Biochimica et Biophysica Acta 819 (1985) 1–9
Elsevier

BBA 72726

A rapid isolation procedure of plasma membranes from human neutrophils using self-generating Percoll gradients. Importance of pH in avoiding contamination by intracellular membranes

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(Received April 10th, 1985)

Key words: Plasma membrane; Endoplasmic reticulum; Neutrophil; Percoll gradient; 5′-Nucleotidase; (Human)

In this study we report an overall procedure for the isolation of both human polymorphonuclear neutrophils and their plasma membrane, by means of self-generating Percoll gradients. After efficient purification (40% yield), neutrophils were lysed by nitrogen cavitation and cellular structures quickly isolated in a one-step procedure. Plasma membrane recovery was monitored by [^{3}H]concanavalin A and 5′-nucleotidase (EC 3.1.3.5) activity. We showed the latter activity is indeed present in human neutrophils. The procedure resulted in a good yield of plasma membrane, since 45% and 55% of total 5′-nucleotidase and [^{3}H]concanavalin A activity, respectively, were recovered within two gradient fractions. Depending on the final pH of the Percoll gradient medium, endoplasmic reticulum markers contaminated either the plasma membrane or the granule fractions. At pH 9.05, NADH-ferricyanide reductase activity clearly separated from plasma membrane markers and displayed the same profile as CDPcholine:diacylglycerolcholine phosphotransferase (EC 2.7.8.2), a typical enzyme of endoplasmic reticulum. These results emphasize the need for strict monitoring of the pH of the gradient medium in subcellular fractionation of neutrophils.

Introduction

Chemotaxis, endocytosis and initiation of a respiratory burst are major steps in the antibacterial activity of the polymorphonuclear leukocyte. These membrane-mediated processes are triggered by the interaction of extracellular stimuli with the plasma membrane, and they are associated with the activation of the contractile proteins. Precise study of the molecular events occurring at this level requires preparation of highly purified membranes. Several methods are presently available to purify plasma membranes from either animal or human neutrophils [1–7]. As usually in this field, they are generally a compromise between the degree of purity, the yield of membrane material and the length of the separation procedure. The latter parameter might reveal critical specially with neutrophils, owing to the abundance of proteases, which often implies the use of specific inhibitors [1–8]. Also the risk of lipid damage by peroxidative cleavage might well be increased during time-consuming procedures of membrane isolation.

Among the methods described so far, the best purity of plasma membranes is generally attained by using isopycnic centrifugation [1–7].

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In previous studies dealing with Krebs II ascite cells [9] and human platelets [10,11], centrifugation on self-generating Percoll gradients was found rather convenient to quickly separate a light density fraction containing plasma membranes and intracellular membranes (endoplasmic reticulum) from a high density fraction containing secretion granules, lysosomes and mitochondria. Moreover, increase the pH to alkaline values revealed that intracellular membranes acquired an apparently higher density, provoking their shift to the bottom of the gradient, whereas plasma membranes still displayed the same light density. Based on this finding, the separation of plasma membrane from other intracellular membranes or organelles of Krebs II cells was successfully achieved using a single alkaline Percoll gradient [9].

We describe here a simple, one-step, and rapid procedure, effective in producing a high-yield purification of human neutrophil plasma membrane. Cellular disruption was carried out by nitrogen cavitation, and self-generating Percoll gradients were used both for isolation of the cells and for separation of subcellular particles. Two important points are revealed by the present data: (1) the existence of a high 5'-nucleotidase activity in human neutrophils; (2) the possible contamination of either plasma membrane or granules by the reticulum, depending on the pH of the Percoll gradient.

Material and Methods

Materials. [^{3}H]Concanavalin A (60 Ci/mmol), sodium boro[^{3}H]hydride (5.0 Ci/mmol), and [^{3}H]AMP (15 Ci/mmol) were purchased from the Radiochemical Center, (Amersham International) Amersham, U.K., Plasmagel® from Laboratoire Roger Bellon, Neuilly, France, Percoll® from Pharmacia, Uppsala, Sweden, and *Vibrio cholerae* neuraminidase (5 mU/mg) from Merck, Darmstadt, F.R.G. Galactose oxidase (200 U/mg) and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Krebs-Ringer buffer contained: 121 mM NaCl, 4.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 16.5 mM Na_2HPO_4 and was adjusted to pH 7.4 using 1 M HCl.

