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Isolation of human neutrophil plasma membranes employing neutrophil cytoplasts and changes in the cell-surface proteins upon cell activation

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A plasma membrane fraction, highly enriched in 5′-nucleotidase activity, was prepared from human neutrophils by disruption of previously formed neutrophil cytoplasts (enucleated neutrophils), which were devoid of intracellular organelles. This plasma membrane fraction shows an extremely low contamination by specific and azurophilic granule markers as compared to previous reported preparations. Nevertheless, a novel tertiary granule (Mollinedo, F. and Schneider, D.L. (1984) J. Biol. Chem. 259, 7143–7150), unlike specific and azurophilic granules, fuses partially with the cell surface under the experimental conditions used for cytoplast preparation. Comparison between the external cell-surface proteins in resting neutrophils and neutrophil cytoplasts by lactoperoxidase-catalyzed iodination showed some differences both in deletion and in addition of proteins. In resting cells, iodine was incorporated into at least 13 proteins ranging in size from over 200 to 30 kDa. A 140 kDa polypeptide, representing the major labeled surface component in resting neutrophils, was absent from cytoplasts. Furthermore, high-molecular-weight proteins (110 and over 160 kDa were more exposed to iodination after cytoplast preparation. Activation of human neutrophils by *N*-formylmethionylleucylphenylalanine induced some alterations in the pattern of labeled cell-surface proteins, which correlated to a certain degree with those observed during cytoplast preparation.

Introduction

Neutrophilic polymorphonuclear leukocytes (neutrophils) are short-living cells, involved in protecting the host organism against invading microbes and in most acute immunologically induced tissue injury. These cells, upon activation by many different stimuli, perform a number of very specialized functions [1–4]: chemotaxis, cell adhesion, aggregation, phagocytosis, generation of reactive oxygen metabolites and secretion of granule components. In order to accomplish these functions the neutrophil surface must contain specific receptors, enzymes, transport systems and a machinery able to generate a signal responsible for the stimulus-response coupling. However, little information is available on the structural organization and biochemical characterization of the neutrophil plasma membrane. The major difficulty in these studies stems in the isolation of purified plasma membranes. For cells rich in lysosomes, such as neutrophils, the release of

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Abbreviations: ATPase, adenosine triphosphatase; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FMLP, *N*-formylmethionylleucylphenylalanine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecyl sulfate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

lysosomal hydrolases hampers the purification of intact plasma membranes.

A potentially new tool for studying different aspects of neutrophil function has been recently reported by Roos et al. [5] with the preparation of neutrophil cytoplasts. Biochemical and ultrastructural studies [5,6] indicate that these cytoplasts contain about one-third of the plasma membrane (outside-out) present in intact neutrophils, no nucleus and hardly any specific and azurophilic granules. On these grounds, cytoplasts might be an ideal starting material for plasma membrane isolation, avoiding the degradative action of the hydrolytic enzymes present in the neutrophil granules. Neutrophil cytoplasts provide also an ideal system to study neutrophil plasma membrane functions and cell activation processes. As neutrophil cytoplasts are able of opsonin recognition, superoxide anion generation, aggregation and cell adhesion, it is assumed that fusion of granules with plasma membrane is not required for the expression of these functions [5,6]. However, questions arise as to whether granule fusion with the plasma membrane has already taken place during cytoplast preparation. Thus, it cannot be ruled out the putative presence of membrane granule components on the cytoplast surface, which may play a key role in some neutrophil functions as postulated for superoxide anion generation [7–11].

In this paper, we have further characterized biochemically the neutrophil cytoplasts and used them to prepare highly purified plasma membranes from human neutrophils. Moreover, we have studied comparatively the cell-surface proteins from both resting neutrophils and neutrophil cytoplasts by lactoperoxidase-catalyzed iodination, in order to elucidate at what extension the resting neutrophil surface remains intact after the enucleation process.

Materials and Methods

Isolation of neutrophils

Heparinized fresh venous blood was obtained from healthy adult donors. Mononuclear cells were separated from granulocytes by centrifugation through Ficoll/Hypaque [12]. Neutrophils were subsequently purified by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes [13]. Cells were suspended in a buffered salt solution (Hepes buffer) comprising 150 mM NaCl/5 mM KOH/10 mM Hepes/1.2 mM $MgCl_2$/1.3 mM $CaCl_2$ (pH 7.5) or processed for cytoplast preparation.

