Specific and Azurophilic Granules from Rabbit Polymorphonuclear Leukocytes. II. Cell Surface Localization of Granule Membrane and Content Proteins before and after Degranulation

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ABSTRACT The compositional relationship between the cell surface of rabbit polymorphonuclear leukocytes (PMNs) and the membranes of PMN cytoplasmic granules has been investigated. Heterophilic PMNs obtained from peritoneal exudates contained 13 cell surface polypeptides ranging in molecular weight from 220,000 to 12,000 daltons as determined by lactoperoxidase-catalyzed protein iodination and gel electrophoresis. Of these, four polypeptides co-migrated with proteins identified as the major constituents of specific (SpG) and azurophilic (AzG) granule membranes. The most notable of these were cell surface proteins of 145,000 and 96,000 daltons that co-migrated with SpG membrane proteins and a 48,000-dalton protein that was also a major component of AzG membranes. Also, four iodinated cell surface proteins co-migrated with proteins identified as granule content proteins released from PMNs during exocytosis. Extensive washing did not remove these proteins from the cell surface.

Iodination of PMNs after the release of SpG and AzG contents by calcium ionophore-induced exocytosis revealed that there was not a dramatic qualitative change in the proteins on the cell surface. Instead, there were large, quantitative increases in the relative amounts of ^{125}I that were incorporated into several pre-existing cell surface proteins; all of these cell surface proteins co-migrated as a set with those polypeptides identified as either granule membrane or content proteins. Although nearly all of the major polypeptides of SpG and AzG had counterparts on the cell surface of freshly isolated peritoneal exudate PMNs, there were several polypeptides that were unique to the cell surface. Thus, the PMN has at least three membrane compartments with strikingly different protein compositions.

Numerous morphological studies have demonstrated that in secretory cells membrane-bounded granules fuse with the plasma membrane during secretion (exocytosis), resulting in the mixing of these membranous compartments (1). In addition, recent evidence suggests that components of secretory cell plasma membranes may recycle back to the Golgi complex to be reutilized for secretory granule formation (2, 3). Moreover, there are indications that in some cells newly added secretory granule membranes may recycle, intact, from the plasma membrane back to the cytoplasm (4). Even with this information, the compositional relationship between the plasmalemma and secretory granule membranes is not well documented, owing to the difficulty in obtaining plasma and granule membranes from the same cell type. Rabbit polymorphonuclear heterophils (PMNs), since they exist as single cells in vivo, possess a readily accessible plasma membrane, two well-defined populations of cytoplasmic granules, and a capacity for secreting the contents of these granules into the extracellular space. Thus, these cells provide an excellent model for studying the relationship between the plasma membrane and secretory granule membranes.

In the preceding paper (5) we reported on the separation of rabbit PMN SpG and AzG into membrane and content subfractions and enumerated the major proteins of each fraction. In this report we compare these proteins to plasma

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 APRIL 1983 1040-1046

membrane proteins accessible to surface iodination before and after degranulation. We demonstrate that although several of the dominant iodinatable proteins on the surface of resting and degranulating PMNs co-migrate with the major membrane and content proteins of the two granules, there are major differences in the protein compositions of these three membrane compartments.

MATERIALS AND METHODS

Materials: Trypsin-TPCK (I.U.B. No. 3.4.21.4) and soybean trypsin inhibitor (SBTI) were obtained from Worthington Biochemical Corp. (Freehold, NJ). All other reagents were as described in the preceding report (5).

Secretion of PMN Granule Contents: Most techniques used in this work, except as noted below, have already been described in the preceding paper (5). PMNs were induced to secrete SpG and AzG contents as described (5) and, immediately after addition of the calcium ionophore A23187, 5-ml aliquots of the cell suspension were dispensed into siliconized 50-ml polycarbonate centrifuge tubes (conical bottoms). After incubating the cells for up to 30 min at 37°C in these tubes, secretion was stopped by adding 4 volumes of ice-cold HBSS. Cells were harvested by centrifugation at 2,500 *g* (3,300 rpm) for 3 min (IEC CRU-5000 centrifuge, Damon/IEC Division, Needham, MA), washed twice in ice-cold HBSS, and resuspended in 2 ml of 0.15 M NaCl containing 5 mM glucose. Each sample was split into two equal portions: one portion was frozen and thawed before iodination and the other was kept on ice.