Isolation of neutrophils. Human peripheral blood was obtained from healthy adult volunteers by venipuncture (100 to 150 ml). The blood was anticoagulated in plastic syringes with disodium-EDTA (0.5%). A 1/4 volume of Plasmagel was added, and the syringes were placed obliquely at 37°C for 20 min. Thereafter, the whole procedure was carried out at 4°C. The leukocyte-rich supernatant was collected in a plastic tube, and centrifuged at $200 \times g$ for 10 min after adding a double volume of 0.16 M ammonium chloride. The cell pellet was washed and resuspended in Krebs-Ringer buffer (pH 7.4), containing 2 mg/ml glucose. Two ml of cell suspension were layered on a previously prepared mixture (pH 7.4) of 15.4 ml Percoll, brought to isotonicity with 6.6 ml 0.49 M NaCl, 0.033 M Tris-HCl (pH 7.4). Neutrophils were then separated by centrifugation at $48\,000 \times g$ for 10 min ($3.48 \cdot 10^9$ rad$^2 \cdot$ s^{-1}) in a fixed-angle rotor (Beckman, rotor 60 Ti). This procedure resulted in the formation of two clearly visible cell bands: the upper band contained platelets and other leukocytes, whereas neutrophils were concentrated in the lower band. The latter were carefully collected with a siliconized Pasteur pipette and washed twice in Krebs-Ringer buffer. The number of neutrophils was calculated from the leukocyte count (Model ZF Coulter Counter) and the differential on May-Grünwald-Giemsa-stained smears. Cell viability was assessed by the Trypan blue exclusion technique.

Neutrophil subcellular fractionation. All procedures were carried out at 4°C on ice. Neutrophil disruption was performed by the nitrogen cavitation technique, as previously described [9]. Briefly, cells were suspended to a concentration of 10% (v/v) in an isotonic lysis buffer consisting of 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 25 mM Tris-HCl (pH 9.6), with 0.02 mM phenylmethylsulphonylfluoride as a protease inhibitor. They were subjected to a pressure of 40 bars of N_2 for 20 min in a Kontes pressure homogenizer (Kontes, Vineland, NJ, U.S.A.), followed by dropwise release in a plastic tube. The lysate was centrifuged at $1000 \times g$ for 10 min to sediment nuclei and undisrupted cells.

Plasma membrane separation. Four ml of the supernatant were loaded on the top of a previously

prepared mixture of 11 ml Percoll, 2.2 ml distilled water buffered with 4.8 ml 400 mM KCl, 20 mM $MgCl_2$, 400 mM Tris-HCl (pH 9.6), so that final conditions were isotonic [9]. However, due to a non-negligible buffering capacity of the Percoll gradient, the final pH of the medium under these conditions was only 8.8. In order to bring the pH up to the desired value (e.g. 9.6, which was the highest value tested in this study), the 2.2 ml volume of distilled water was replaced by a mixture of 1.2 ml of 0.1 M NaOH and 1 ml water. This procedure prevented the flocculation observed when NaOH was directly added to the Percoll. At pH 9.2, the osmolarity of the gradient was 330 mosM.

After centrifugation ($1.29 \cdot 10^{10}$ $rad^2 \cdot s^{-1}$, i.e. $160\,000 \times g$ for 10 min at the plateau in a Beckman rotor 60 Ti), 2 ml fractions were harvested from the top of the gradient. The density was decreased by adding 4 ml of 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4) and Percoll was removed by centrifugation at $200\,000 \times g$ for 45 min (Beckman rotor 50 Ti), as previously described [9].

Cell surface labelling. Lectin labelling of neutrophils was performed with tritiated concanavalin A. About 10^8 cells in 10 ml Tris-HCl buffer (pH 7.4) were incubated with 2 μCi of [^{3}H]concanavalin A for 10 min at 20°C with gentle agitation. Cells were washed twice in the same buffer and resuspended in the lysis buffer (pH 9.6). Approximately 60% of the added [^{3}H]concanavalin remained bound to the cells. At this concentration, the lectin produced neither respiratory burst, nor degranulation of the neutrophils.

The galactose-oxidase tritiated sodium borohydride method [12] was also used to label neutrophil surface glycoproteins. $5 \cdot 10^7$ neutrophils in 1 ml Krebs-Ringer buffer (pH 7.4) were treated with 25 U/ml of *Vibrio cholerae* neuraminidase for 15 min at 37°C. Neutrphils were then washed twice in Krebs-Ringer buffer, followed by incubation with 5 U/ml of galactose oxidase for 30 min at 37°C. The cell suspension was made up to 10 ml by the addition of Krebs-Ringer buffer and centrifuged. The pellet was washed twice and resuspended in 0.5 ml Krebs-Ringer buffer. Sodium boro[^{3}H]hydride (0.5 mCi) was added to galactose oxidase-treated neutrophils and the mixture incubated at 20°C for 30 min. The cells were washed three times in Krebs-Ringer buffer and then resuspended in the lysis buffer.