Neutrophil cytoplasts preparation

Cytoplasts were prepared as described by Roos et al. [5] in the presence of proteinase inhibitors. In brief, neutrophils ($260 \cdot 10^6$ cells) were suspended in 2.5 ml phosphate-buffered saline (2.7 mM KCl/1.5 mM KH_2PO_4/137 mM NaCl/8 mM Na_2HPO_4 (pH 7.4)) containing 12.5% (w/v) Ficoll 70, 5 μg/ml cytochalasin B and the following proteinase inhibitors: 33 μM leupeptin, 35 μM antipain, 35 μM pepstatin A, 24 μg/ml chymostatin and 0.48 units aprotinin. Cells were preincubated for 5 min at 37°C and then the suspension was layered on a prewarmed (20 min at 37°C), discontinuous density gradient of 5 ml of 16% (w/v) Ficoll 70 on top of 5 ml of 25% (w/v) Ficoll 70, both in phosphate-buffered saline and containing 5 μg/ml cytochalasin B. Gradients were centrifuged at $83000 \times g$ (middle of the tube) for 30 min without refrigeration in a SW27 swing-out rotor, previously prewarmed for 2 h at 37°C. After centrifugation, three fractions were obtained: band I, corresponding to enucleated neutrophils or neutrophil cytoplasts (at the interface of the 12.5% and 16% Ficoll solutions); band II, corresponding to some intact neutrophils and cell debris (at the interface of the 16% and 25% Ficoll solutions); and band III, corresponding to plasma membrane vesicles filled with nuclei and granules (at the bottom of the tubes). Band I was diluted to 4-times its volume with Hepes buffer and centrifuged at $1200 \times g$ for 15 min. The resulting pellet (neutrophil cytoplasts) was resuspended again in 10 ml Hepes buffer and centrifuged at $300 \times g$ for 15 min.

Plasma membrane isolation

Neutrophil cytoplasts prepared as above were resuspended to a final volume of 2 ml in 25 mM Tris-HCl (pH 7.5) containing the following mixture of proteinase inhibitors: 25 μM leupeptin, 37 μM antipain, 33 μM pepstatin A, 65 μM TLCK, 25 μg/ml chymostatin, 0.11 units aprotinin, 5 mM EDTA. The cytoplast suspension was frozen at

−20°C, thawed, diluted with 5 ml of 25 mM Tris-HCl (pH 7.5) and homogenized by 15 strokes with a Potter-Elvehjem homogenizer. Membranes were collected by centrifugation at 77000 × *g* (middle of the tube) for 90 min at 4°C, resuspended in 7 ml of 25 mM Tris-HCl (pH 7.5) and rehomogenized by 14 strokes. Plasma membranes were finally pelleted by recentrifugation at 77000 × *g* (middle of the tube) for 90 min at 4°C, resuspended in 1 ml of 25 mM Tris-HCl (pH 7.5) and used for enzymatic analysis.

Cell-surface labeling by lactoperoxidase-catalyzed iodination

Resting and activated human neutrophils were resuspended to $24 \cdot 10^6$ cells/ml in ice-cold Hepes buffer containing 5.5 mM glucose. 1 ml of this cell suspension was incubated with 10 μg lactoperoxidase, 2 μg glucose oxidase and 100 μCi carrier-free $Na^{125}I$. To minimize pinocytosis and internalization of label, iodination was carried out for 10 min at 4°C. Reaction was terminated by addition of 7 ml phosphate-buffered saline supplemented with unlabeled 0.5 mM NaI and 0.5 mM $Na_2S_2O_5$. Cells were collected by centrifugation at 250 × *g* for 7 min and washed twice with 8 ml phosphate-buffered saline supplemented with unlabeled 0.5 mM NaI and 0.5 mM $Na_2S_2O_5$. Finally, cells were washed with 4 ml of phosphate-buffered saline and pelleted by centrifugation at 250 × *g* for 7 min. Samples to be analyzed by SDS-polyacrylamide gel electrophoresis were treated as described below. Samples to be assayed for acid-precipitable radioactivity were stored frozen as pellets. To determine trichloroacetic acid-insoluble counts, 500 μg of bovine serum albumin in 1 ml of water was added as carrier to cell pellets and the samples were stirred by vortex and subsequently precipitated with 200 μl of 40% (w/v) trichloroacetic acid. After incubation at room temperature for 20 min, pellets were obtained by centrifugation and radioactivity was counted in sediment and supernatant.

Polyacrylamide gel electrophoresis and autoradiography

Electrophoresis was performed on slab gels according to the method of Laemmli [14]. Samples were heated at 100°C for 5 min in a buffer comprising 50 mM Tris-HCl (pH 7.0)/2% SDS/5% 2-mercaptoethanol/0.012% Bromophenol blue/10% glycerol. The acrylamide concentration of the stacking gel was 3.2% and the separating gel was 7%. Electrophoresis was carried out at a constant current of 10 mA. To visualize proteins, gels were fixed and stained with 0.25% Coomassie blue R250 in 50% methanol/7% acetic acid and decolorized in 40% methanol/7% acetic acid. Gels were dried onto Whatman 3MM filter paper and exposed to Kodak X-OMAT-S film and autoradiographs were developed on a commercial X-ray film processor. Densitometric records of the autoradiographs were obtained by using a Joyce Loebl Cromoscan 3.

Activation of neutrophils and neutrophil cytoplasts

Human neutrophils were resuspended to $24 \cdot 10^6$ cells/ml in Hepes buffer containing 5.5 mM glucose. This cell suspension was incubated with cytochalasin B (5 μg/ml) at 37°C for 5 min. Subsequently, the activating agent was added at the final concentration indicated in the legend of the figure and the suspension was incubated at 37°C for an additional 5 min. Then, cells were pelleted by centrifugation at 250 × *g* for 7 min, washed twice with 2 ml of Hepes buffer containing 5.5 mM glucose and the final pellet was resuspended in 1 ml of the same buffer and processed for labeling as described above. Cell activation was performed in the presence of the following proteinase inhibitors: 7.4 μM antipain, 21.9 μM pepstatin A, 15 μg/ml chymostatin and 0.3 units aprotinin. These proteinase inhibitors, used at the concentrations above indicated, do not affect neutrophil activation [15], but may inhibit proteolytic damage on cell-surface proteins by secreted proteinases.

