Characterization of Human Platelet Surface and Intracellular Membranes Isolated by Free Flow Electrophoresis*

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High voltage free flow electrophoresis has been applied to the separation of human platelet membranes. After short treatment with neuraminidase at the whole cell level, three membrane vesicle subpopulations have been isolated.

Using a surface label (125I-labeled Lens culinaris lectin), the marker enzyme NADH-cytochrome c reductase, and lipid analysis, two of the fractions have been identified as of surface origin and the other consists of intracellular membrane elements. The distribution of adenylate cyclase, leucyl aminopeptidase, 5'-nucleotidase and Ca²⁺-ATPase has also been investigated, and their usefulness as markers for the different membrane fractions has been evaluated. All three fractions are vesicular but differ in size and character. Their phospholipid and cholesterol contents have been determined, and the cholesterol/phospholipid ratios of the two surface fractions are over twice that of the intracellular membrane, which also has a significantly lower microviscosity as determined by fluorescence polarization using diphenyl hexatriene. The polypeptide profiles from sodium dodecyl sulfate-polyacrylamide gel electrophoresis are particularly distinctive, with actin present in the two surface membrane fractions and absent from the intracellular membranes. Myosin, confirmed by its ATPase characteristics, is almost exclusively localized in one of the surface membrane fractions, and actin-binding protein is a prominent feature of the other.

The hemostatic properties of blood platelets depend upon the responsiveness of the plasma membrane to surface signals, which are then transduced in some way to the interior of the cell to activate a sequence of cytoplasmic events which result in shape changes, aggregation, and the secretion of granule-stored procoagulants and other constituents.

In order to investigate at the molecular level the various surface membrane constituents involved in these signal-response mechanisms, the isolation of plasma membranes free from intracellular membrane contamination is a desirable prerequisite. Despite considerable research efforts, however, by many groups using a variety of different technical approaches, such separations have proved extremely difficult to achieve, and the many procedures and problems have been well reviewed by Sixma and Lips (1).

Some of the technical difficulties certainly lie in the small size of the platelet and its unusual resistance to mechanical forces for disruption. At their best, most present procedures result in a single mixed membrane fraction containing surface and intracellular elements or a number of subfractions variously enriched with surface-oriented components but also containing membrane material of intracellular origin. An additional problem, too, is that unlike other cells, normal platelets have a considerable proportion of their plasma membrane invaginated as a surface-connected canicular membrane system. Such domains may differ significantly in composition from the rest of the boundary membrane which is contiguous with them.

In this paper, we describe a new approach to the differential isolation of platelet surface and intracellular membranes using high voltage free flow electrophoresis. The technique was first introduced by Hannig and Heidrich (2, 3). It has been used successfully for the isolation of membrane subfractions from erythrocytes (4) and mitochondria (5), lysosomes (6), and renal tubules (7), but to our knowledge, it has not been previously applied to platelet membrane fractionation.

With this procedure, we have been able to isolate, on a preparative scale, piste membrane subfractions from homogenates of fresh human platelets in which the surface charge had been modified by pretreatment with neuraminidase at the whole cell level. Three membrane vesicle subpopulations are produced of clearly different electrophoretic mobilities and which also differ significantly from each other analytically, enzymatically, in polypeptide and lipid composition, and in microviscosity. One subpopulation is believed to consist of membrane material of intracellular origin, and the other two are believed to represent different surface membrane domains.

MATERIALS AND METHODS

Neuraminidase (Clostridium perfringens) was the affinity-purified Sigma type IX preparation, diluted 1:50 with 0.1 M acetate buffer, pH 5.5, and frozen in small aliquots. Lens culinaris lectin was a gift from Dr. M. Crompton (Imperial Cancer Research Fund Laboratories, Lincoln’s Inn Fields, London). The lectin was trace-labeled with 125I using the chloramin-T procedure (8). The specific activity at the time of preparation was 5 × 10⁶ cpm/mg of lectin. Carrier-free Na⁺-⁵¹ was obtained from The Radiochemical Centre, Amersham, U. K. All other chemicals were commercial preparations of analytical grade.

Isolation of Platelets—Platelets were isolated from fresh buffy coats and processing was started within 3-4 h of collection. The isolation and preparation of washed platelets was carried out at room temperature. The buffy coats were centrifuged at 200 × g for 15 min and the resulting platelet-rich plasma was diluted 1:1 with 0.15 M NaCl, 4 mM EDTA, and the platelets were sedimented by centrifugation at 2000 × g for 20 min.