PMN secretory products were collected from cytochalasin B, A23187-treated cells as described in the preceding report (5).

Iodination of Cells: Rabbit PMNs obtained before or after degranulation were resuspended to 2×10^6 cells/ml in ice-cold 0.15 M NaCl containing 5 mM glucose (see Fig. 1 for experimental design). To these suspensions were added in sequence $Na^{125}I$, lactoperoxidase (LPO) and glucose oxidase (GO) to final concentrations per ml of 75 μCi, 10 μg, and 2.0 μg, respectively. After incubation for 10 min on ice the reactions were stopped with 8 volumes of 0.15 M NaCl, 0.5 mM $Na_2S_2O_5$; cells were collected by centrifugation at 2,000 *g* (3,000 rpm) for 3 min at 4°C in siliconized 15-ml glass centrifuge tubes (IEC-CRU 5000 centrifuge) and washed three times by centrifugation in fresh stop solution. The sedimented cells were resuspended in 1 ml of 0.15 M NaCl, samples were removed for determination of incorporated radioactivity, and the remaining cells were solubilized in electrophoresis sample buffer. In control experiments, cell suspensions were frozen and thawed before labeling. Radioiodination was carried out as described for intact cells except that the reactions were stopped by addition of an equal volume of 20% TCA containing 0.2 M KI and processed as were disrupted granules.

To establish that ^{125}I incorporation required both LPO and GO, control experiments were done in which one or the other or both enzymes were omitted from the reactions. A suitable volume of carrier solution was added in the absence of an enzyme. The amount of ^{125}I-incorporation was determined as described (5).

PAGE: ^{125}I-labeled cells were analyzed by SDS PAGE on 6–16% linear polyacrylamide gels as described (5). After exposure of the X-ray film, some of the dried gels were photographed and lanes were sliced into 1-mm thick fractions that were used for radioactivity determinations. The molecular weights of iodinated cell surface proteins were determined by linear regression analysis using the $\log_{10}$ of the molecular weights of standard proteins (see Fig. 3) and the relative migratory distances from the top of the resolving gel. The assignment of molecular weights was based on averaged data from 10 experiments.

Biochemical Methods: Procedures for determining enzyme activities and protein concentration were as described in the previous report (5).

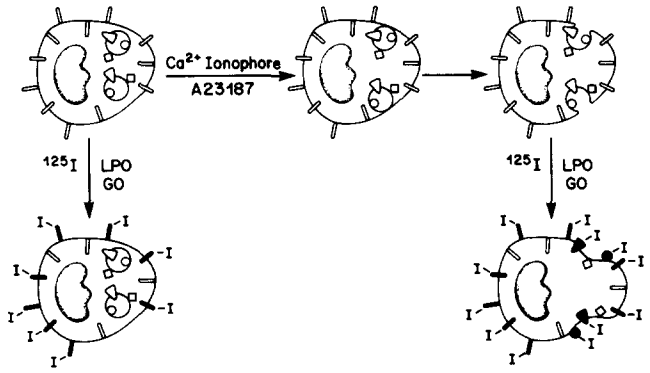

FIGURE 1 A schematic diagram of the experiments designed to identify proteins of granule membranes that were transferred to the plasma membrane following degranulation. Cells radioiodinated before and after degranulation were then analyzed by SDS PAGE and autoradiography.

RESULTS

Secretion by Ionophore-treated PMNs

In the preceding report (5) it was determined that with the conditions used in these experiments ~85% and ~65% of the