Enzyme assays

Lactate dehydrogenase [16], lysozyme [17] and peroxidase [18] were assayed as described previously. β-Glucuronidase was assayed at pH 4.5 with phenolphthalein glucuronidate as substrate as described [19]. *N*-acetyl-β-D-glucosaminidase was determined at pH 4.5 using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate [20]. Mg^{2+}-dependent ATPase and DCCD sensitive ATPase were assayed in the absence and in the presence of

0.1 mM DCCD as previously described [21]. Oligomycin-sensitive ATPase was determined by preincubating the samples with 1 μg/ml oligomycin for 5 min at 37°C before measuring ATPase activity as described [21]. 5′-Nucleotidase (AMPase) was measured after 2 h incubation in the presence of AMP as described [22]. Alkaline phosphatase (orthophosphoric monoester phosphorylase) was assayed with 5 mM *p*-nitrophenyl phosphate as substrate in 1 ml of 1% Triton X-100/5 mM $MgCl_2$/200 mM Tris (pH 10.45) as described [23].

Gelatinase activity was measured using radiolabeled gelatin by slight modification of the described method [24]. Briefly, samples were preincubated for 15 min at 37°C with 3.7 mM diisopropylfluorophosphate and 0.1 mM Ep-475 to inhibit serine and thiol proteinase [25]. Then, 1.7 mM *p*-aminophenylmercuric acetate was added to activate the latent form of gelatinase [26,27]. After 1 h at room temperature, radioactive gelatin was added and the assay was performed in the presence of 7.2 mM $CaCl_2$. The mixture was incubated for 1 h at 37°C, and then the reaction was stopped by addition of 1 mg bovine serum albumin and 13% (w/v) trichloroacetic acid. After 5 min centrifugation in a Beckman Microfuge, radioactivity was measured both in sediment and supernatant.

One unit of enzyme activity is defined as that amount which liberates 1 μmol of product per min.

Measurement of protein concentration

Protein was determined after precipitation with trichloroacetic acid using the Folin reagent [28] as described [29].

Statistical analysis

Results are expressed as mean ±S.E. of the number of experiments indicated.

Results

Biochemical analysis of human neutrophil cytoplasts

The cell content of protein and several enzymes measured in resting human neutrophils is listed in Table I. Due to the heterogeneity reported in azurophilic granules [18,30], we have used three markers for these organelles, namely, peroxidase, β-glucuronidase and *N*-acetyl-β-D-glucosaminidase.

TABLE I

CELL CONTENT OF PROTEIN AND ENZYMATIC MARKERS IN HUMAN NEUTROPHILS

Neutrophils were highly purified by Ficoll-Hypaque centrifugation as described in Materials and Methods. Data represent mean ±S.E. for the number of independent preparations indicated in parentheses.

Component	Specific content (nmol/min per mg protein)
Alkaline phosphatase	27.3 ± 2.7 (11)
5′-Nucleotidase	0.72 ± 0.13 (9)
Lactate dehydrogenase	386.1 ±38.5 (14)
Peroxidase	139.1 ± 7.31 (3)
β-Glucuronidase	1.73 ± 0.27 (13)
N-Acetyl-β-D-glucosaminidase	49.6 ± 5.6 (10)
Mg^{2+}-dependent ATPase	19.3 ± 1.5 (8)
DCCD-sensitive ATPase	8.7 ± 1.3 (7)
Oligomycin-sensitive ATPase	1.60 ± 0.57 (3)
Lysozyme	26.4 ± 4.1 [a] (14)
Protein	52 ±12 [b] (9)

[a] μg equiv./mg protein.
[b] μg/10^6 cells.

Centrifugation of neutrophils on the Ficoll/cytochalasin B gradient, as described in Materials and Methods, rendered three bands, named I, II and III (Fig. 1). Band I, corresponding to the cytoplast fraction, was found to be slightly enriched in 5′-nucleotidase (1.45-fold), a widely accepted plasma membrane marker [31–33]. Other enzymatic activities were also enriched in this fraction, such as alkaline phosphatase (1.05-fold), Mg^{2+}-dependent ATPase (1.53-fold), DCCD-sensitive ATPase (1.65-fold) and the cytosolic marker lactate dehydrogenase (1.75-fold). On the other hand, granule markers were negatively enriched in this fraction: lysozyme (specific granules), 0.19-times; β-glucuronidase, *N*-acetyl-β-D-glucosaminidase and peroxidase (azurophilic granules), 0.15-; 0.12- and 0.05-times, respectively. Taking into consideration that cytoplasts accounted for about 31% of the total cell protein, the above results indicated that this fraction was practically devoid of granules, whereas it contained 54% of the total cell lactate dehydrogenase and 45% of the total 5′-nucleotidase activity. Interestingly, cy-