Preparation and Homogenization of Control and Neuraminidase-
treated Washed Platelets—The platelets were resuspended in 10 mM HEPES buffer, pH 6.2, containing 0.150 M NaCl, 4 mM KCl, 3 mM EDTA, and 0.1% bovine serum albumin and incubated at 37 °C for 10 min. The platelet suspension (usually 20 ml containing approximately 10^10 platelets/ml) was divided into two equal parts: 100 μl of neuraminidase (in 0.1 M acetate buffer, pH 5.5) was added to one half, giving a final concentration of 0.05-0.06 U/ml neuraminidase (in the platelets). An equal volume of acetate buffer, pH 5.5, was added to the other half as a control (platelet platelets). Both platelet suspensions were incubated for 20 min at 37 °C. At the end of the incubation, the suspensions were rapidly diluted 1:3 with 10 mM HEPES buffer, pH 7.2, containing 0.150 M NaCl, 4 mM KCl, 3 mM EDTA, 0.1% bovine serum albumin (washing buffer), and immediately centrifuged at 1800 × g for 15 min. The control and treated platelets were washed three times by resuspension in the washing buffer and finally resuspended in 10 mM HEPES buffer, pH 7.2, containing 0.3 M sorbitol (homogenizing buffer) to a ratio of 4 ml of buffer/g wet weight of cells. Each suspension was sonicated at setting 6 for 10 s at 4 °C using a Dawa sonicator with a 4-mm diameter probe. The sonicate was centrifuged at 2000 × g for 20 min at 4 °C, the supernatant was retained, and the pellet of broken cells and debris was resuspended in the homogenizing buffer and resonicated. After centrifugation, the two supernatants were combined and constituted the starting homogenate for all subsequent subfractionation.

Preparation of Platelet Membranes—Platelet homogenate (~6 ml) was layered onto a 15-ml linear sorbitol gradient prepared from 1 M and 3.5 M sorbitol solutions containing 1 mM EDTA and buffered to pH 7.2. The upper 5 ml of the gradient were then centrifuged for 20 min at 100,000 × g for 15 min. The buffer for the labeling membrane was eluted by sucrose. The tubes were centrifuged in a swing-out rotor (3 × 25 ml) at 42,000 × g for 90 min at 4 °C. Platelet membranes which located in the upper of the two discrete particulate zones were removed with a Pasteur pipette and were centrifuged at 90,000 × g for 90 min.

Free Flow Electrophoresis of Platelet Membranes—Free flow electrophoresis was carried out on an Elphor Varis apparatus (Bender & Hobein, Munich) according to Hannig and Heidrich (3). The electrode buffer was that recommended by Heidrich and Leutner (4) and consisted of 100 mM triethanolamine, 100 mM acetic acid, adjusted to pH 7.2 with NaOH. The buffer for the separating chamber was 10 mM EDTA, 0.1% bovine serum albumin (washing buffer), and immediately centrifuged at 1800 × g for 15 min. The control and treated platelets were washed twice with cacodylate buffer. The pellets were then fixed for 15 min at 4 °C in 2% OsO4, dehydrated in alcohol including a step containing uranyl acetate in 100% ethanol, and then propylene oxide to Epon/Araldite mixture in which they were embedded. Thin sections were mounted on grids and stained with Reynolds lead citrate and 5% aqueous uranyl acetate. Specimens were viewed in a Philips 400T electron microscope.

RESULTS AND DISCUSSION

Electrophoretic Profiles of the Membrane Preparations—Fig. 1 (top) shows full voltage electrophoresis from control platelets. Two subfractions are resolved (C1 and C2) of clearly different electrophoretic mobilities. However, both fractions were labeled with the 125I-labeled lectin being somewhat more enriched. The activity of NADH-cytochrome c reductase showed a broad peak predominantly associated with the most electrophoretic fraction (C1), but this activity overlapped considerably with the C2 fraction.

Pretreatment of platelets with neuraminidase, which under our conditions removes 30–40% of the total cell sialic acid as determined after acid hydrolysis, resulted in a reduction in the electrophoretic mobility of a large proportion of the membrane (Fig. 1, bottom). These membranes now separated into three discrete fractions, N1, N2, and N3, N1 and N3 being respectively the least and most affected by neuraminidase when their mobilities were compared with fractions from platelets not treated with neuraminidase. The 125I-labeled lectin was now only associated with fractions N2 and N3, being substantially higher in N3. Fraction N2 carried virtually no lectin label but the NADH-cytochrome c reductase was located predominantly in this fraction with only a small proportion of activity associated with N2 and none with fraction N1.
As an example of the reproducibility of the procedure, Fig. 2a shows the superimposed protein profiles of the separated membrane fractions from three experiments with different platelet preparations all pretreated with neuraminidase.