Enzyme markers. Membrane enzymatic activities were determined as alrelady reported [9] except that 5'-nucleotidase (EC 3.1.3.5) was measured at pH 9.0 with 0.1 mM AMP (specific radioactivity 2.7 μCi/μmol). NADH-dehydrogenase (EC 1.6.99.3) with ferricyanide as electron acceptor was determined at 340 nm since Percoll had previously been removed. The same activity with cytochrome *c* as acceptor (EC 1.6.99.3) was also determined, as well as CDPcholine:1,2-diacylglycerolcholine phosphotransferase (EC 2.7.8.2) [13].

For the secretory granules, lysozyme (EC 3.2.1.17) was assayed by the microbiological method, using *Micrococcus lysodeikticus* as a substrate and hen egg white lysozyme as a reference. β-Glucuronidase (EC 3.2.1.31) was tested using Sigma kit. Cytosolic lactate dehydrogenase (EC 1.1.1.27) was also assayed with Sigma kit. Succinate dehydrogenase (EC 1.3.99.1) and monoamine oxidase (EC 1.4.3.4) for mitochondria were tested by the methods of King [14] and Stadt et al. [15], respectively. Galactosyltransferase (EC 2.4.1.38), used as a marker for the Golgi membranes, was determined according to Verdon and Berger [16].

Electron microscopy. Pelleted subcellular fractions were fixed with 1.25% glutaraldehyde in phosphate buffer for 30 min at 4°C. They were then washed in 0.1 M isotonic phosphate sucrose buffer (pH 7.4), and postfixed in 1% osmic acid solution (pH 7.4). Specimens were dehydrated through graded ethanol and propylene acid and embedded in epoxyresin. Thin sections were doubly stained with lead citrate and uranyl acetate and examined with a Hitachi H300 electron microscope.

Analytical methods. Proteins were determined by the method of Lowry et al. [17] in the presence of sodium dodecyl sulphate (0.07% w/v). DNA was assessed by the method of Giles and Myers [18], using calf thymus DNA as standard. Radioactivity was evaluated with a Packard Tri-Carb 4530 spectrometer equipped for automatic quenching correction, using Picofluor as scintillation fluid (Packard, U.S.A.).

Results

Cell isolation

Neutrophil isolation was performed on a self-generating Percoll gradient. The high density band, containing neutrophils, was clearly differentiated from that of platelets and other leukocytes on the top of the gradient. That band contained 98% of neutrophils, and less than one platelet per 10^3 neutrophils. More than 96% of neutrophils excluded Trypan blue, and no morphological modification was detected by transmission electron microscopy. In most cases, approximately $8 \cdot 10^7$ neutrophils were recovered from 100 ml of blood (40% yield).

Low speed fractionation of the cell lysate

After centrifugation of the lysate at $1000 \times g$ for 10 min, the supernatant contained 90% of the total protein and more than 70% of [^{3}H]concanavalin A, 5'-nucleotidase, NADH-ferricyanide reductase, β-glucuronidase, and lysozyme activities. Succinate-cytochrome *c* reductase and monoamine oxidase activities were not detected. Confirming data from microscope examination (not shown), over 90% of lactate dehydrogenase remained in the supernate, indicating almost total disruption of neutrophils. On the other hand, the pellet contained over 97% of DNA, therefore nuclear membranes did not appear to be disrupted. The $1000 \times g$ supernatant was thus used for further fractionation.

Among the various enzymes, significant 5'-nucleotidase activity was detected. However, this was strongly dependent on pH, and, as illustrated in Fig. 1, a very narrow peak appeared at pH 9.0. Moreover, addition of 1 mM *p*-nitrophenyl phosphate to the medium did not significantly alter the activity, suggesting that it was not supported by a non-specific phosphatase.

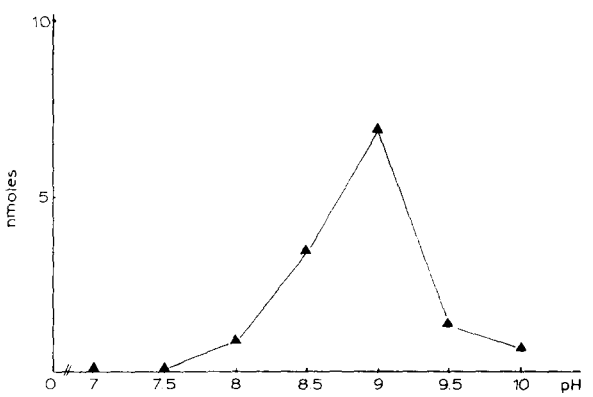

Fig. 1. pH dependence of the 5'-nucleotidase activity in human neutrophils. Results corresponding to nmoles produced at 37°C for 30 min. Substrate: 25 nCi 5'-[^{3}H]AMP/ml, 0.10 mM 5'-AMP. 1 mM *p*-nitrophenyl phosphate. Tris-HCl buffer: 100 mM Tris/0.18 mM $MgCl_2$.