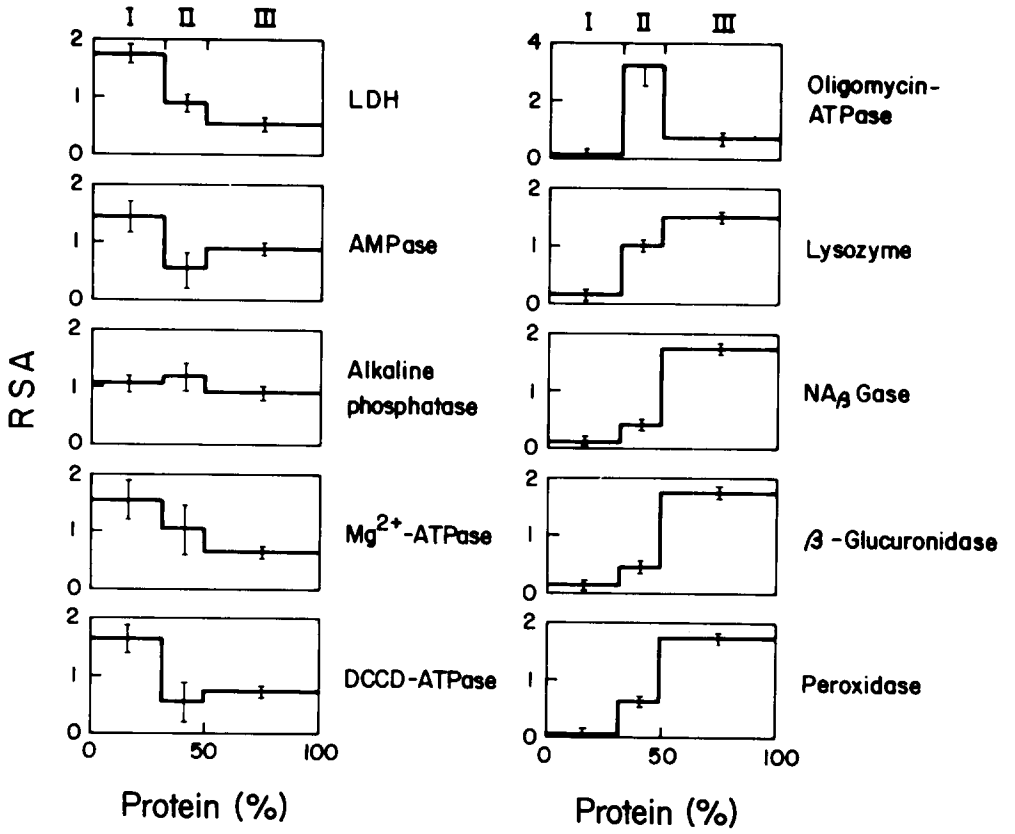

Fig. 1. Distribution patterns of enzymes after cytoplast preparation from human neutrophils. Cells were processed as described in Materials and Methods and three fractions (I, II and III) were obtained. RSA (relative specific activity) is calculated by dividing the percent activity in a fraction by the percent protein in that fraction. Values are shown as mean values ±S.E. of at least three independent determinations. The percentages (±S.E.) of recovered activities were: lactate dehydrogenase (LDH), 98% (±9); 5′-nucleotidase (AMPase), 109% (±22); alkaline phosphatase, 96% (±4); Mg^{2+}-ATPase, 102% (±19); DCCD-sensitive ATPase, 94% (±16); lysozyme, 85% (±9); *N*-acetyl-β-D-glucosaminidase (NAβGase), 71% (±4); β-glucuronidase, 67% (±8); peroxidase, 110% (±31).

toplasts accounted for about 50% of the total ATPase, an enzymatic activity localized in the plasma membrane [21,34,35] as well as in the membranes of the cytoplasmic granules [21,35,36]. In contrast, band III accounted for the 76% of the total lysozyme and for the 88% of the total β-glucuronidase, *N*-acetyl-β-D-glucosaminidase and peroxidase activities.

Despite the well-known scarcity of mitochondria in mature neutrophils [7,21,31,35–38], we assayed oligomycin-sensitive ATPase as a marker for mitochondria. Oligomycin used at 1 μg/ml, which inhibits more than 90% of the mitochondrial ATPase [39], had little effect on neutrophil and cytoplast ATPase activity, 8.3% (±3.0) and 3.1% (±1.6) inhibition, respectively. Furthermore, as shown in Fig. 1, cytoplasts accounted for less than 5% of the total oligomycin-sensitive ATPase, demonstrating that the ATPase activity localized in neutrophil cytoplasts was not of mitochondrial origin.

Fusion of intracellular granules with the cell surface during neutrophil cytoplast preparation

As shown in Table II, fusion of specific and azurophilic granules with the plasma membrane does not take place under the experimental conditions used for preparation of cytoplasts. However, a novel tertiary granule has been recently reported in human neutrophils [21,27], which readily fuses with the cell surface. This tertiary granule contains gelatinase as a soluble marker [21,27] and cyto-

TABLE II

EXTRACELLULAR RELEASE OF CYTOPLASMIC GRANULE MARKERS DURING CYTOPLAST PREPARATION FROM HUMAN NEUTROPHILS

Human neutrophils were incubated at 37°C for 5 min under the experimental conditions described in Materials and Methods for cytoplast preparation in the absence of protease inhibitors. Cells were then pelleted and enzymatic activities were measured in sediment and supernatant. Values are shown as mean values ±S.E. of at least three independent determinations.