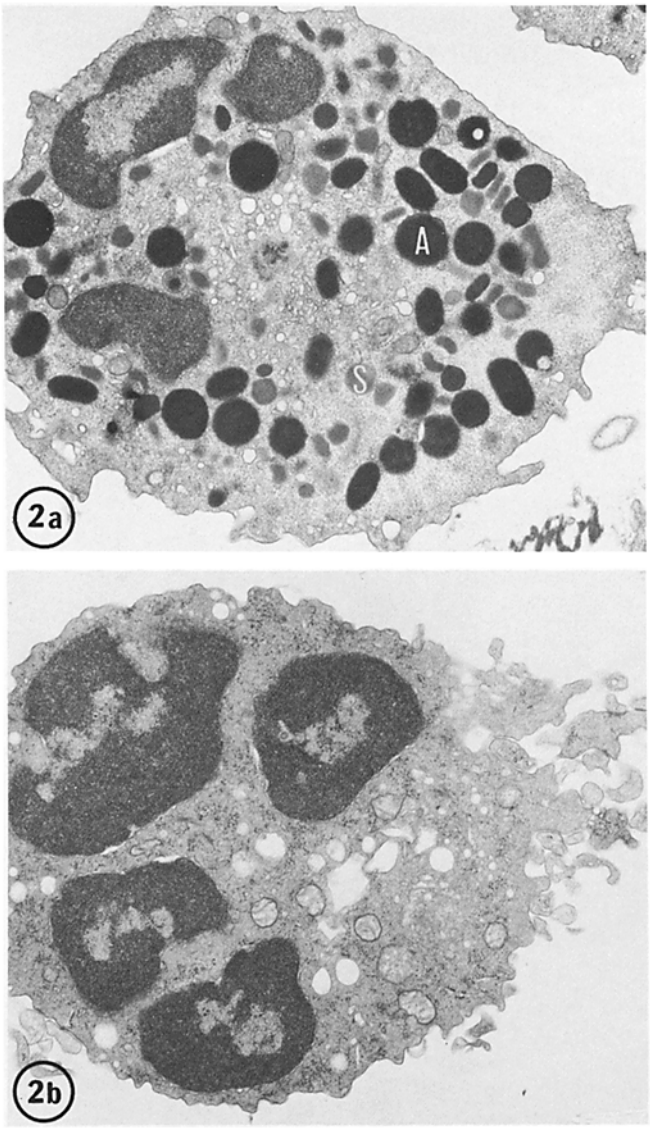

FIGURE 2 Electron micrographs of rabbit peritoneal heterophils before and after treatment with the calcium ionophore A23187. (*a*) Electron micrograph of a typical exudate PMN demonstrating numerous specific (*S*) and azurophilic (*A*) granules. (*b*) Electron micrograph of a typical PMN after a 30-min incubation with 10^{-6} M A23187. After incubation, cells were vigorously washed three times in PBS and then fixed and processed for electron microscopy. By this time, most cells had few, if any, granules but did have numerous empty vacuoles (presumably the result of membrane retrieval from the cell surface) and an elaborate expanse of plasma membrane extensions. Often, these membranous structures were located on the side of the cell opposite the nucleus. As with resting exudate cells, the cell surface of degranulating PMNs, by and large, had no identifiable granule content material. *a*, × 14,000; *b*, × 12,900.

total cellular lysozyme and myeloperoxidase, respectively, were released upon ionophore stimulation. These biochemical data indicated that extensive SpG and AzG fusion with the plasma membrane had occurred, and electron microscopic observations (Fig. 2) of degranulating cells confirmed these results. Whereas the resting cells had the normal complement of both types of granules, most of the degranulating cells contained few, if any, granules. In addition the plasma membranes of the degranulating cells, like those of resting cells, did not have detectable attached granule matrix material. Additional evidence that cells were secreting granule contents in response to A23187 was obtained by comparing the SDS PAGE protein profiles of cells before and after ionophore treatment. As shown by the protein staining patterns in Fig. 3, untreated PMNs contained numerous polypeptides including major proteins of 82, 80, 23, 22, 17, 16, and 15 kdaltons mol wt which co-migrated with the major SpG and AzG content proteins. However, following ionophore treatment, these proteins were lost or depleted in whole cell preparations and as shown in Fig. 3, were recovered in the medium as secretory products.

Identification of ^{125}I-labeled Cell Surface Proteins

To assess cell surface changes resulting from degranulation, externally disposed plasma membrane proteins were radioiodinated with the LPO-GO method before and after ionophore treatment and analyzed by SDS PAGE. To permit direct comparisons, an equal number of cells from each treatment was loaded onto the gels. SDS PAGE and autoradiography of cells iodinated before ionophore treatment showed that 13 polypeptides ranging in molecular weight from 220 to 12 kdaltons were radiolabeled on intact PMNs. Proteins most consistently labeled are indicated in Fig. 3 by the molecular weight markers. When we compared SDS PAGE patterns of these labeled surface proteins to patterns from SpG and AzG we found that they were surprisingly similar: about one-half of the iodinated cell surface proteins co-migrated with granule membrane or content proteins. The proteins common to both types of membranes were of mol wt 145, 96, 48, and 27 kdaltons. The 23-, 15-, and 12-kdaltons proteins co-migrated

