Preparation of secretory vesicle-free plasma membranes by isopycnic sucrose gradient fractionation of neutrophils purified by the gelatin method

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Abstract

Isolated human neutrophils serve as a model for the *in vitro* study of host defensive processes as well as the cell biology and biochemistry of primary human cells. We demonstrate that the requirements of the gelatin-based procedure for neutrophil isolation from whole blood induces the complete loss of secretory vesicles from *in vitro* isolated populations, whereas isolation by a dextran-based methodology results in the preservation of this organelle. Following density fractionation of cellular cavitates, examination of commonly employed plasma membrane marker activities yielded subcellular localization patterns that were indistinguishable between dextran- or gelatin-isolated populations, indicating both populations to be otherwise comparable in terms of the relative complexity and large-scale organization of plasma membranes. Given that the cell surface upregulation of secretory vesicles is implicated as an initial requirement of neutrophil activation as well as an intrinsic feature of neutrophil priming, we show that dextran and gelatin-isolated neutrophils may be considered to occupy functionally nonactivated and primed cellular states, respectively. These differences in phenotype can be exploited in specific ways. We suggest that the gelatin method has technical advantages with regard to the study of neutrophil plasma membranes. In particular, results from this study indicate the gelatin method to be a reliable and effective preparatory technique appropriate for tandem use with density fractionation procedures to achieve rapid isolation of plasma membranes that are uncontaminated by secretory organelles.

Abbreviations: AMP-5' NT – adenosine mono-phosphate-dependent-5' nucleotidase; AP – alkaline phosphatase; HLA – human leukocyte antigen; LF – lactoferrin; Mg^{++} ATPase – magnesium dependent adenosine tri-phosphatase; MPO – myeloperoxidase

Introduction

Neutrophils are widely recognized as the principle effectors of the innate immune response. The rapid recruitment and activation of circulating, quiescent leukocytes poses a unique battery of cellular, physiological and pathophysiological requirements on responding effector granulocytes, including the

secure sequestration, maintenance, and regulated release of preformed microbiocidal proteins and peptides. Circulating, quiescent neutrophils house three distinct granule subsets, including primary (azurophil), secondary (specific) and tertiary (gelatinase enriched) granules, as well as a stable endosomal subpopulation referred to as secretory vesicles (Borreagaard and Cowland 1997). The

mobilization of granules and secretory vesicles is differentially regulated, with the cell surface upregulation of each subset governed independently by specific thresholds of cellular activation. Pattern of release is, from the most-to-least readily mobilized, secretory vesicles > tertiary > secondary > primary granules (Sengelov et al. 1993). Selective and sequential mobilization confers progressive changes in cellular phenotype that underlie hallmark features of neutrophil defensive function *in vivo* (Faurschou and Borregaard 2003). Initial cell surface upregulation of secretory vesicles is required for neutrophil extravasation and is thus implicated as a general condition of neutrophil activation (Sengelov et al., 1995; Borregaard et al., 1994).

Secretory vesicles are plasma membrane-derived organelles formed during the latter stages of neutrophil development (Borregaard 1997). As would be expected, the physical properties of this organelle substantially overlap those of surface membranes and prevent purification of either fraction on basis of relative density (Borregaard et al. 1990). Detailed studies examining the biology of secretory vesicle dynamics within human neutrophils suggest that this organelle is inherently unstable within *in vitro* isolated cells and readily lost following exposure to various environmental (Borregaard et al. 1987) and/or physiologic factors (Borregaard et al. 1994). Thus, in many fields of neutrophil research that require extensive *in vitro* manipulation of isolated populations, including the study of priming (Condliffe et al. 1998), secretory vesicles are widely assumed to be uniformly upregulated to the cell surface of intact cells under study. However, this assumption, although convenient and justifiable, may not be as absolute as currently considered. For example, high-resolution electron microscopy techniques based on combined immuno- and enzyme-cytochemistry have been applied to visualize the dynamic behavior secretory vesicles within intact fMLF-stimulated human neutrophil populations and indicate the mobilization of this organelle to surface membranes may be more intricate and complex than is generally appreciated (Kobayashi and Robinson 1991). Other studies indicate cell surface mobilization kinetics of secretory vesicles can be substantially altered in a stimulus-specific manner (Ward et al. 2000). These studies suggest an unexpected complexity and variability associated with secretory organelle exocytosis and

underscore the importance of accounting for secretory vesicle loss in various experimental systems. Thus, a detailed accounting of the fate of this organelle following neutrophil isolation by a common gelatin-based procedure (Henson and Oades 1975), which is known to promote an intermediate stage of neutrophil activation, is appropriate. Findings in this regard could provide insight into the physiologic changes induced in neutrophils isolated by this method and may, as a result, additionally contribute to the clarification of previous controversial findings in neutrophil research.