Marker	Release (%)
β-Glucuronidase	0.88 ± 0.44
N-Acetyl-β-glucosaminidase	2.25 ± 0.86
Peroxidase	0.85 ± 0.21
Lysozyme	9.55 ± 3.27
Gelatinase	37.70 ± 5.76

chrome *b*, Mg^{2+}-dependent ATPase and DCCD-sensitive ATPase in its membrane [21,36]. As shown in Table II, a significant amount of gelatinase activity is released during cytoplast formation.

Plasma membrane preparation from neutrophil cytoplasts

Disruption of cytoplasts was carried out as described in Materials and Methods. Measurements of lactate dehydrogenase activity indicated that more than 99.6% of the cytoplasts were disrupted following this process. As shown in Table III, the final plasma membrane fraction contained only 1.6% of the total cell protein and about 27% of the total 5′-nucleotidase activity, achieving an overall enrichment of about 17-fold. *N*-Acetyl-β-D-glucosaminidase, β-glucuronidase and lysozyme were negatively enriched, 0.14-;, 0.16- and 0.5-times respectively. These data indicate that the enrichment of 5′-nucleotidase (i.e., plasma membrane) with respect to the markers for azurophilic and specific granules was 120- and 37-times, respectively. The plasma membrane protein represents 6.7% (±0.9) of the total protein found in neutrophil cytoplasts.

Lactoperoxidase-catalyzed iodination of resting human neutrophils and neutrophil cytoplasts

We studied the external cell surface proteins in resting and enucleated neutrophils by lactoperoxidase-catalyzed iodination in order to find out more about the relationship between both surfaces. Viability of cells and cytoplasts after labeling was 95% and 90% respectively, as checked by Trypan blue exclusion.

Since neutrophils contain in their azurophilic

TABLE III

ISOLATION OF PLASMA MEMBRANE FRACTION BY NEUTROPHIL CYTOPLAST DISRUPTION

Values are percentages of total cell activity ±S.E. from three different preparations. Recoveries (±S.E.) of cytoplast preparation from resting human neutrophils and of plasma membrane isolation from cytoplasts were, respectively: protein, 93 (±8) and 85% (±6); alkaline phosphatase, 96 (±4) and 91% (±5); 5′-nucleotidase, 96 (±4) and 94% (±6); lactate dehydrogenase, 93 (±11) and 92% (±11); β-glucuronidase, 70 (±7) and 82% (±17); *N*-acetyl-β-D-glucosaminidase, 73 (±6) and 80% (±5); Mg^{2+}-ATPase, 102 (±19) and 83% (±1.5); DCCD-sensitive, Mg^{2+}-ATPase, 94 (±16) and 82% (±9) lysozyme, 80 (±9) and 84% (±23).

Enzyme	Percentage of total enzyme activity				
	Neutrophil	Cytoplasts	(Enrichment) (×)	Plasma membrane	(Enrichment) (×)
Protein	(100)	31.0 ±2.0		1.61 ± 0.21	
Alkaline phosphatase	(100)	31.8 ±0.9	(1.03)	22.8 ±0.1	(14.2)
5′-Nucleotidase	(100)	38.8 ±6.5	(1.25)	27.1 ±0.4	(16.8)
Lactate dehydrogenase	(100)	53.6 ±3.8	(1.73)	0.21 ± 0.02	(0.13)
β-Glucuronidase	(100)	3.14 ± 1.19	(0.10)	0.27 ± 0.06	(0.16)
N-Acetyl-β-D-glucosaminidase	(100)	3.74 ± 1.81	(0.12)	0.23 ± 0.07	(0.14)
Mg^{2+}-ATPase	(100)	42.6 ±3.3	(1.37)	23.0 ±2.5	(14.3)
DCCD-sensitive, Mg^{2+}-ATPase	(100)	42.6 ±5.4	(1.37)	25.1 ±0.9	(15.6)
Lysozyme	(100)	4.82 ± 1.46	(0.15)	0.77 ± 0.14	(0.47)

granules myeloperoxidase, which is able of catalyzing the covalent bonding of halogens to proteins [40], it was essential to demonstrate the requirement of exogenously added enzymes for labeling. The results shown in Table IV indicate that addition of exogenous enzymes is necessary for cell labeling, in spite of the capacity of these cells to trap free iodine. We found in eight different experiments that only 17.1% (±1.4) of the cell-associated ^{125}I was trichloroacetic-acid-precipitated, even after repeated washing the cells with excess unlabeled iodine.