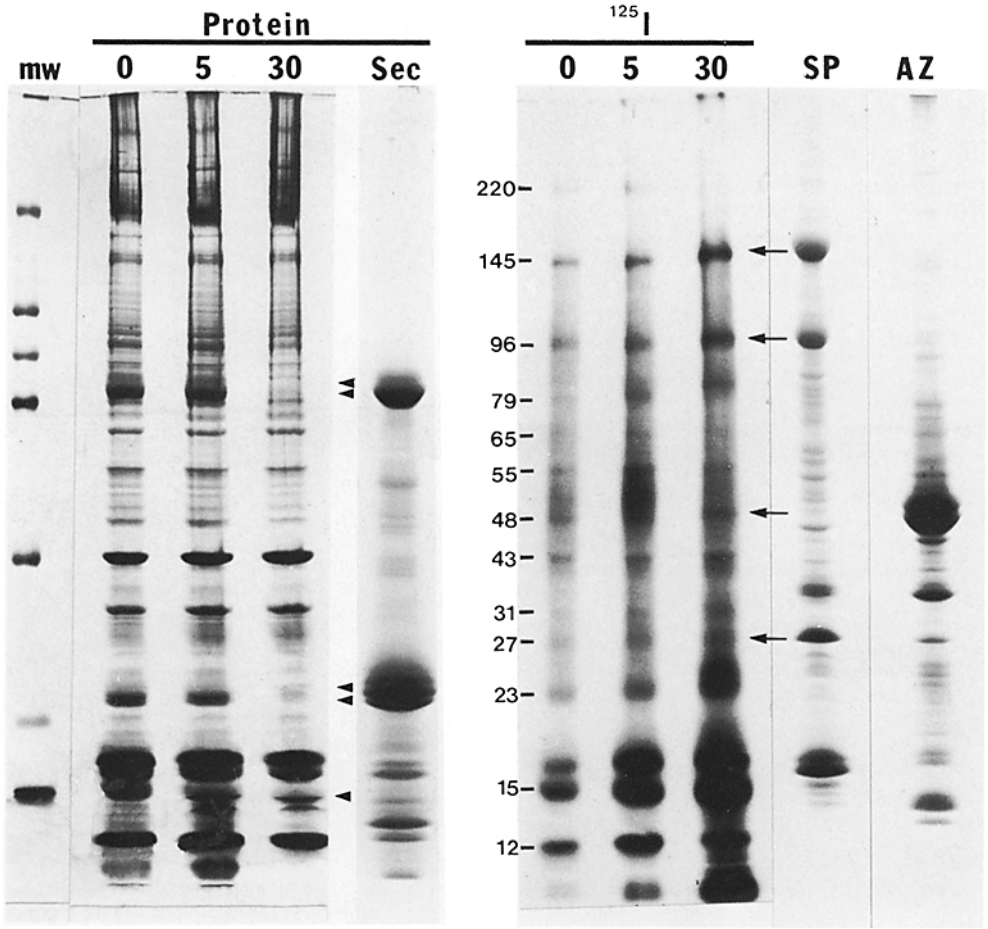

FIGURE 3 SDS PAGE analysis on 6–16% gradient gels of rabbit peritoneal PMNs surface labeled by LPO-catalyzed iodination before and after induction of degranulation by the calcium ionophore A23187. Shown are the silver-stained proteins and corresponding autoradiographs of cells before (0), 5 min (5), and 30 min (30) after A23187 treatment. An equal number of cells were loaded for each time point to permit direct comparisons of cell surface changes. The molecular weight markers indicate the most consistently labeled proteins on the surface of resting cells. The protein-stained gels of whole cell preparations showed the loss or depletion of proteins (indicated by the arrowheads) from degranulating cells that co-migrated with the major secretory proteins collected from the media in which degranulating cells were suspended (*Sec*). The corresponding autoradiographs of surface-labeled PMNs demonstrated the appearance of several proteins that co-migrated with proteins enriched in specific (*SP*) and azurophilic (*AZ*) granule membranes (arrows), as well as proteins that co-migrated with secretory proteins. The lane marked (*mw*) contained molecular weight markers: myosin, 200 kdaltons; β-galactosidase, 115 kdaltons; phosphorylase B, 96 kdaltons; ovalbumin, 43 kdaltons; soybean trypsin inhibitor, 21 kdaltons; and lysozyme, 14 kdaltons.