In this study, we compare the differential dynamics of secretory vesicle mobilization induced following neutrophil isolation using two preparative methods that differ substantially. Procedures employing either gelatin or dextran to achieve primary separation of erythrocytes from leukocytes were used to isolate neutrophil populations from whole blood. Each method has distinct advantages that allow isolation of neutrophil populations in very different, if not reciprocal, cellular activation states (Stie and Jesaitis, submitted). In particular, the dextran-based approach is a relatively gentle method that largely preserves secretory organelles within isolated populations (Borregaard et al. 1990). Thus, neutrophils isolated by the dextran method may be considered to occupy a nonactivated or unprimed cellular state and were useful in this study as a control for examining the extent of secretory vesicle upregulation and the uniformity of their integration in surface membranes. In contrast, the conditions imposed by the gelatin procedure should promote LPS- as well as temperature-dependent changes in neutrophil physiology that could, in turn, induce loss of the secretory compartment in isolated populations. We demonstrate that the methodological requirements of the gelatin protocol are indeed sufficient to induce complete mobilization of secretory vesicles as well as their uniform integration into existing cell surface structure. Such populations may be considered operationally primed based on the complete absence of secretory vesicles (Faurschou and Borregaard, 2003; Borregaard et al., 1994). An additional novel aspect of this study includes the subcellular characterization of intact secretory vesicles relative to plasma membranes and major granule subsets by isopycnic sucrose density gradient sedimentation.

Materials

Blood separation plastics and chemicals

- (1) Conical sedimentation tubes (50 ml) (Cat. No.05-539-6).⁸
- (2) Conical sedimentation tubes (250 ml) (Cat. No.430776).⁶
- (3) Ammonium chloride (Cat. No.A5666).²⁰
- (4) Dextran T-500 (Cat. No. 1700320-02).¹
- (5) Dextrose (Cat. No. BP350).⁸
- (6) DFP, diisopropyl fluorophosphate (Cat. No. 30967).⁴
- (7) DPBS, Dulbecco's phosphate buffered saline
- (8) DPBS⁴⁺, DPBS supplemented with 0.9 mM MgCl₂, 0.5 mM CaCl₂, 0.1% BSA and 0.1% dextrose.
- (9) EDTA, ethylene-diaminetetraceticacid-disodium-salt-dihydrate (Cat. No. ED255).²⁰
- (10) Ficoll-Hypaque (Cat. No. 17-1440-02).¹
- (11) Gelatin (Cat. No. G7).⁸
- (12) Hemocytometer
- (13) Separatory funnel, 1 L (Cat. No. A-06126-60).¹⁶
- (14) Sodium bicarbonate (Cat. No. S-5761).²⁰
- (15) WFI (water-for-injection)-grade distilled water (Cat. No. SH30221).¹¹

Cell treatment and density gradient chemicals

- (16) EDTA, ethylene-diaminetetraceticacid-disodium-salt-dihydrate (Cat. No. ED255).²⁰
- (17) HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (Cat. No. H3375).²⁰
- (18) Iodine-125, carrier-free (Cat. No. IMS30).¹
- (19) PMSF, phenyl-methylsulphonyl-fluoride (Cat. No. 52332).⁴
- (20) Protease inhibitor cocktail (Cat. No. P8340).²⁰
- (21) Sucrose, ultrapure (Cat. No. 821721).¹²
- (22) WGA, wheat-germ-agglutinin (Cat. No. WO125).²⁰

Density gradient materials and equipment

- (23) Fraction collector
- (24) Gradient maker (Cat. No. SG50).¹⁰
- (25) Peristaltic pump
- (26) Sedimentation tubes, 30 ml polycarbonate (Cat. No. 355631).²
- (27) Swinging bucket rotor

Enzyme and protein assay materials, reagents and equipment

- (28) ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Cat. No. A1888).²⁰

- (29) Ammonium molybdate (Cat. No. A674).⁸
- (30) AMP, adenosine-mono-phosphate (Cat. No. A1752).²⁰
- (31) ATP, adenosine-tri-phosphate (Cat. No. A9187).²⁰
- (32) BCA (bicinchoninic acid) assay kit (Cat. No. 23228).¹⁸
- (33) BSA, bovine-serum-albumin (Cat. No. A2153).²⁰
- (34) Citric acid, monohydrate (Cat. No. C-1909).²⁰
- (35) Diethanolamine (Cat. No. D-8885).²⁰
- (36) Histidine (Cat. No. H8125).²⁰
- (37) Hydrogen peroxide, 30% solution (Cat. No. H323).⁸
- (38) Malachite green (Cat. No. M-9636).²⁰
- (39) Microtiter flat-bottom enzyme assay plates, Costar (Cat. No. 3370).⁶
- (40) Microtiter plate reader
- (41) *p*-Nitrophenylphosphate (Cat. No.1040).²⁰
- (42) Polyvinyl alcohol (Cat. No. P8136).²⁰
- (43) Triton-X-100 (Cat. No. T9284).²⁰