With respect to the distribution of membrane protein between the fractions, N_I generally accounts for 25-35% of the material applied to the electrophoresis chamber, and N_II and N_III together, account for the remainder. In addition to the EDTA routinely included as a Ca^{2+}-dependent protease inhibitor at various stages in the preparation, a study was made of the value of adding a wide range protease inhibitor mixture during the preparation. Fig. 2b shows the electrophoresis protein profile for the separated membrane fractions prepared from platelets processed in the presence of phenylmethylsulfonyl fluoride, leupeptin, and pepstatin. The membrane separation was essentially the same as those prepared in the absence of these inhibitors (Fig. 2a), and no differences were revealed in the polypeptide patterns prepared under the two experimental conditions.

Enrichment of Markers in Membrane Subfractions—The membrane fractions from the neuraminidase-treated cells were pooled across the peaks according to the protein profiles as indicated in Fig. 1 (bottom). The specific activities of the homogenate and the electrophoretically separated membrane subfractions for the ^{125}I-lectin label and NADH-cytochrome c reductase activity are shown in Fig. 3. It can be seen that the membrane subfraction pools N_I and N_III were both significantly enriched in the lectin label (N_I, 2.9-3.7-fold; and N_III, 4.8-5.7-fold) with respect to homogenate, and the N_I fraction showed no lectin enrichment. In contrast, this latter fraction had the highest enrichment for NADH-cytochrome c reductase (12.8-13.4-fold) with no enrichment of this enzyme in fractions N_II and N_III. A number of other enzyme activities were investigated for their localization and possible suitability as markers in the characterization of the membrane fractions. These have been selected for inclusion in Fig. 3 on the basis of Fig. 1. Distribution of protein ( ), ^{125}I-lectin ( ), and NADH-cytochrome c reductase ( ; NADH cyt-c-red.) in the electrophoretically separated membrane subfractions from control (top) and neuraminidase-treated (bottom) platelets. Pooled fractions (N_I, N_II, and N_III) were taken as indicated for analytical studies.
that the enzyme enrichment value with respect to homogenate in a particular membrane subfraction was accompanied by depletion in the other two fractions. Leucyl aminopeptidase and 5'-nucleotidase appear to be useful markers for the Nᵢ fraction, though their lower enrichment values may suggest multisite location in the platelet. Adenylate cyclase is predominantly associated with the Nᵢ and Nᵢᵢ fractions, and the Ca²⁺-ATPase is almost exclusively located in fraction Nᵢ with 11-13-fold enrichment. This ATPase measured under high ionic strength conditions could be readily eluted from the membrane with 0.6 M KCl, and the activity showed a pH profile with two peaks at pH 5.8 and above pH 9.0. In the presence of 3 mM Ca²⁺, it was half-maximally inhibited by 25 μM Mg²⁺. These features are characteristic for the Ca²⁺-ATPase of myosin. Studies of the activity of alkaline p-nitrophenyl phosphatase and phosphodiesterase activity towards either bis(p-nitrophenyl phosphate) diester or the corresponding fluorescent substrate bis(methylumbelliferyl) phosphate (23) revealed that they had no value as marker enzymes for these human platelet membrane fractions. This is in contrast to earlier studies with pig platelet surface and intracellular membrane fractions (24), where two phosphodiesterase activities showed differential localization with good enrichments.

The cholesterol and phospholipid contents and cholesterol/phospholipid ratios for the homogenate and the pooled membrane fractions are presented in Table I. The three membrane subfractions differ significantly in a number of respects. The highest cholesterol/phospholipid ratio is found in fraction Nᵢᵢᵢ being approximately 2.5 times greater than in fraction Nᵢ. This ratio difference is accounted for entirely by the high cholesterol content of Nᵢᵢᵢ, since the phospholipid contents of Nᵢ and Nᵢᵢ are substantially the same. Fig. 4 shows a typical fluorescence polarization study of the three membrane subfractions using the probe diphenylhexatriene. The derived microviscosity (η) values have been plotted against temperature, and it is clear that the membrane vesicles of fraction Nᵢ have a very significantly lower microviscosity than the vesicles of the Nᵢᵢ and Nᵢᵢᵢ fractions. This finding is consistent with the much lower cholesterol content found in this fraction, a feature which is now considered to at least partially account for higher fluidity characteristics of certain cell membranes.

Fig. 5 shows the SDS-polyacrylamide gel separations of the mixed membrane fraction and the three membrane subpopulations from a typical free flow electrophoretic separation. The mixed membrane fraction contains between 30 and 40 discrete polypeptides, the most prominent being two which have mobility characteristics corresponding to actin (Mr = 43,000) and the heavy chain of myosin (Mr = 200,000). These two proteins co-migrated with standard preparations of rabbit muscle actin and myosin. The most significant difference between the fractions was the complete absence of these two contractile proteins in fraction Nᵢ. Both proteins were well
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Data from a typical experiment are shown using membrane fractions \( N_I, N_{II}, \) and \( N_{III} \) labeled with diphenyl hexatriene.

**Fig. 4.** Plot of microviscosity against the reciprocal of the absolute temperature. Data from a typical experiment are shown using membrane fractions \( N_I, N_{II}, \) and \( N_{III} \) labeled with diphenyl hexatriene.

