNs. Most other major proteins of the cell were also not labeled. However, when the cells were first homogenized and then labeled, proteins that migrated like myosin and actin, as well as many other cell proteins, did become labeled (results not shown). Therefore, the lack of label in polypeptides from iodinated cells reflected the inaccessibility of these proteins to lactoperoxidase and not the lack of iodinatable sites.

**ENZYME SENSITIVITY OF LACTOPEROXIDASE-LABELED MATERIAL:** To obtain information on the chemical nature and surface disposition of material labeled on intact cells, the iodinated cells were exposed to various enzyme treatments. After all treatments, cell viability was assayed by trypan blue exclusion, and in all experiments reported here the cells showed >95% viability. In one experiment, after 20 min of protease treatment

| Band No. | Approx. mol wt | Periodic acid-Schiff (PAS) stain† | Protease sensitivity‡ | Present in plasma membrane fraction |
|----------|----------------|----------------------------------|-----------------------|-------------------------------------|
| 1        | >200,000       | −                                 | +                     | +                                   |
| 2        | 150-180,000    | +                                 | +                     | +                                   |
| 3        | 120,000        | −                                 | +                     | −                                   |
| 4        | 90,000         | −                                 | +                     | +                                   |
| 5 a, b   | 66-68,000      | −                                 | −                     | −                                   |
| 6 a, b   | 60-62,000      | −                                 | +                     | −                                   |
| 7        | 45,000         | −                                 | −                     | +                                   |
| 8        | 35,000         | −                                 | ±                     | +                                   |
| 9        | 25,000         | −                                 | −                     | −                                   |
| 10       | >20,000        | −                                 | +                     | +                                   |
| 11       | >18,000        | +                                 | −                     | −                                   |

* Mol wt for bands 3–9 were obtained from the linear portion of a plot of log Mol wt vs. Rf using standards listed in Materials and Methods. Band 1 has a lower mobility than myosin (200,000), band 2 had a lower mobility than immunoglobulin G (150,000), and bands 10 and 11 have lower mobilities than myoglobin (17,500).
† These bands have the same Rf as PAS-positive bands.
‡ See Results and Fig. 2.
at 23°C (10 μg/ml of trypsin or 50 μg/ml of pronase), 30–40% of acid-insoluble radioactivity was released from the cells (Table IV). SDS-PAGE of digested cells from another experiment is depicted in Fig. 2. In this experiment, digestion was more extensive. Similar protease sensitivities were seen at 4°C. The distribution of bulk proteins was unaffected by digestion (Fig. 2, right). Both proteolytic enzymes produced two cleavage products (Fig. 2, arrows) that remained associated with the cells. Soybean trypsin inhibitor could completely prevent hydrolysis of the labeled proteins by trypsin. In the experiments shown in Fig. 2 (wells e and f), however, some trypsin activity escaped inhibition, and band 3, which seems to be especially sensitive to proteolytic digestion, was partially hydrolyzed and a small amount of cleavage product was produced. This suggests that the latter may be the cell-bound portion of the band.

**TABLE IV**

| Treatment                                      | % of zero time TCA precipitable radioactivity remaining on cells |
|------------------------------------------------|---------------------------------------------------------------|
| 1. PBS                                         | 100                                                           |
| 2. Trypsin 10 μg/ml in PBS                     | 66                                                            |
| 3. Trypsin 10 μm/ml + soybean trypsin inhibitor 10 μg/ml in PBS | 99                                                            |
| 4. Pronase 50 μg/ml in PBS                     | 62                                                            |

PMNs were labeled, washed three times, and resuspended at 0°C at a final concentration of 2.8 × 10⁷ cells/ml in PBS containing the above enzymes. The cells were transferred to a rolling apparatus at 23°C, and at 0 and 20 min 1.4 × 10⁶ cells were removed into ice-cold 2 mM PMSF in PBS. The cells were centrifuged, washed two times with PBS, and the cell pellets were processed for measurement of TCA-precipitable radioactivity.