ELISA/Immunoblot materials, reagents and equipment

- (44) Acrylamide-bisacrylamide solution, 40% (Cat. No. BP 1408-1).⁸
- (45) Goat serum (Cat. No. 642921).¹⁵
- (46) HRP (horseradish-peroxidase-conjugated)-goat-anti-mouse IgG antibody (Cat. No. 170-6516).³
- (47) HRP (horseradish-peroxidase-conjugated)-goat-anti-rabbit IgG antibody (Cat. No. 170-6515).³
- (48) Maxi-sore immuno-plates (Cat. No. 439454).¹⁷
- (49) Microtiter plate reader
- (50) Mouse monoclonal anti-human CD 16 antibody (Cat. No. C2267-29).²¹
- (51) Mouse monoclonal anti-human HLA-class I antibody (Cat. No. H6098-39A).²¹
- (52) PVDF, polyvinylidene-difluoride, immobilon (Cat. No. IPVH00010).¹³
- (53) Pre-stained molecular weight standard proteins (Cat. No. 50550).⁵
- (54) Rabbit polyclonal anti-LF antibody (Cat. No. 670581).¹²
- (55) SDS, sodium-dodecyl-sulfide (Cat. No. BP166).⁸
- (56) Sheep anti-human β_2 microglobulin antisera (Cat. No. CR2010SAP).⁷
- (57) Supersignal chemiluminescent detection kit (Cat. No. 34080).¹⁸
- (58) Tween-20 (Cat. No. P1379).²⁰

Procedures

Neutrophil isolation

The dextran method of neutrophil isolation is performed essentially as described by Borregaard et al. (1983) with minor modification. One unit whole blood is gently mixed in a 1:1 volumetric ratio with 150 mM NaCl solution containing 3% dextran made with WFI-grade distilled water within an autoclaved 1 L separatory funnel. After 30–45 min at room temperature, the resulting suspension is centrifuged ($740\times g$, 10 min, brake off, 25 °C chamber) and pellets are resuspended in several milliliters of room temperature 150 mM NaCl solution and transferred into sterile 50 ml conical tubes. Additional saline (25 °C) is added to a final volume of 35 ml, and the mixture underlaid with 10 ml Ficoll-Hypaque medium for gradient sedimentation ($415\times g$, 25 min, brake off, 25 °C chamber). Neutrophil-rich pellets are resuspended in 25 ml ice-cold distilled water (WFI-grade) and agitated by repeated up/down pipetting for 20–25 s to facilitate lysis of residual red cells. Isotonicity is restored by addition of an equal volume of ice-cold 300 mM NaCl solution. One-to-two cycles are required to fully eliminate red cells. Cells are then sedimented ($360\times g$, 10 min, brake on, 4 °C chamber) and pellets finally resuspended in 25 ml DPBS⁴⁺. Suspensions are transferred to ice for 15 min treatment with 3 mM DFP. Prior to DFP addition, we remove a small aliquot of cells for cell count determination by hemocytometer. 3 mM DFP is then applied followed by 15-min incubation period on ice. DFP-treated suspensions are sedimented ($360\times g$, 10 min, brake on, 4 °C chamber), washed 1–2-times in the same buffer, and either resuspended in 10 ml DPBS⁴⁺ for radiolabeling (see below) or 5 ml cavitation buffer (0.34 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 0.1 mM MgCl₂, 1 mM ATP, 0.1 mM PMSF, pH 7.4, and protease inhibitor cocktail, added as recommended by manufacturer). We employ N₂ cavitation for cell disruption at 450 psi for 15 min at 4 °C.

Nuclei and cellular debris are removed from cavitates by low speed sedimentation ($890\times g$, 10 min, brake on, 4 °C chamber). In some cases, we additionally extract nuclear pellets in an iced Dounce homogenizer with 5 strokes of a type A pestle; the nuclear extract is re-sedimented as

above and the soluble fraction added to the appropriate low speed supernatant. Low speed supernatants from each population are separately pooled into a final volume of 5 ml and layered on top of an already prepared linear sucrose gradient (see below).

The gelatin method of neutrophil isolation is performed essentially as described by Henson and Oades (1975) with minor modification. One unit of donor blood is transferred into two 250 ml sedimentation tubes and sedimented at low speed ($260\times g$, 20 min, brake off, 25 °C chamber) to separate plasma and non-granular leukocytes from PMN/red cell-enriched pellets. PMN/red cell-enriched pellets are then transferred into a sterile 1 L separatory funnel. 500 ml (per unit blood) gelatin solution (2% gelatin in 150 mM NaCl), pre-warmed to 37 °C, is then added, some of which is used to recover remaining cells from sedimentation tubes. The separatory funnel is tightly sealed, rotated gently end-over-end, positioned upright with the aid of funnel holder, and transferred to a 37 °C incubator. After 30 min incubation, the separatory funnel is removed from the incubation chamber, the lid is loosened, and red cell pellets are removed through a stopcock fitted at the lower end. The upper phase of the suspension is transferred into two 250 ml sedimentation tubes, centrifuged ($360\times g$, 10 min, brake on, 25 °C chamber), and the cell pellets resuspended and pooled in a total volume of 75 ml ice-cold ammonium chloride lysis buffer (155 mM NH₄Cl, 9.4 mM NaHCO₃, pH 7.4, and 130 μ M EDTA). The cell suspension is then transferred to ice and incubated 5 min under mild agitation produced by periodically pipetting the mixture up and down. Treated suspensions are sedimented ($360\times g$, 10 min, brake on, 4 °C chamber), washed 1–2 times in 25 ml DPBS⁴⁺, and finally transferred to a sterile 50 ml conical tube for DFP treatment in the same buffer (see above). Cell counts, cell lysis, and preparation of low speed supernatants are as described above. It is essential that plastic-ware directly contacting blood during the course of gelatin isolation is not re-used during consecutive steps of this procedure (see Results and discussion Section).