**Fig. 5.** SDS-Polyacrylamide gel electrophoretic separations of the polypeptides of the mixed membrane fraction (M) and subfractions \( (N_I, N_{II}, \) and \( N_{III} ) \). The positions of myosin, actin, and other molecular weight standards are indicated.

represented in the membrane fraction \( N_{III} \), but while actin featured most prominently in the \( N_{III} \) fraction, the myosin band was barely detectable. This almost exclusive association of myosin with the \( N_{III} \) membrane fraction is substantiated by the specific localization of the Ca\(^{2+}\)-ATPase activity referred to earlier which displayed characteristic myosin-like properties. Above the position of myosin, there is a faint band in \( N_{III} \) which is more prominent in \( N_{III} \) and absent from \( N_I \). Although the identity of this polypeptide has not been firmly established, it migrates with a mobility closely corresponding to that of actin-binding protein \( (M_w = 260,000) \) and this is being further characterized. The electron microscopy of the mixed membrane fraction showed vesicles of widely varying diameters (Fig. 6). The membrane subfraction \( N_I \) contained a fairly homogeneous population of quite small vesicles, whereas \( N_{II} \) and \( N_{III} \) consisted of vesicles of considerably greater diameters, with very large vesicles predominating in fraction \( N_{III} \).

It is our view that fraction \( N_I \) consists of membrane vesicles of predominantly intracellular origin, since in addition to significant difference from \( N_{II} \) and \( N_{III} \) in lipid composition, microviscosity, electron microscopic appearance, and a characteristic polypeptide profile, it represents membrane regions which at the whole cell level are inaccessible to both the lectin label and neuraminidase (Fig. 1). The enrichment and almost exclusive localization of antimycin-insensitive NADH-cytochrome c reductase in the \( N_I \) fraction (Fig. 1, bottom, and Fig. 3) suggests that these vesicles have some of the characteristics of endoplasmic reticulum fractions prepared from other cells. The presence of, and enrichment of 5'-nucleotidase in the \( N_I \) fraction is particularly interesting, since despite its wide use as a plasma membrane marker, it has been identified in endoplasmic reticulum (25) and in microsomal fractions (26) of other cells, and previously we have found that this enzyme, with AMP as substrate, is not well expressed in surface membrane-enriched fractions prepared from pig platelets (27).

Concerning fractions \( N_{II} \) and \( N_{III} \), the location of the lectin, the absence of reductase activity and vulnerability to neuraminidase attack suggests that both of these fractions are rich in surface-oriented components and may represent two different domains of the plasma membrane. Our results suggest, too, that \( N_{II} \) is somewhat less accessible to both the neuraminidase and lectin label than \( N_{III} \), and may represent invaginated regions of the platelet surface. The microviscosity profiles for \( N_{II} \) and \( N_{III} \) are closely similar, as are also the polypeptide patterns, with the one major exception that \( N_{II} \) is very much enriched in myosin. This myosin is readily eluted from the vesicles with 0.6 M KCl, and it is not known whether its presence depends upon association with actin or some other membrane component. The relatively low lipid content of this \( N_{II} \) fraction, expressed in terms of membrane protein, may of course be accounted for by the rich content of cytoskeletal proteins.

In conclusion, we believe that high voltage free flow electrophoresis has some significant advantages over existing procedures for the differential isolation of platelet membranes. With this technique, we believe we have been able to separate surface and intracellular membrane vesicles from a human platelet mixed membrane fraction. Appropriate markers for the membrane subfractions show good enrichment and negligible cross-contamination, and the procedure is reproducible and can be carried out on a preparative scale. Additionally, the surface membrane separates into two discrete subpopulations which differ significantly in morphological and molecular criteria. We believe these two fractions may represent different plasma membrane domains, and the possible site of origin in the intact platelet of all three membrane subfractions is being investigated.
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**FIG. 6.** Electron micrographs of mixed membrane (a) and membrane subfractions N_I, N_II, and N_III (b, c, and d, respectively). The bars equal 1 μm.

**Acknowledgments**—We wish to thank Dr. A. Sturk and Prof. J. C. White for their helpful advice, and Drs. R. J. Haslam and M. Lagarde for the many stimulating discussions during the course of this work. We are also grateful to Dr. Gilliam Bullock and Mr. Brian Kemmenoe of Ciba-Geigy Ltd., Horsham, for kindly preparing the electron micrographs. The fluorescence polarization studies were made by H. W. in the laboratory of Dr. M. Shinitzky at The Weizmann Institute of Science, Rehovot, Israel. We are grateful for his hospitality. Our thanks are also due to the National Blood Transfusion Service Laboratory at Tooting, London, S.W.17, for supplying fresh buffy coats.

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