*FIGURE 2* Protease sensitivity of lactoperoxidase-labeled proteins of rabbit peritoneal PMNs. After the treatments indicated below, labeled whole cells were analyzed by SDS-polyacrylamide gel electrophoresis. The samples on the autoradiograph are designated a–h, while those on the Coomassie blue-stained gel, a′–h′. (a, a′) PBS for 10 min. (b, b′) Trypsin 10 μg/ml in PBS for 10 min. (c, c′) Trypsin 10 μg/ml in PBS for 10 min. (d, d′) Trypsin 50 μg/ml in PBS for 20 min. (e, e′) Trypsin 10 μg/ml + soybean trypsin inhibitor 10 μg/ml for 10 min. (f, f′) Trypsin 50 μg/ml + soybean trypsin inhibitor 50 μg/ml for 10 min. (g, g′) Pronase 10 μg/ml for 10 min. (h, h′) Pronase 10 μg/ml for 20 min. For further details of enzyme treatments see legend to Table IV.
3 protein. Phospholipase C at 50 μg/ml, the highest concentration which did not affect cell viability, failed to release any of the polypeptides.

Lectin Binding

Certain lectins show considerable cell surface receptor specificity and, therefore, when radiolabeled provide highly sensitive probes for the cell surface. WGA was chosen for these studies because it does not dissociate during fractionation in sucrose gradients (4), it binds to N-acetylglucosamine and sialic acid, common plasma membrane components, and it does not have the extensive membrane-perturbing effects of other lectins such as Con A (32). The concentration-dependence of the binding of iodinated WGA to rabbit peritoneal neutrophils is shown in Fig. 3. The binding approaches saturation at 0.55 μg lectin bound/10^6 cells, which is equivalent to 1.4 × 10^7 molecules lectin bound/cell. Assuming a surface area of 250 μm²/cell (15), there are 6 × 10^7 molecules/μm², which is the approximate concentration of binding sites on fibroblasts and fat cells (6, 22). Unlabeled WGA competed on an equal basis for the iodinated lectin, indicating that the probe was not altered during its radioiodination. Over 90% of the bound lectin could be competed with ovomucoid (2 mg/ml) or cold lectin (200 μg/ml) at all lectin concentrations tested. Only 80% was competed by 0.2 M N-acetyl-D-glucosamine.

Plasma Membrane Isolation from Resting PMNs

To compare the total cell surface proteins of PMNs with those parts of the surface that are either interiorized or remain external during phagocytosis, it was essential to develop a procedure that would permit concomitant isolation of both plasma membrane and phagocytic vesicles. Isolation of a well-characterized membrane fraction from granulocytes of any species has not yet been reported. Plasma membrane isolation proved to be extremely difficult because of the resistance of the cells to breakage. We were unable to produce cell ghosts following membrane hardening in hypotonic solutions containing Zn²⁺, Tris-Mg²⁺, glutaraldehyde, or fluorescein mercuric acetate (FMA) (28, 41). Even in the absence of hardening agents, the cells could be broken neither by a Dounce homogenizer nor by N₂ cavitation under conditions used to break lymphocytes. A Potter-Elvehjem homogenizer with a motor-driven pestle proved satisfactory for cell breakage, but nuclear disruption was extensive. However, hypertonic sucrose reduced nucleus and lysosome lysis, and the presence of BSA at 500 μg/ml helped further to stabilize these organelles. It was necessary to remove nuclei and whole cells early in the procedure to prevent their adsorption of surface membranes. The resulting supernate was then fractionated by centrifugation through layers of varying sucrose concentrations (in BSA) as described in Materials and Methods. A partially purified plasma membrane fraction at the 35%-45% sucrose interface was obtained that was enriched both for lactoperoxidase-labeled proteins and for¹²⁵I-WGA bound to the cell surface before homogenization.