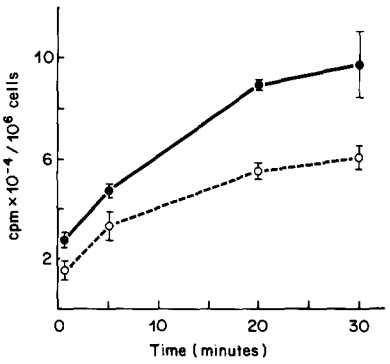

FIGURE 4 Changes in the amount of LPO-catalyzed ^{125}I incorporation into PMN surface proteins before and after initiation of degranulation. To induce secretion, PMNs were treated with the calcium ionophore A23187 for various periods of time, as described in Materials and Methods, and then subsequently radiolabeled by LPO-catalyzed iodination procedures. The amount of TCA-insoluble radioactivity for each sample of cells was determined and plotted against the time of incubation in A23187. (●) Nonpretreated cells; (○) cells pretreated with trypsin before ionophore stimulation. Trypsinization of cells was accomplished by incubating 5×10^6 cells/ml in 0.15 M NaCl containing 10 μg/ml trypsin-TPCK for 20 min at 23°C. The reactions were stopped by adding to cell suspensions an equal volume of ice-cold 0.15 M NaCl containing 20 μg/ml soybean trypsin inhibitor (SBTI). Cells were washed twice with 0.15 M NaCl, 10 μg/ml SBTI by centrifugation at 1,000 *g* for 10 min at 4°C, prepared for degranulation by resuspension in HBSS-cytochalasin B preincubation solutions, and subsequently treated as were nontrypsinized cells.

with granule content proteins. Examination of the SDS PAGE pattern of the cells after 30 min of ionophore treatment showed that although there was a fourfold increase in the amount of ^{125}I incorporated per cell (Fig. 4) there were few, if any, new proteins that appeared. With the exception of proteins of mol wt 79, 65, 55, and 43 kdaltons, which did not change and the 220-kdalton protein, which disappeared, the increased labeling appeared in proteins that were iodinatable before degranulation. By determining the cpm in gel slices it was found that the relative amounts of radioactivity associated with the 27-, 96-, and 145-kdalton mol wt proteins had increased two to threefold by 30 min after ionophore stimulation (Fig. 5), whereas several other cell surface proteins, i.e., the 79-, 65-, 55-, and 43-kdalton species maintained a constant level of ^{125}I-incorporation. In addition to the proteins of mol wt 27, 96, and 145 kdaltons, a protein of 48 kdaltons, which co-migrated with the 48-kdalton major AzG membrane protein, incorporated three times more ^{125}I by 30 min after ionophore treatment.

As a separate means to establish that the appearance of these proteins at the cell surface was induced by degranulation, resting PMNs were trypsinized to remove cell surface, trypsin-sensitive proteins, treated with ionophore A23187 to release SpG and AzG, and surface iodinated at various times after ionophore stimulation. That trypsin-pretreated cells were able to respond to ionophore stimulation was indicated in three ways: (*a*) SDS PAGE analysis of these trypsinized, ionophore-treated cells showed them to have reduced amounts of granule-associated secretory proteins (Fig. 6); (*b*) biochemical analysis revealed that trypsinized cells had released 81% and 71%,