TABLE IV

DEPENDENCE OF ^{125}I INCORPORATION INTO NEUTROPHILS ON EXOGENOUSLY ADDED ENZYMES

$24 \cdot 10^6$ neutrophils were incubated in 1 ml Hepes/glucose buffer containing 100 μCi ^{125}I with or without enzymes and processed as described in Materials and Methods.

	cpm/10^6 cells	%
Complete reaction mixture	1254460	100
Minus lactoperoxidase	186914	14.9
Minus glucose oxidase and lactoperoxidase	27450	2.2

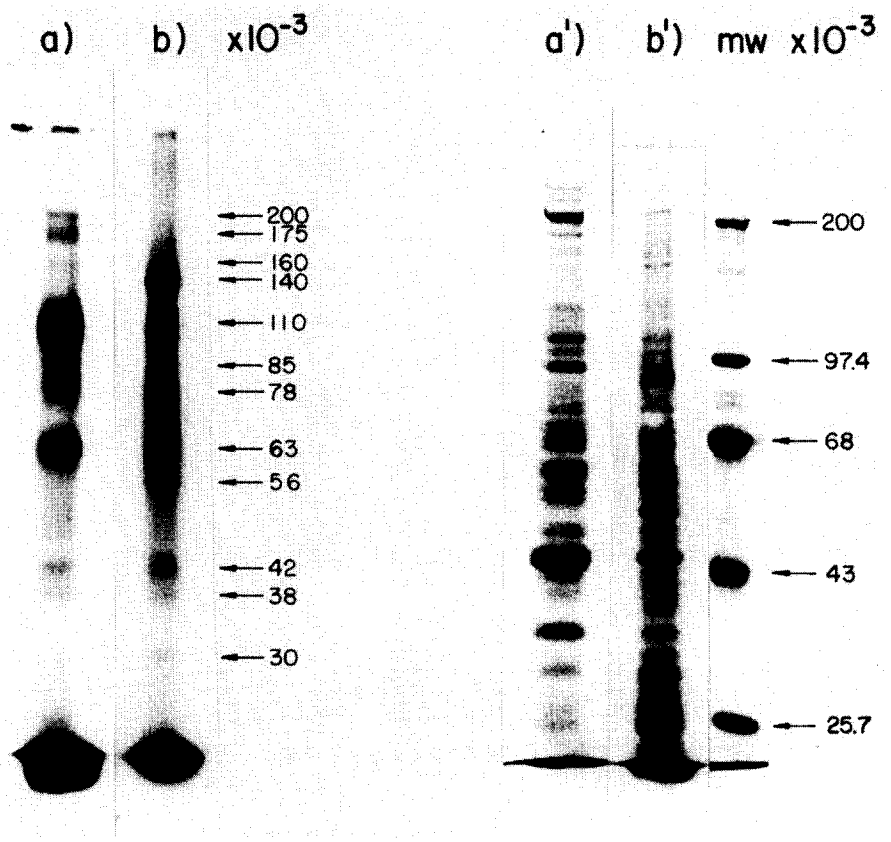

Fig. 2. SDS-polyacrylamide gel electrophoresis of human neutrophils and neutrophil cytoplasts labeled by lactoperoxidase-catalyzed iodination. Whole cells and cytoplasts were prepared, labeled and electrophoresed as described in Materials and Methods. The samples on the autoradiograph are designed a–b, while those on the Coomassie blue-stained gel, a′–b′. (a, a′) Resting neutrophil cytoplasts prepared in the presence of proteinase inhibitors. (b, b′) Resting human neutrophils. An equal number of cells and cytoplasts were loaded on each lane to allow direct comparisons of cell-surface changes. The lane marked mw contained the following molecular weight markers: myosin (H chain); 200000; phosphorylase *b*, 97400; bovine serum albumin, 68000; ovalbumin, 43000; α-chymotrypsinogen, 25700. For further details see Materials and Methods.

Extraction of the trichloroacetic-acid-insoluble radioactivity with chloroform/methanol (2 : 1, v/v) at 25°C released only 14% of the labeled material, indicating that very little iodine was incorporated into lipid.

A comparative analysis of labeled proteins from resting human neutrophils and neutrophil cytoplasts by SDS-gel electrophoresis is shown in Fig. 2. The molecular weights of the labeled cell surface proteins in resting human neutrophils from four different experiments were: > 200 000; 200 000; 175 000; 160 000; 140 000; 110 000; 88 000–85 000; 78 000; 63 000; 56 000; 42 000; 38 000 and 30 000. Homogenization of neutrophils prior to iodination rendered a very different pattern of labeled proteins (results not shown), being the iodine incorporated mostly into low-molecular-weight proteins, with a prominent band that migrated like actin. These results, together with the fact that major cell proteins stained by Coomassie blue were not labeled by lactoperoxidase-catalyzed iodination, indicated that only the external surface of neutrophils was iodinated.