Fig. 4 shows the sedimentation profiles on the postnuclear supernates of cells labeled with either¹²¹I-WGA or lactoperoxidase. The arrows indicate the 35%-45% interface. Between 30 and 50% of the counts applied to the discontinuous gradients banded at this density (see Table V also). This material showed a 10- to 12-fold enrichment in specific activity (cpm/mg protein) relative to labeled whole cells. Because we permitted at least 20% of the starting cells to survive homogenization...
FIGURE 4  Sedimentation of the postnuclear supernate from cells labeled either with $^{125}$I-WGA or by lactoperoxidase-catalyzed iodination. 10$^8$ cells were labeled with (a) $^{125}$I-WGA, or (b) lactoperoxidase-catalyzed iodination as described in Materials and Methods. The cells were homogenized as described for plasma membrane isolation and the postnuclear supernate (1.5 ml) was layered on a discontinuous sucrose gradient containing 1.5 ml each of 35, 45, 50, and 60% wt/vol sucrose and 2 ml each of 65 and 70% wt/vol sucrose. Fractions were collected from the top of the centrifuge tube and passed through a UV-absorbance monitor (Fraction 1 is the top). Fractions from $^{125}$I-WGA-labeled cells were counted directly; fractions from lactoperoxidase-iodinated cells were TCA precipitated and counted. The arrow indicates the 35%-45% interface. 48% of $^{125}$I-WGA recovered from gradient (a) was found in this fraction, while 40% of the lactoperoxidase-labeled material applied to gradient (b) was found here.

to prevent breakage of nuclear membranes and lysosomes, a large fraction of the surface label was recovered in the low speed pellet (Table V). Also contributing to the radioactivity of the low speed pellet was its enrichment for a unique set of surface peptides that are described in the following section. As a result, the total amount of lactoperoxidase-iodinated material or $^{125}$I-WGA recovered in the plasma membrane fraction from starting homogenate ranged from as low as 7% in the poorest experiment to an average of 20% (Table V).

The plasma membrane fraction contained 4 and 7%, respectively, of the homogenate activity of two lysosomal enzymes, acid phosphatase and β-D-glucuronidase (Table VI). Alkaline phosphatase is present in specific granule membranes and may also be present in the plasma membrane: 22% of the homogenate activity for this enzyme was present in our plasma membrane fraction. The fractions of high absorbance in the gradients shown in Fig. 4 represent granule populations. These sedimented to the bottom of the tube when the 60 and 70% sucrose layers were omitted in other experiments.

**SDS-PAGE of Subcellular Fractions of Lactoperoxidase-Labeled PMNs**

The 35%-45% interface was enriched for ~20 Coomassie blue staining polypeptides of >30,000 daltons (Fig. 5c'). Proteins which co-migrate with actin and myosin were found in this fraction (A, M).

Examination of the autoradiograph (Fig. 5c and

| TABLE V | Recovery of Labeled Plasma Membrane During Cell Fractionation |
|----------|-------------------------------------------------------------|
|          | Lactoperoxidase-labeled cells | $^{125}$I-wheat germ agglutinin-labeled cells |
| % of starting homogenate | % of postnuclear supernate | % of starting homogenate | % of postnuclear supernate |
| Homogenate | 100 | 100 |
| Low speed pellet | 40 | 55 |
| Low speed supernate | 60 | 100 | 45 | 100 |
| Total counts recovered from gradient | 47 | 79 | 37 | 82 |
| Counts at 35%-45% | 19* | 32 | 18 | 39 |

For details of fractionation, see Materials and Methods and legend of Fig. 4.

*96% of this radioactive material was sedimentable at 27,000 g for 45 min.
**DISCUSSION**

**TABLE VI**

*Activity of Granule Enzymes in Isolated Plasma Membrane Fraction*

| Enzyme          | Total activity* per 6 x 10⁶ cells | Plasma mem- | % in plasma mem- |
|-----------------|----------------------------------|brane frac-|brane fraction  |
| Acid phosphatase| 90                               | Homogenate| 4.0            |
| β-glucuronidase  | 50                               | 3.4       | 6.8            |
| Alkaline phospha-| 180                             | tase      | 39.0           |

* For acid and alkaline phosphatase, activity is expressed as nmol p-nitrophenol produced from p-nitrophenylphosphate/min. β-glucuronidase activity is expressed as nmol p-nitrophenol produced from p-nitrophenyl-β-D-glucuronide/h.