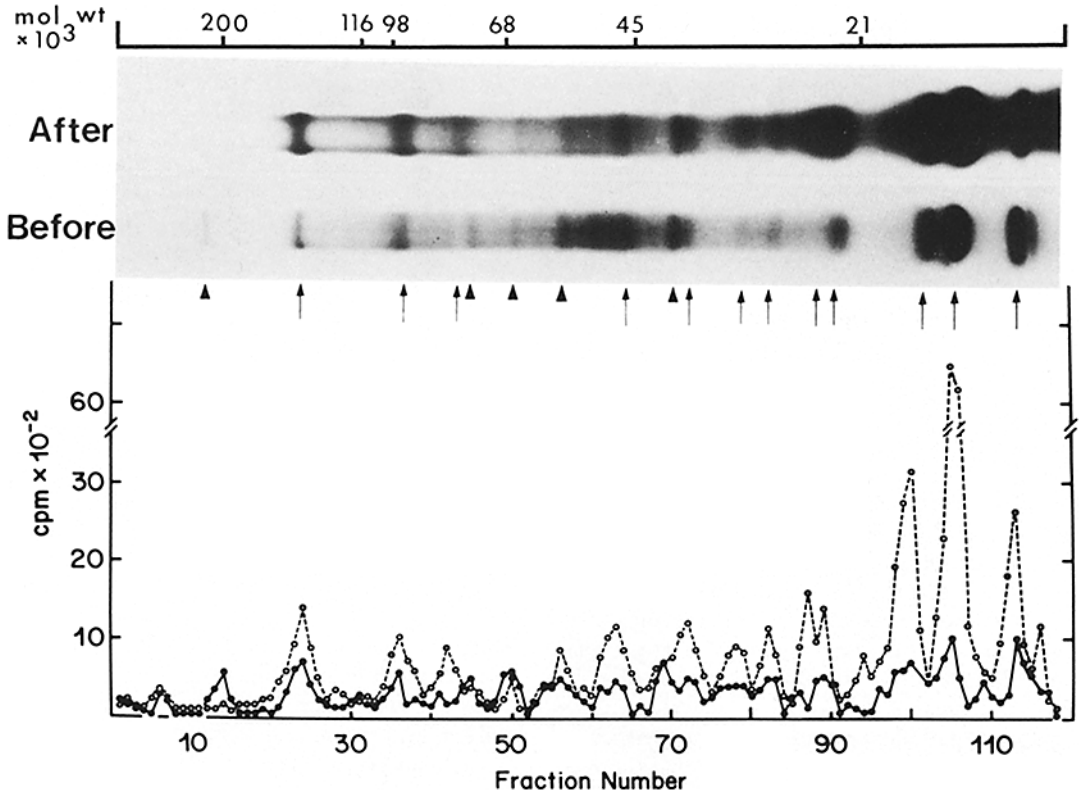

FIGURE 5 SDS PAGE analysis on 6–16% gradient gels of PMNs iodinated before (●) and after (○) a 30-min incubation in 10^{-6} M A23187. Equal numbers of cells were radioiodinated before and after degranulation and subsequently analyzed by SDS PAGE. Gels from these experiments were dried between dialysis membranes, photographed, used to make autoradiographs, and sliced into 1-mm thick fractions, and each fraction was counted directly for ^{125}I-incorporation. Arrows point to proteins found in increased amounts on the cell surface following secretion, and arrowheads point to cell surface proteins which remained the same or decreased.

respectively, of the amount of lysozyme and myeloperoxidase that nontrypsinized cells had under similar conditions, and (*c*) trypsinized PMNs showed an increase in the number of CPM of ^{125}I per cell following a 30-min treatment with A23187 (although only ~60% of that attained by non-trypsinized cells, Fig. 4). SDS PAGE analysis shown in Fig. 6 demonstrated that trypsin treatment of cells before degranulation rendered both the 96- and 145-kdalton proteins much less accessible to iodination. However, ionophore-stimulation of these trypsin-pretreated cells led to the reappearance on the cell surface of both the 96- and 145-kdalton proteins as judged by surface iodination.

Concomitant with the appearance of granule content and membrane proteins on the cell surface was the disappearance of one major cell surface protein of ~220 kdaltons mol wt. This polypeptide, while always labeled on untreated PMNs, varied greatly in the relative amount of ^{125}I incorporated. In several experiments it was the dominant iodinated surface protein. Nevertheless, degranulation invariably led to the apparent disappearance of this protein from the surfaces of secreting cells (Fig. 3). This protein was also highly trypsin-sensitive as evidenced by its loss from the surface of trypsinized, resting cells (Fig. 6).

Controls

The results shown in Table I demonstrated that omission of either LPO or GO from the reaction mixtures led to an ~1,000-fold decrease in the amount of TCA-precipitable radioactivity, indicating that exogenously added enzymes were required for ^{125}I-incorporation. In addition, the absence of ^{125}I-incorporation into several intracellular proteins showed that iodination occurred only on the cell surface. For example, proteins of mol wt 200 kdaltons (myosin?), 45 kdaltons (actin?), and 32 kdaltons were all major cellular proteins, were neither secreted nor depleted from cells following ionophore treatment, and were not radioiodinated under the conditions used for surface labeling. Moreover, cells lysed before radioiodination gave a completely different labeling pattern than did intact cells and the 45 kdalton mol wt protein, which is probably actin, became highly labeled (data not shown).