Isopycnic sucrose gradient sedimentation

Sucrose solutions are made with 10 mM HEPES, pH 7.4. Linear gradients are formed using a gra-

dient maker¹⁰ with 11 ml 20% (w/w) and 11 ml 55% (w/w) sucrose solutions in the flow and mixing columns, respectively. Prior to pouring gradients, a 3 ml cushion of 60% (w/w) sucrose is added to the sedimentation tube on the receiving end. A peristaltic pump is employed to insure smooth, continuous flow during gradient formation. Gradients are formed at 4 °C and maintained at this temperature for 16–24 h prior to use. Low speed supernatants are layered on top of gradients and sedimentation tubes transferred into a Beckman² SW28 rotor and sedimented 3.5 h ($8.3 \times 10^4 \times g$, 3.5 h, 4 °C chamber). We then employ an Auto Densi-Flow IIC fraction collector⁹ at 4 °C to fractionate gradients. Fractions can be stored at –70 °C immediately following fractionation or maintained at 4 °C for immediate analysis. Fractions stored at –70 °C must be rapidly thawed in the presence of 0.1 mM PMSF and protease inhibitor cocktail, added as recommended by manufacturer, to prevent proteolysis, and subsequently equilibrated on ice prior to analysis.

Cell surface labeling

Iodine-125-conjugated wheat germ agglutinin [¹²⁵I] WGA was prepared as described previously (Jesaitis et al. 1983). Briefly, cells are suspended in 10 ml DPBS⁴⁺ and radiolabeled using 70 µg WGA per 5×10^8 cells with a total applied cpm of 2.5×10^3 per population. After 5 min on ice, cells are washed twice in the same buffer, cavitated, and fractionated as described above. We assessed the [¹²⁵I] WGA content of gradient fractions directly using a gamma counter (10/200 Plus¹²). The concentration of active [¹²⁵I] WGA applied per population in this study (1×10^4 cpm) represents the minimal amount to obtain acceptable signal/noise ratio and is well below previously reported levels applied without altering normal subcellular distribution pattern of plasma membranes (Quinn et al. 1989; Jesaitis et al. 1982).

Biochemical assays

Protein measurements are made using the BCA assay kit with BSA as a protein standard. The

sucrose density of gradient fractions is calculated by refractive index measurement.

Costar flat-bottom microtiter plates are used for the following biochemical assays. Mg⁺⁺ ATPase activity is determined by a modified approach based on two previously described methods (Henkel et al., 1988; Jesaitis et al., 1982). A 10 µl sample volume is added to 35 µl reaction buffer (40.5 mM histidine, 162 M NaCl, 16.2 mM KCl, and 17.4 mM MgCl₂) and assayed in the presence or absence of 5 mM ATP for 22 min at 37 °C. Termination is by addition of 200 µl per well of a 1:1:2:2 ratio of 5.72% ammonium molybdate, w/v, in 6 M HCl, 2.32% (w/v) polyvinyl alcohol, 0.0812% (w/v) malachite green, and distilled water, respectively. Reaction initiation/termination is staggered at 10 s intervals to permit synchronization. Terminated samples are allowed to stabilize 5 min at room temperature prior to colorimetric quantitation of free phosphate (630 nm). Measurements are acquired using a microtiter plate reader (SpectraMax 250).¹⁴ The amount of inorganic phosphate released is calculated based on analysis of a standard series of phosphate solutions with linear range of detection between 1 and 10 nanomoles phosphate.

AMP-5' NT activity is assayed as described by Emmelot and Bos (1966) but modified to allow timed initiation of 50 µl reactions by final addition of 11 mM AMP. Synchronization, reaction termination, and phosphate quantitation are as described above.

MPO activity is directly measured from 40 µl sample volumes following addition of 25 µl reaction buffer (50 mM citric acid, pH 4.2, 15 µM hydrogen peroxide, 0.0275% ABTS) by microtiter plate reader¹⁴ at wavelength 405 nm.

AP activity is assayed using *p*-nitrophenylphosphate as substrate (DeChatelet and Cooper 1970) in the presence or absence of 0.1% Triton X-100. 20 µl samples are added to microtiter plate wells containing 30 µl of a reaction buffer consisting of 0.1 M diethanolamine-HCl buffer, pH 9.75, with 0.33 mM MgCl₂. Reactions are initiated by adding 50 µl of 8 mM *p*-nitrophenylphosphate solution, prepared in the same buffer, and transferred to a 37 °C incubator. Reactions are terminated after 20 min at 37 °C by addition of 100 µl 2 M NaOH and analyzed at wavelength 405 nm by microtiter plate reader.¹⁴

ELISA detection assays

Maxisorp immuno-plates are used for ELISAs. HLA activity is assayed by sandwich ELISA according to the method of Bjerrum and Borregaard (1990) using affinity purified sheep anti-human β_2 microglobulin and mouse anti-human HLA class I as capture and detection antibodies, respectively.