† Total enzyme activity in plasma membrane was obtained by dividing the enzyme activity measured in this fraction by the yield of lactoperoxidase-incorporated radioactivity in this fraction.

(1) confirmed the enrichment of iodinated proteins at the 35%-45% interface relative to whole cells. Bands 1, 2, 4, and 7-11 were present in this fraction. Band 10, which usually appeared as a very minor protein of whole cells, was now enriched. Bands 3, 5, and 6 were absent. These differences can be seen more clearly in optical absorbance scans of these autoradiographs, shown in Fig. 6. The curves were normalized for equivalent amounts of band 1. Thus, the 35%-45% fraction, although highly enriched for plasma membrane, was not representative of the entire surface as defined by lactoperoxidase-catalyzed iodination. Bands 3, 5, and 6 were concentrated in the low speed pellet (Fig. 5 b). No new peptides were generated during the course of membrane isolation.

**Iodination of Neutrophils**

External labeling procedures such as lactoperoxidase-catalyzed iodination provide an important tool for the study of membrane components in cells, such as neutrophils, which do not incorporate macromolecular precursors. Under appropriate conditions, the procedure ensures study of only those molecules localized on the cell surface. Neutrophils, however, are not ideal cells for surface labeling because of: (a) the presence of lysosomal myeloperoxidase, a peroxide-dependent halogenating enzyme; (b) the presence of peroxide-generating cyanide-insensitive oxidase on the cell surface (3) and in lysosomes (31); (c) the ability of polymorphonuclear neutrophils to actively accumulate iodine (24); (d) the presence of ectoproteases, i.e., enzymes on the cell surface which hydrolyze extracellular proteins (29) as well as lysosomal proteases; and (e) the capacity for bulk membrane internalization. However, most of these properties are activated under conditions which stimulate phagocytosis, and labeling at 4°C in the absence of phagocytosis prevented or inhibited all except intracellular iodine accumulation. Therefore, iodine was made the limiting reagent in the system and under these conditions lactoperoxidase-dependent incorporation of ¹²⁵I into acid-insoluble material accounted for 96% of the labeling observed. The viability (as measured by trypan blue exclusion) and biological activity (as measured by phagocytic activity [44]) of the neutrophils were found to be unaffected by the presence of 5,000 atoms of iodine/cell. Less than 10% of the acid-insoluble label was extracted by organic solvents, indicating that the bulk of incorporated iodine was not in lipid.

**FIGURE 5**

Electrophoretic analysis of various cell and sucrose gradient fractions. 7.5 x 10⁶ lactoperoxidase-labeled cells were fractionated as described in Materials and Methods. Each sucrose-containing gradient fraction was diluted with ~5 vol of 10 mM HEPES, 2 mM PMSF to permit recovery of membranous material by centrifugation at 27,000 g for 45 min. The samples on the autoradiograph are designated a-d, while those on the Coomassie blue-stained gels, a'-c'. (a, a') Whole cells, (b, b') Low speed pellet, (c, c') 35%-45% sucrose interface. (d) A dilution of the sample shown in (c). M and A indicate the position of marker rabbit skeletal myosin and actin; BSA indicates the position of contaminating BSA which was included in the sucrose gradient to aid fractionation.
Labeled Proteins

SDS-slab gel electrophoresis of labeled whole cells revealed ~13 labeled polypeptides ranging from 250,000 to 18,000 daltons in weight. These labeled proteins did not appear to correspond with any of the predominant Coomassie blue staining bands. This was also the case for L-cell surface proteins (17), suggesting that membrane proteins exposed to the extracellular milieu represent a very small fraction of total cell protein.

The bulk of the bands from labeled intact cells were released by mild proteolytic digestion, supporting their disposition at the cell surface. In the presence of small amounts of protease, one of the bands (No. 3) appeared to be especially sensitive. Concomitant with its hydrolysis was the appearance of a membrane-bound cleavage product. This may represent the protease resistant core of band 3.