DISCUSSION

In this report we have demonstrated by use of SDS PAGE that about one-half of the major iodinatable proteins of the PMN cell surface co-migrated, as a set, with membrane and content proteins of SpG and AzG isolated from these cells. When we began these studies we anticipated that the non-ionophore-treated, resting cells would show one set of labeled proteins, and that degranulating cells would show a new set of granule-derived proteins. Unexpectedly, it was found that the surface of the resting cells was dominated by proteins that co-migrated with granule proteins. After degranulation, few new proteins appeared; instead, labeling of several of the previously existing surface proteins was increased.

The surface labeling pattern of resting peritoneal PMNs was similar but not identical to that recently obtained by Willinger and Frankel (6). Of particular interest were the three highly labeled proteins of mol wt 27, 96, and 145 kdaltons, which we suspect are the cell surface proteins identified by Willinger and Frankel (6) as bands 2 (mol wt ~150 kdaltons), 4 (mol wt ~90 kdaltons), and 9 (mol wt 25 kdaltons). When compared by SDS PAGE, we found that these highly labeled, cell surface proteins co-migrated with the 27-kdalton protein and the 96- and 145-kdalton glycoproteins of SpG membranes.

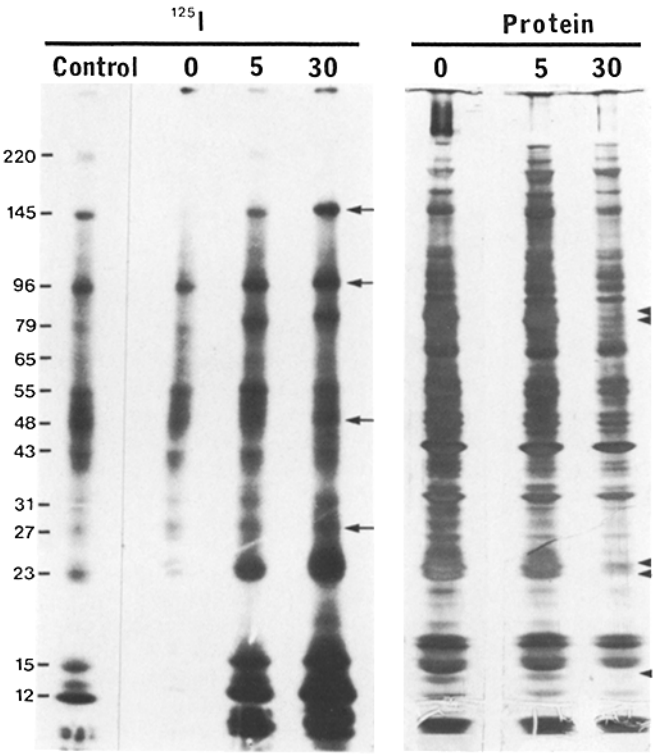

FIGURE 6 SDS PAGE analysis of degranulating PMNs trypsinized before degranulation. Cells were trypsinized, induced to secrete, and surface labeled with LPO-catalyzed ^{125}I as described in the legend for Fig. 4. The lane marked *Control* demonstrates the pattern of ^{125}I-labeled proteins on cells radioiodinated before degranulation and is compared to lanes showing pretrypsinized cells radiolabeled before (0), 5 min (5), and 30 min (30) after induction of degranulation. Lanes marked *Protein* show the corresponding silver-stained proteins of trypsinized, iodinated cells; arrowheads point to secretory proteins lost during degranulation. Arrows indicate cell surface proteins that showed increased labeling and that co-migrated with membrane proteins of specific and azurophilic granules.

TABLE I

Requirement of Exogenously Added Enzymes for Iodination of Rabbit PMNs

	TCA-precipitable cpm/10^6 cells	% Control
Complete reaction mixture	138,000	100
Minus lactoperoxidase	7,038	5.1
Minus glucose oxidase	1,900	1.4
Minus both enzymes	1,200	0.9

Rabbit peritoneal PMNs were incubated with or without enzymes, washed three times, TCA-precipitated, and ^{125}I-incorporation was determined.