For the CD 16 assay, immuno-plate wells were coated with 45 μ l sample volume for 16–32 h at 4 °C. Because the predominant cellular fraction of this protein is housed within intact secretory vesicles, individual gradient fractions are freeze-thawed according the method of Philips et al. (1991) in the presence of 100 μ M PMSF and protease inhibitor cocktail, added as recommended by manufacturer, prior to initial coating. Coated wells are continuously rinsed for 5 min in wash buffer containing 150 mM NaCl and 10 mM HEPES, pH 7.4, and then exposed to 2 μ g CD 16 monoclonal antibody, diluted in wash buffer supplemented with 0.5% BSA, for 60 min at room temperature with agitation. Plate wells are washed as described above, and a 1:2000 dilution of HRP-conjugated secondary antibody, diluted as above, is applied for 60 min, followed by continuous washing. Reaction buffer (50 mM citrate, pH 4.2, 15 μ M hydrogen peroxide, 0.0275% ABTS) is added and signal quantified spectrophotometrically at wavelength 405 nm. Additional controls applying primary and secondary antibodies to buffer-coated wells are employed to measure associated background noise, which is then subtracted from initial activity readings. Parallel controls run without primary and secondary antibody demonstrate that the HRP-substrate solution is color-stable under these assay conditions. The efficacy of granule lysis by the freeze-thaw method employed (Philips et al. 1991) is evaluated by measuring the relative increase in detergent-free AP activity (see above) before and after treatment of gradient fractions from dextran isolated neutrophil populations. When compared to AP activity released from control fractions assayed in parallel in the presence of 0.1% Triton-X-100, vesicle disruption by this method is found to be ~97% efficient. A detergent-free approach to vesicle disruption is preferred because, inclusion of detergent during the initial coating of immuno-plate wells significantly reduces sensitivity of antigen-detection.

Electrophoresis and immunoblotting

Gradient fractions are solubilized and denatured in 6 $\times$ SDS sample buffer in the presence of 0.1 mM PMSF and protease inhibitor cocktail, added as recommended by manufacturer, by transferring lightly vortexed samples to a heating block, pre-warmed to 90 °C, for 5 min. Samples are again vortexed, allowed to cool to room temperature, and loaded on 10% polyacrylamide slab gels. The electrophoretic mobility of gradient samples is compared to that of pre-stained molecular weight standard proteins. Protein from gels is electro-transferred onto PVDF membranes and treated as described below.

Immunoblotting is performed at 22 °C as previously described (Jesaitis et al. 1988). Following electrotransfer, PVDF membranes are incubated in blocking solution (0.5 M NaCl, 10 mM HEPES, pH 7.4, 3% BSA, 10% goat serum) for 16–32 h at 4 °C and exposed to 0.5 μ g of rabbit polyclonal anti-LF antibody, diluted in DPBS with 3% goat serum, 1% BSA and 0.2% Tween 20, for 60 min (25 °C), rinsed five times in wash buffer (0.25 M NaCl, 10 mM HEPES, pH 7.4, 0.2% Tween 20) for a total of 25 min, and blotted with a 1:20,000 dilution of HRP-conjugated secondary antibody in the same buffer used for the primary antibody. After rinsing membranes as above, signal is measured using Supersignal chemiluminescent detection kit. Signal is then quantified from optical scans of exposed film using ImagQuant software (Version 3.3).¹

Results and discussion

Relative differences in cell surface topography can significantly alter the otherwise fundamental biochemical and biophysical properties generally attributed to mammalian cell plasma membranes (DePierre and Karnovsky 1973). Similarly, surface membrane irregularities resulting from incomplete cell surface integration of various organelle subsets following their mobilization to plasma membranes may be indicated by global alterations in peak sedimentation density of plasma membrane-specific marker activities (Mukherjee et al. 1994). We sought therefore to verify the compositional and structural integrity of plasma membranes from gelatin-isolated neutrophil populations, which may be regarded as

LPS-primed (Guthrie et al. 1984; Haslett et al. 1985), by analyzing the subcellular distribution profile of a panel of commonly employed plasma membrane-specific marker activities.

Reference marker activities

Experiments examining gelatin- and dextran-isolated neutrophil populations were performed in parallel under identical conditions of cavitation, sedimentation and fractionation. Because, sucrose content of gradient fractions rose linearly from 11 to 60% (wt/wt; data not shown), marker activities are expressed in terms of percent sucrose. Comparative analysis of subcellular protein distribution obtained from density fractionated cavitates of gelatin- and dextran-isolated neutrophils were indistinguishable and demonstrate the majority of cellular protein localized within cytosol with minor peaks corresponding to the subcellular location of primary and secondary granules (Figure 1a). Reference markers, LF and MPO, were employed to identify gradient regions enriched in secondary and primary granules, respectively (Figure 1b). As shown in Figure 1b, the subcellular distribution of granule marker activities closely corresponded in gradients formed from either gelatin- or dextran-isolated neutrophil populations.