A class of iodinatable, large, protease-sensitive glycoproteins of 230–250,000 daltons has been detected on many cells in culture (19, 20). Our band 1 may be a member of this class. However, we did not detect carbohydrate co-migrating with this band when tested by the PAS stain. This method, however, is insensitive, especially in slab gels. We did find that band 2, of 150–180,000 daltons, was PAS-positive.

Myosin and actin represent ~1 and 10%, respectively, of total granulocyte or macrophage protein (36, 37). In our studies, neither was labeled on intact rabbit neutrophils. This observation contrasts with those of Olden et al. (30) using antibody to myosin and employing enzymatic iodination of fibroblasts. We did find that unlabeled proteins co-migrating with myosin and actin were enriched in the plasma membrane fraction of granulocytes.

**Isolation of a Plasma Membrane Fraction from Neutrophils**

Some of the difficulties that we encountered in membrane isolation have already been described in Results. In systems in which it is possible to isolate intact membrane ghosts, the iodinated protein profile of whole cells has been more or less identical to that of the ghosts (14, 16, 17). However, difficulties arise when membranes are isolated by fragmentation and differential centrifugation, as was necessary in the case of neutrophils. Under these conditions, heterogeneous membrane vesicles are produced, and it becomes more difficult to unambiguously identify a plasma membrane fraction. Therefore we sought an independent method, to complement surface iodination, to permit identification of the granulocyte cell surface.

Many investigators have used enzymatic activities as membrane markers. However, their use requires that the activity not be modified during fractionation and, more importantly, that the activity be localized to the plasma membrane of the particular cells being examined. For example, 5'-nucleotidase, while restricted to the plasma membranes of guinea pig PMN (9), has been identified by cytochemical techniques in the primary granules of rabbit neutrophils (1, 2). Indeed, only in exceptional cases have such “marker” enzymes been shown to be located uniquely on plasma membranes. At the present time there are no reports of well-characterized enzymes associated uniquely with the plasma membrane of rabbit PMNs. We are pursuing the possibility that Mg²⁺-dependent ATPase may be such a membrane marker, because in preliminary tests we found a large fraction of the activity in the 35%–45% interface.
We have chosen, therefore, to use \(^{125}\)I-labeled WGA, a molecule that binds to N-acetyl glucosamine and sialic acid residues and was found to label exclusively the plasma membrane of a variety of cell types (4), as a further probe for the cell surface fraction of rabbit neutrophils. These cells bound \(^{125}\)I-WGA with high affinity, and the lectin did not dissociate during the course of cell fractionation. Because the lectin had a high specific activity, the assay for plasma membrane was sensitive. At the low concentrations of lectin used and at the low temperature during binding, cell agglutination did not occur. These are conditions that should prevent WGA internalization (4); however, we did not directly test this. The sucrose gradient profiles at 280 nm of lectin-labeled and unlabeled homogenates were identical, indicating that gross cross-linking of organelles had not occurred.

The recoveries of \(^{125}\)I-WGA and acid-insoluble radioactivity introduced by lactoperoxidase were parallel throughout the membrane isolation procedure. Approx. 40% of both labels sedimented with nuclei and whole cells. A large fraction of unopened cells was tolerated because we wished to avoid damaging nuclei and lysosomes and the consequent possibility of contamination of the plasma membrane fraction with membranes from those organelles. 30–50% of the remaining label banded at the 35–45% interface. This fraction was enriched relative to whole cell protein ~10- to 12-fold both for lactoperoxidase-incorporated iodine and for \(^{125}\)I-WGA, based on the amount of Coomassie blue-stained or UV-absorbing protein. Electron microscopy of this fraction showed it to be composed of heterogeneous vesicles. Contamination by specific granules and glycogen granules was evident, although only 4 and 7% of the lysosomal enzymes, acid phosphatase and \(\beta\)-glucuronidase, respectively, were present in the fraction. We find 22% of the homogenate alkaline phosphatase activity in this fraction, suggesting specific granule membrane contamination. However, all of the activity of this enzyme is not unambiguously localized to specific granule membranes, and some may be associated with the plasma membrane. The material at the 35–45% interface may be similar to a membrane fraction isolated from rabbit peritoneal neutrophils by Woodin and Wieke (45). In addition to the plasma membrane found at the 35–45% interface, 20–30% of the iodinated cell surface label applied to the gradients was found at the 11.6–35% interface (see Fig. 4b, fractions 8 and 9). Analysis of this material by gel electrophoresis showed it to have essentially the same iodinated peptide composition as the material at the 35–45% interface. It presumably represents either small vesicles of plasma membrane which have failed to sediment to the proper density position, or large vesicles which contain a high proportion of entrapped aqueous phase and therefore have a slightly reduced density.