To our knowledge, there have been few studies comparing cell surface proteins and secretory granule membrane proteins (3, 7–9), and even fewer in which the proteins were compared before and after exocytosis. In the course of this work, another report on cell surface changes in degranulating rabbit PMNs was published (10). Thrall et al. (10) found that radioiodination and electrophoretic analysis of rabbit peripheral blood PMNs resolved only four cell surface proteins compared to the 13 identified here and by Willinger and Frankel (6). Cell surface

changes that resulted from calcium ionophore treatment included the loss of a 44-kdalton protein and appearance of a 54-kdalton protein (10). We did not observe these changes. As we interpreted the gels from their experiments (Fig. 4, reference 10), however, there were two high molecular weight proteins of approximately 145 and 96 kdaltons that appeared on the cell surface during degranulation. (These are our own estimates based on the molecular weight markers available in this figure). We suspect that these may be similar to the granule-associated proteins identified in our study.

There are several explanations as to why the PMN cell surface might have many proteins in common with secretory granule membrane proteins. One is that granule and plasma membranes may be assembled from common precursors. Another is that as PMNs migrate from the bloodstream to the peritoneal cavity in response to chemotactic stimuli they release a portion of their granules, thereby incorporating granule membranes into the plasmalemma. Gallin and co-workers and others have demonstrated that treatment of cells with chemotactic peptides, which stimulate both human and rabbit PMNs to secrete specific granule contents (11–13), leads to an increase in the number of cell surface receptors for these chemotactic peptides after stimulation (14). These workers have also shown that isolated SpG contain chemotactic peptide receptor activity (15); and Wright and Gallin (16) have demonstrated that human PMNs obtained from peritoneal exudates contained more chemotactic receptors on the cell surface than did peripheral blood PMNs. Thus, it would appear that specific granule membranes are added to the plasmalemma during PMN chemotaxis. A similar mixing of secretory granule and plasma membranes has been reported in other cell types (9, 17).

It should be emphasized that the plasmalemma of rabbit peritoneal PMNs, even after addition of extensive amounts of granule membranes during exocytosis, had at least four or five iodinated cell surface proteins for which there were no identifiable counterparts in purified SpG and AzG membranes. The quantitative data indicated that each of these proteins incorporated a uniform amount of ^{125}I before and after secretion. One interpretation of these results is that these proteins were unique to the plasma membrane; and, therefore, even after secretion, the plasma membrane was not simply the sum of SpG and AzG membrane components. Since others have shown that PMNs and other cell types internalize large amounts of plasma membrane to compensate for the addition of secretory granule membranes (18–20), these observations suggest that in order to retain a constant number of some cell surface polypeptides a nonrandom internalization of membranes following secretion must occur.

Although our experimental approach is somewhat different from that used by Muller et al. (21) in their studies on membrane recycling in macrophages, our results, in one respect, are quite similar. By use of one-dimensional SDS PAGE analyses of radiolabeled membranes, these investigators determined that phagolysosomal membrane proteins were essentially identical to those of the plasma membrane. As those authors pointed out, it was somewhat surprising that no new proteins derived from lysosomal membranes appeared in the phagolysosomes. One explanation for their results was that the proportion of lysosomal membrane to plasma membrane in the phagolysosomes was so low that the lysosomal proteins would be difficult to detect against the high background of other proteins. Another possibility, which would be consistent with our results, is that the membrane proteins of the primary lysosomes are similar to plasma membrane proteins.

Finally, the presence of putative granule content proteins on the cell surface deserves mention. The possibility that binding of these content proteins was due to nonspecific adsorption cannot be ruled out; the alterations in the cell surface, however, resulting from the adhesion of content proteins may not be trivial but could reflect specific functions of these proteins. For example, it has been shown that a variety of PMN granule content proteins, including cationic proteins (22–24) and lysozyme (25), are capable of modulating PMN responses to inflammatory stimuli. Therefore, secreted granule proteins could serve as mediators of feedback control by interacting with the cell surface.

We thank Drs. Richard G. W. Anderson, Anthony Bretscher, and Fred Grinnell for helpful discussions and criticisms, and Ms. Helen Patterson and Ms. Alicia Benitez for their excellent help in preparation of the manuscript.

The research was supported in part by grants from the U.S. Public Health Service (GM 25661 to W. J. Snell) and the Veterans Administration Medical Research Service (to W. A. Shannon).

This work constitutes a part of a doctoral thesis submitted by William J. Brown to the faculty of The University of Texas Health Science Center, Dallas, Texas.

Received for publication 19 August 1982, and in revised form 29 November 1982.

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