Plasma membrane marker activities

We initially examined Mg^{++} ATPase activity (Harlan et al. 1977) to determine the distribution of plasma membrane-enriched vesicles following density fractionation of cellular cavitates. Figure 1b shows the typical bimodal localization pattern of this activity clearly indicating preferential enrichment within plasma membranes as well as specific granules and is consistent with previous work (Jesaitis et al. 1982). A small shift in cellular Mg^{++} ATPase activity from specific granules to plasma membrane fractions in gelatin-prepared cells, relative to dextran-prepared cells, was consistently observed, suggesting an additional basis of phenotypic distinction between gelatin- and dextran-isolated populations (see below).

Analysis of HLA, AMP-5' NT, and detergent-free AP activity yielded localization patterns that were superimposable between gradients made

from either dextran- or gelatin-isolated neutrophils (Figure 1c). Furthermore, these profiles closely correspond with those of Mg^{++} ATPase obtained from gelatin and dextran populations in the ~30% range of sucrose densities (compare Figure 1b and 1c). These data suggest that the physical properties of bulk phase plasma membranes derived from gelatin-isolated neutrophil populations are not significantly different as a result of possible complexities associated with cell-surface upregulation of secretory vesicles or previously observed perturbing effects that can potentially result from LPS-plasma membrane interactions (see below).

AP Activity

AP is a glycosphosphatidylinositol (GPI)-linked protein that is enriched in secretory vesicles. Quantitative measurement of this marker activity in the presence or absence of detergent can therefore serve as a primary indicator for extent of secretory vesicle loss within *in vitro* isolated neutrophil populations (Borregaard et al. 1990). Following density fractionation of cellular cavitates, dextran-isolated neutrophils were found to retain from 60 to 70% of their secretory vesicle complement (Figure 2a). Gelatin-isolated neutrophils, however, completely lacked this organelle (Figure 2a), as AP activity showed no sensitivity to detergent in these populations. The absence of error bars for the gelatin-isolated populations analyzed in Figure 2a is due to the lack of detectable latent AP activity. These results are highly reproducible, but only when certain precautions are observed. Specifically, we have found that re-use of various plastics, including sedimentation tubes, pipettes, and separatory funnels, throughout the gelatin-isolation procedure promotes from 20% to 40% retention of secretory vesicles within neutrophil populations (Figure 2b). It is possible that the repeated exposure of cells to plasma-coated surfaces modulates cellular activation potential during the course of isolation. This explanation is consistent with the immunoregulatory effects of certain plasma components on neutrophil behavior described in previous studies (Balke et al. 1995; Tschesche et al. 1994) as well as our own observations of complete secretory organelle loss being contingent on the use of clean, fresh materials during each isolation step. The

Figure 1. Subcellular distribution of protein, plasma membrane, specific granule, and azurophil granule marker activities from neutrophil populations prepared by dextran- (black lines) and gelatin- based (gray lines) procedures. (a) Subcellular distribution of protein in fractions obtained from cavitates of dextran-prepared cells (■) and gelatin-prepared cells (▲). Protein content of gradient fractions is plotted in units of mg/ml as a function of percent sucrose. Protein recovery from gradient fractions ranged from 89% to 95%. (b) Subcellular distribution of plasma membrane-enriched vesicles identified by measuring Mg^{++} ATPase activity in fractions prepared from dextran (▲) and gelatin (▲) prepared cells. PM indicates plasma membrane. As indicated, Mg^{++} ATPase is additionally enriched in specific granules. LF content of gradient fractions from dextran (·····) and gelatin (·····) isolated populations was measured by immuno-blot analysis and used to confirm the subcellular distribution of specific granules (dextran ····; gelatin ····) was identified by measurement of MPO activity, assessed by enzymatic analysis. Marker recovery from gradient fractions exceeded 90%. Data are representative of comparable experiments from 8 gradients, four dextran and four gelatin. Right and left ordinate axes are indicated by 'R' and 'L,' respectively. (c) Subcellular distribution of HLA (■), AMP-5' NT (▲), and detergent-free alkaline phosphatase (▼) activities, indicating plasma membrane enrichment. The data shown are representative of similar results obtained from six gradients (three from dextran-, three from gelatin-isolated neutrophil populations). Marker recovery from gradient fractions was $\geq 84\%$ of the applied activity.

application of new or clean plastic-ware during neutrophil isolation by the gelatin protocol is therefore essential to the successful reproduction of results described in this study.