Although most of the labeled proteins present in resting cells were visualized also in cytoplasts, several differences are observed in Fig. 2. These

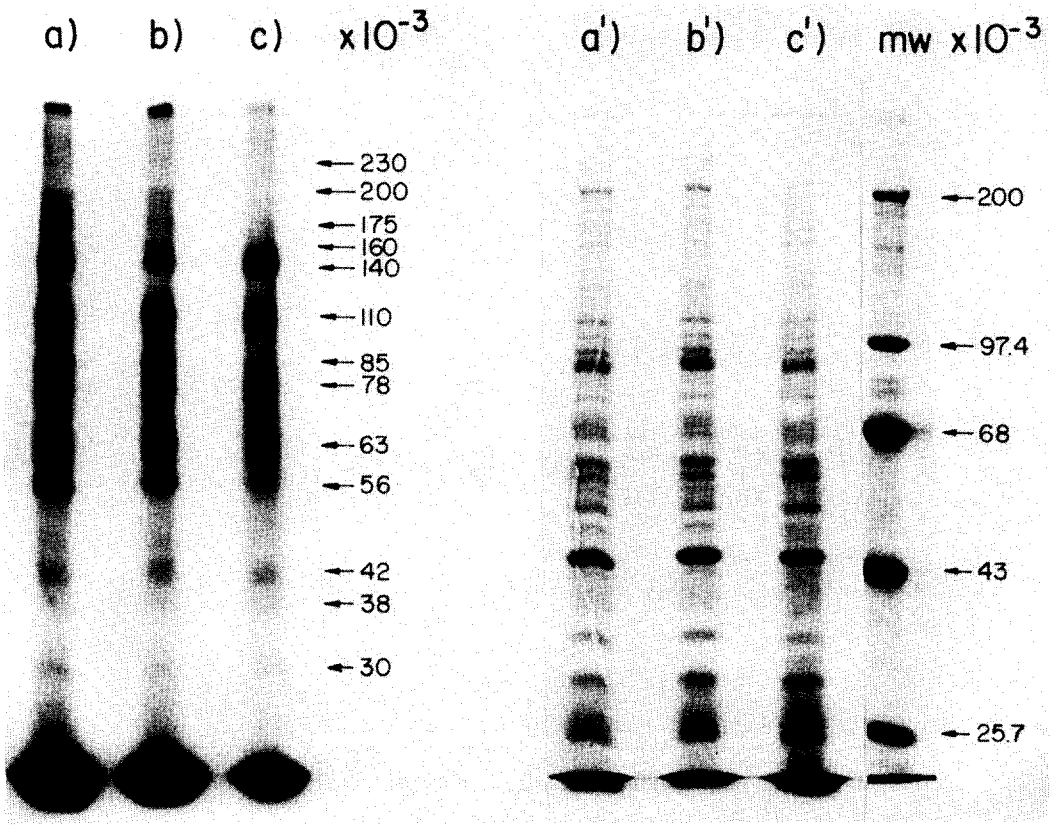

Fig. 3. SDS-polyacrylamide gel electrophoresis of resting and stimulated human neutrophils labeled by lactoperoxidase-catalyzed iodination. Whole treated and untreated cells were prepared, labeled and electrophoresed as described in Materials and Methods. The samples on the autoradiograph are designated a–c, while those on the Coomassie-blue-stained gel, a′–c′. (a, a′) Neutrophils activated with 10^{-7} M FMLP in the presence of cytochalasin B (5 μg/ml). (b, b′) Neutrophils activated with $1 \cdot 10^{-9}$ M FMLP in the presence of cytochalasin B (5 μg/ml). (c, c′) Resting neutrophils. An equal number of cells were loaded on each lane to allow direct comparisons of cell-surface changes. The lane marked mw contained the following molecular weight markers: myosin (H chain), 200 000; phosphorylase *b*, 97 400; bovine serum albumin, 68 000; ovalbumin, 43 000; α-chymotrypsinogen, 25 700. For further details see Materials and Methods.

latter can be summarized as follows: (a) the most intense band in the autoradiograph of labeled resting cells (M_r 140000) was absent or highly diminished in cytoplasts; (b) the main labeled component in cytoplasts corresponded to a protein of M_r 110000; (c) high molecular proteins of $M_r > 160000$ were consistently more labeled in cytoplasts than in resting cells.

Coomassie-blue-stained gels indicated that cytoplasts were to a great extent devoid of low-molecular-weight polypeptides (compare Figs. 2a′ and 2b′), which represent most of the cytoplasmic granule constituents in human neutrophils.

Alterations in plasma membrane proteins upon cell activation

Full activation of human neutrophils with $1 \cdot 10^{-7}$ M FMLP [41] induced several changes in the labeling pattern of cell-surface proteins (Figs. 3 and 4): (a) decrease in the intensity of the M_r 140000 band; (b) the intensities of the bands with M_r 56000, 88000–85000 and 110000 were noticeably increased; (c) high-molecular-weight proteins ($M_r > 160000$) were more exposed to iodination after cell stimulation. Interestingly, similar changes occur in a minor extension even at $1 \cdot 10^{-9}$ M FMLP (Fig. 3b), under conditions where only chemotaxis is induced [41].

Unlike whole cells, activation of neutrophil cytoplasts with $1 \cdot 10^{-7}$ M FMLP did not induce any change in the labeled banding pattern (Mollinedo, F., unpublished data).