Our procedure for the isolation of a plasma membrane-enriched fraction from neutrophils was useful because (a) it permitted concomitant isolation of phagocytic vesicles (44), (b) it was quick, reducing the chance of membrane protein proteolysis, and (c) small amounts of cells and isotope could be used. However, the fraction clearly was not pure, and we have sacrificed yield for the benefits just indicated. Consequently, although we can rationalize the bulk of the losses as arising from unbroken cells during homogenization and the sedimentation of variable-sized plasma membrane vesicles to positions in the sucrose gradients other than the 35–45% interface, it is possible that a select fraction of plasma membrane was examined. In fact, the results discussed below show that the 35–45% interface is not representative of the total cell surface proteins. Furthermore, the enrichment of band 10 in this fraction relative to whole cells suggests the existence of an additional membrane component deficient in band 10.

A Dense Surface Component of Neutrophils

Available evidence suggests that all proteins labeled by enzymatic iodination are plasma membrane proteins. As indicated in Table III, all the lactoperoxidase-labeled proteins of rabbit peritoneal neutrophils except bands 3, 5, and 6 were located in the predominant membrane-enriched fraction. Bands 3, 5, and 6, however, were enriched in the low speed pellet containing nuclei and whole cells. They were never found in the sucrose gradient of the postnuclear supernate. It is unlikely that these are intracellular proteins because they were sensitive to protease treatment of the intact cell. If they are external components the following possibilities exist: (a) Bands 3, 5, and 6 may be membrane components of another cell type having similar sedimentation characteristics as intact neutrophils but which are not disrupted during homogenization. However, these proteins, as well as the remaining eight, are present in a labeled cell population which adhered to tissue culture dishes within 30 min and therefore have the properties of PMNs. (b) Bands 3, 5, and 6 may belong to a
unique membrane fragment which is dense because of its chemical composition (low in lipid, rich in carbohydrate and protein) or its structure (sheets of membranes). (c) These proteins may reside in a unique segment of plasma membrane which remains attached to the nucleus during homogenization. Preliminary results rule out this possibility because bands 3, 5, and 6 also sediment at low forces from heparinized homogenates in which the nuclei were completely disrupted. (d) These proteins, as a membrane unit or individually, bind to chromatin.

Evidence exists in other systems for the second possibility. All lactoperoxidase-labeled proteins of NIL8 fibroblasts except a large external iodinated glycoprotein (LETs protein) sediment with conventional plasma membrane rich in sialic acid, cholesterol, and Mg++. ATPase (14, 20). LETs protein is concentrated in a high density particle \( p = 1.253 - 1.259 \) which is mainly carbohydrate and protein. S'-Nucleotidase is present in both this fraction as well as the major plasma membrane fraction. LETs protein remains associated, however, with Zn++-hardened ghosts, confirming its surface localization. Chang et al. (4) have also identified a dense membrane fraction from liver cells which sediments slightly slower than mitochondria and is enriched for glucagon-stimulated adenylcyclase activity.

Fragmentation of the plasma membrane into specialized regions can occur only if unique domains exist on the surface, i.e., if all components are not random. This is probably the case for tissue cells such as liver from which bile fronts and gap junctions can be isolated (15). It would be particularly significant if specialized regions could be isolated from single cells that otherwise appear unspecialized in morphology. DePierre and Karnovsky (8) have discussed the possibility that the neutrophil surface may be heterogeneous in order to perform such functions as phagocytosis, adhesion, and chemotaxis. We hope to isolate and further characterize the fraction rich in bands 3, 5, and 6 to establish its origin and function.

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