The incubation requirements for gelatin-mediated red cell sedimentation in the presence of LPS very likely underlie the complete surface upregulation of secretory vesicles in isolated populations. Accordingly, exposure of neutrophils to as little as 50 pg LPS for 30 min at 37 °C induces notable and multiple priming effects on treated populations (Dahinden et al. 1983) and is consistent with the complete upregulation of secretory vesicles in the populations under study (Borregaard et al. 1994). Alternatively, direct neutrophil-LPS interactions occurring during the course of 37 °C incubation may substantially delay (Ward et al. 2000) the otherwise rapid temperature-dependent mobilization of secretory vesicles (Borregaard et al. 1987). These latter observations are consistent with results obtained from high-resolution single cell studies, which suggest that intracellular behavior of this organelle may affect their differential and incomplete integration within plasma membrane structure (Kobayashi and Robinson 1991). In addition, previously observed nonspecific unsaturable adsorption of LPS by plasma membranes (Wilson et al. 1982) may also contribute to irregularities in cell surface organization. Collectively, these studies raise questions concerning the extent of secretory organelle loss in gelatin-isolated neutrophil populations and further suggest possible nonspecific alterations in the surface membrane organization, shape, and/or structure of this population. We have addressed these questions, in part, by quantitatively demonstrating the absence of secretory vesicles in gelatin-isolated neutrophils (see above).

A prominent concern highlighted by one of the reports discussed above (Kobayashi and Robinson 1991) is the apparent capacity of neutrophil secretory vesicles to undergo a type exocytosis following cell stimulation that is similar to the compound exocytic behavior of eosinophils (McLaren et al. 1977) and mast cells (Guo et al. 1998). The resulting incomplete surface integration of this organelle subset would be expected to alter both the structural complexity and physical properties of plasma membranes (Scepek and Lindau 1993) and, consequently, affect density sedimentation profile of plasma membrane marker

activities (Mukherjee et al. 1994). We therefore examined the density sedimentation pattern of AP in gelatin-isolated populations relative to dextran-isolated controls. Measurement of this marker activity in either the absence of detergent (dextran populations, Figure 3a) or in the absence and presence of detergent (gelatin-isolated populations, Figures 2c and 3a, respectively) invariably corresponded with each other as well as with other plasma membrane-specific markers, including Mg^{++} ATPase (compare Figures 1b and 3a), HLA and AMP-5' NT (compare Figures 1c and 3a). Accordingly, our data indicate the uniform cell surface integration of AP activity, and thus secretory organelle membranes, following their complete loss in gelatin-isolated populations.

To further verify the surface accessibility of AP activity, neutrophils were labeled with radioiodinated wheat germ agglutinin, [125 I] WGA, a lectin that binds with high affinity to certain cell surface glycoproteins, prior to cavitation. The subcellular distribution of [125 I] WGA consistently corresponded with cellular AP activity of gelatin-isolated (compare Figures 2c and 3a) as well as the detergent-free activity of dextran-isolated populations (Fig 2c), confirming the cell surface localization of this enzymatic activity in both populations. Notably, these data also demonstrate the utility of AP as a plasma membrane marker for subcellular mapping of plasma membrane enrichment in density gradient fractions prepared from gelatin-isolated neutrophil populations, irrespective of detergent usage.

In gradient fractions obtained from dextran-isolated neutrophil populations, peak distribution of AP activity shifted to approximately 3% greater density when assayed in the presence of detergent (Figure 3a). This finding is in agreement with previous work employing Percoll as density medium indicating secretory organelles to be slightly heavier in average density than plasma membrane-enriched vesicles (Borregaard et al. 1990). In contrast, gradient fractions from gelatin-isolated neutrophils gave *indistinguishable activity profiles in the presence and absence of detergent* (Figure 3a). The density-shift in latent activity present in dextran populations was further examined by calculating the subcellular distribution of intact secretory vesicles. Our calculations confirm peak secretory organelle localization at 33% sucrose with substantial overlap at peak

Figure 2. Distribution of cellular alkaline phosphatase activity from dextran and gelatin-isolated neutrophil populations and subcellular localization of latent activity in density fractionated lysates. (a) Gradient fractions were assayed for alkaline phosphatase activity in the absence and presence of 0.1% Triton-X-100 to determine relative surface-localized and total cellular activity, respectively. Data obtained from analysis of eight gradients, four dextran and four gelatin, are shown. (b) Percent of cellular AP activity that is associated with the cell surface following gelatin-isolation with re-use of various plastic-ware during the procedure (see text). Data are from four gradients, each made with neutrophil populations obtained from different donors. (c) Comparison of surface-associated alkaline phosphatase activity from dextran (▲) and gelatin (▲) prepared cells with subcellular distribution of cell surface label, [125 I] WGA, in dextran (○) and gelatin (●) gradients. Right and left ordinate axes are indicated by 'R' and 'L,' respectively. Recovery of [125 I] WGA from gradient fractions ranged from 85% to 93%. Data are representative of comparable experiments from 8 gradients, including four dextran and four gelatin. Error bars show standard error of the mean. Recovery of alkaline phosphatase activity from gradient fractions exceeded 90%.

plasma membrane density of 30% sucrose (Figure 3b). This localization pattern was confirmed by examining the distribution of CD 16, which, like AP, is a GPI-linked protein that is preferentially enriched in secretory organelles (Detmers et al. 1995). As shown in Figure 3b, the peak activity of CD16 coincides with the profile obtained for intact secretory vesicles, thus confirming the subcellular location of this organelle relative to peak plasma membrane activity.