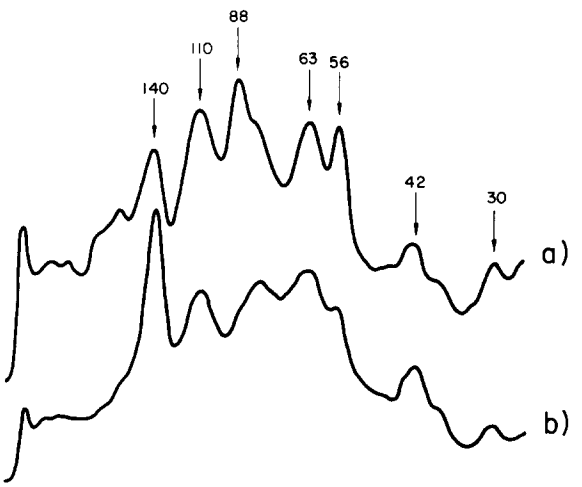

Fig. 4. Comparison of iodinated cell surface proteins in resting and activated cells by SDS-polyacrylamide gel electrophoresis. Densitometric scans are from autoradiographs shown in Fig. 3a and c. (a) Activated neutrophils by 10^{-7} M FMLP in the presence of cytochalasin B (5 μg/ml). (b) Resting neutrophils. Migration was from left to right (+). For further details see Materials and Methods. Numbers indicate molecular weights of the most representative bands shown in the autoradiographs of Fig. 3.

Discussion

The method described here for plasma membrane purification from human neutrophils by disruption of enucleated neutrophils is rapid and simple. This method offers several advantages over previous reported procedures [7,31,42–47]. First, the enrichment of the plasma membrane marker 5′-nucleotidase reaches up to 17-fold, indicating a highly purified preparation. Second, this plasma membrane fraction shows the lowest contamination, so far reported, by granule markers. Enrichment of plasma membrane markers with respect to azurophilic granule markers was over 120-times, whereas previous reported procedures for plasma membrane preparation from neutrophils of different origins rendered relative enrichments of plasma membrane to azurophilic granule markers between 15–50-times [7,45–47].

However, release of gelatinase and appearance of new cell-surface proteins during preparation of cytoplasts suggests that some membrane components of a gelatinase-rich tertiary granule [21,27,36] are incorporated into the plasma membrane. This insertion of tertiary granule membrane into the cell surface could contribute to: (a) the high enrichment of ATPase activity in the cytoplast membrane; (b) the higher recovery of lactate dehydrogenase activity (cytosol) as compared to the plasma membrane marker 5′-nucleotidase in cytoplasts. Despite tertiary granule-membrane fusion occurs during cytoplast preparation, this does not lead in itself to cell activation, and addition of an activating agent is required. In this regard, the results herein reported support the notion that neutrophil activation is accompanied by early fusion of tertiary granules with plasma membrane [9,36], but further events are necessary for full activation.

A high enrichment in alkaline phosphatase was achieved in the plasma membrane fraction.

Whereas alkaline phosphatase has been clearly localized in the specific granules of rabbit neutrophils [48], the subcellular localization of this enzyme in human neutrophils is controversial. Some workers have found this enzymatic activity in a membrane fraction [7,18,30,45], putatively plasma membrane. As a matter of fact, this activity has been used as a marker for plasma membrane in subcellular fractionation [45] and in cytoplast formation [5]. Nevertheless, Peters et al. [49–53], using a combination of analytical subcellular fractionation and electron microscopic cytochemistry, have localized alkaline phosphatase activity to the membrane of a novel low-density cytoplasmic organelle termed phosphasome. Thus, by analogy with tertiary granules, the high enrichment in alkaline phosphatase found in the plasma membrane fraction could be due to partial fusion of those light organelles with the cell surface.

The present study also defines the distribution of cell surface polypeptides of enucleated, resting and activated neutrophils. This paper represents the first comparative study of the cell-surface proteins of resting cells and cytoplasts from human neutrophils. This is of particular importance, since cytoplasts have been recently used to elucidate the role of plasma membrane and granules in different neutrophil functions. These studies have rested on the assumptions that neutrophil cytoplasts are devoid of intracellular organelles and nucleus and also that the cell surface remains intact after cytoplast preparation.

The surface-labeling pattern of resting human neutrophils, with at least 13 proteins of M_r ranging from over 200 000 to 30 000, is very similar to that reported by Willinger and Frankel [54] and by Williams and Becker [42] in rabbit neutrophils. The major labeled band corresponds to a molecular weight of 140 000, which was also found as the main labeled band in the cell-surface labeling of rabbit peritoneal neutrophils [42,54]. Interestingly, we have found that labeling of this high-molecular-weight protein is abolished or greatly diminished after cytoplast preparation. The results herein reported indicate that the cell surfaces from resting and from enucleated neutrophils differ both in qualitative and quantitative terms. The main differences can be summarized as additions (M_r 110 000 and $> 160\,000$) and deletions (M_r 140 000) of proteins during cytoplast formation. It is noteworthy to observe that similar changes in the labeled cell-surface proteins occur during cytoplast formation and cell activation, suggesting that cytoplasts can be considered as a primed state in cell activation. At present, little is known about alterations in neutrophil cell-surface proteins [55–57] upon cell activation, and consequently several mechanisms (internalization of proteins, proteolytic degradation, fusion of granules with plasma membrane, redistribution of plasma membrane proteins, changes in protein accessibility to iodination) might explain the changes observed.

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