Figure 3. (a) Subcellular distribution patterns of total cellular (□) and surface-associated (■) alkaline phosphatase activity from dextran-prepared populations and total cellular alkaline phosphatase activity from gelatin gradients (■). Data are representative of comparable experiments from 8 gradients, four dextran and four gelatin. (b) Comparison of surface-associated (▲) and latent alkaline phosphatase activity (calculated; □) with subcellular distribution of CD16 (△) in gradient fractions obtained from density-fractionated dextran-prepared populations. Data are combined from either six gradients (alkaline phosphatase) or three gradients (CD16). Latent activity was calculated as the difference between surface-associated and total cellular alkaline phosphatase. Recovery of alkaline phosphatase and CD16 activities from gradient fractions was 87–95%. Error bars show standard error of the mean.

As the study of plasma membrane biology is relevant to many fields of neutrophil research, a rapid and convenient method of isolating plasma membranes that is effective and widely accessible would facilitate research in many areas of investigation. The gelatin-isolation procedure thus offers a relatively rapid method for obtaining secretory vesicle-free neutrophil populations for use in density fractionation experiments to obtain purified plasma membrane-enriched vesicle populations. On the other hand, we surmise that the significant and differential impact of various neutrophil isolation methods on cellular complement of secretory vesicles demonstrated in this study could explain conflicting data obtained by different groups investigating various aspects of neutrophil physiology. Studies employing dextran-isolated neutrophil populations, for example, have shown that secretory vesicles house a significant fraction of cellular formyl peptide receptor (FPR) (Sengelov et al. 1994) and cytochrome b_{558} (Sengelov et al. 1992). Secretory organelle loss could therefore contribute, directly and also indirectly by priming cells for further degranulation (Sengelov et al. 1993), to substantial variations in the number of cell surface-associated FPR reported in previous studies from gelatin-isolated (Sklar et al. 1984) vs. dextran-isolated (Williams et al. 1977) neutrophil populations. Other studies examining the effector potential of intact secretory vesicles within 'resting' dextran-isolated neutrophils suggest that, upon stimulation with fMLF, initial activation of the NADPH oxidase occurs predominantly within this compartment and that cell-surface upregulation of this organelle confers on plasma membranes the capacity to generate superoxide (Kobayashi et al. 1998). These findings in combination with data presented in the current study suggest a physiologic basis for conflicting findings regarding the membrane specificity of NADPH oxidase assembly and activation found in neutrophils isolated by the gelatin method (Parkos et al. 1985) vs. the dextran method (Borregaard et al. 1983). In addition, findings from the current study directly address the physiologic relevance of specialized subcellular compartments described previously in the context of FPR regulation (Jesaitis et al. 1986; Jesaitis et al. 1993; Jesaitis et al. 1989; Jesaitis et al. 1988), since the 'unstimulated' neutrophil populations employed in these latter studies were isolated by the gelatin method.

Cell-surface upregulation of secretory vesicles has been intimately and inextricably linked with cellular priming (Morgan et al. 1997; Borregaard et al. 1994; Borregaard et al. 1987). Loss of this organelle in gelatin-isolated neutrophils therefore supports a cellular basis of priming in this population. Residual quantities of LPS in combination with incubation conditions required for gelatin-mediated red cell sedimentation may additionally confer an LPS-primed cellular state (Dahinden et al. 1983; Dahinden and Fehr, 1983; Haslett et al. 1985). Therefore, study of plasma membranes bearing the full complement of secretory vesicle membrane-bound components should prove an important system for examining molecular mechanisms of neutrophil activation and effector-response coupling at the surface membrane level.

Notes

- (1) Amersham Biosciences, Piscataway, NJ, USA
- (2) Beckman Instruments, Palo Alto, CA, USA
- (3) Bio-Rad Laboratories, Hercules, CA, USA
- (4) Calbiochem, La Jolla, CA, USA
- (5) Cambrex, Rockville, ME, USA
- (6) Corning Incorp, Corning, NY, USA
- (7) Cortex Biochem, San Leandro, CA, USA
- (8) Fisher Scientific, Pittsburg, PA, USA
- (9) HBI Technologies, Phoenix, AZ, USA
- (10) Hoefer, Inc., San Francisco, CA, USA
- (11) HyClone, Logan, UT, USA
- (12) ICN Biomedicals, Aurora, OH, USA
- (13) Millipore, Bedford, MA, USA
- (14) Molecular Devices, Sunnyvale, CA, USA
- (15) MP Biomedicals, Irvine, CA, USA
- (16) Nalgene, Rochester, NY, USA
- (17) Nunc, Roskilde, Denmark
- (18) Pierce Biotechnology, Rockford, IL, USA
- (19) Pharmacia, Uppsala, Sweden
- (20) Sigma Chemical Co, St Louis, MO, USA
- (21) US Biological, Marblehead, MA, USA

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