Distribution of various markers on the Percoll gradient

The $1000 \times g$ supernatant was then fractionated on the Percoll gradient at different pH values. In all cases, two visible bands appeared: the light and the high density bands corresponded to membrane

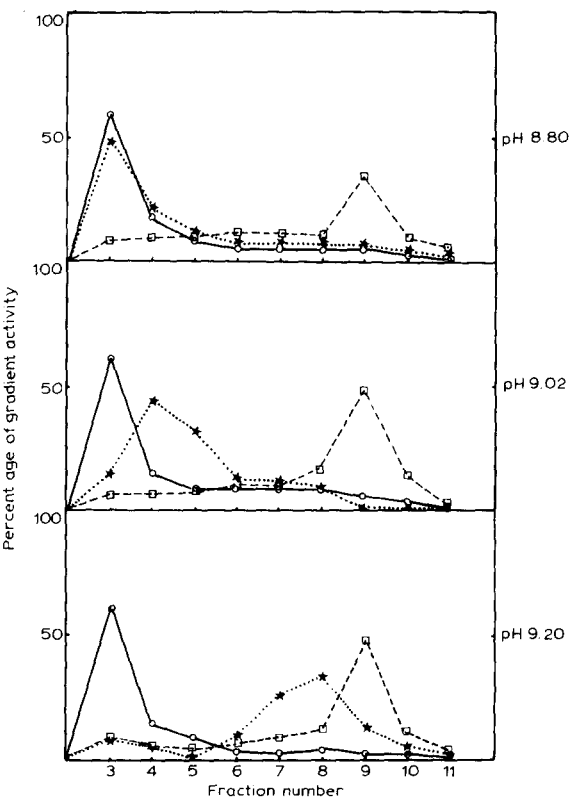

Fig. 2. Localization of markers (percentage of gradient activity) according to the final pH of the Percoll separation gradient. ○——○, [^{3}H]Concanavalin A (plasma membrane marker); ★·····★, CDPcholine:1,2-diacylglycerolcholine phosphotransferase (endoplasmic reticulum marker); □– – –□, β-glucuronidase (lysosome marker).

and granule fractions, respectively (see below). They were separated by a diffuse fraction referred to as the 'intermediate fraction'. As illustrated in Fig. 2, [^{3}H]concanavalin A, used as a plasma membrane marker, peaked in fraction 3, independent of the pH. A similar stability was observed with the granule marker β-glucuronidase, which was maximal in fraction 9. In contrast, pH changes exerted a strong influence on the apparent density of CDPcholine:diacylglycerolcholine phosphotransferase, used as an endoplasmic reticulum marker. As shown in Fig. 2, the latter enzyme was superimposed with [^{3}H]concanavalin A at pH 8.80, whereas increasing the pH to 9.02 and 9.20 induced a remarkable shift of CDPcholine:diacylglycerolcholine phosphotransferase to the lower part of the gradient. The same behaviour was observed for glucose-6-phosphatase and NADH-ferricyanide reductase (not shown).

An intermediate pH of 9.05 was thus routinely used in all further fractionations. As illustrated in Fig. 3, [^{3}H]concanavalin A and 5′-nucleotidase profiles coincided exactly and still peaked at low density (fraction 3). The same profile was also found for boro[^{3}H]hydride labelling (not shown). In contrast, the granule markers lysozyme and β-glucuronidase overlapped at high density (fraction 9). Under these conditions, both CDPcholine:diacylglycerolcholine phosphotransferase and NADH-ferricyanide reductase displayed very similar, if not identical, profiles, with a maximum in the intermediate band (fraction 5).

Specific activities of various markers and protein content of the three major fractions isolated in this way are reported in Table I. Plasma membrane, intermediate and granule fractions represented 4.9%, 4.1%, and 15.4%, respectively, of the total protein from the cell lysate. The remainder was lost in the 1000 × g pellet and with the soluble proteins eliminated during centrifugation of the fractions obtained from the Percoll gradient. Indeed, cytosolic proteins were present all over the gradient using our procedure.

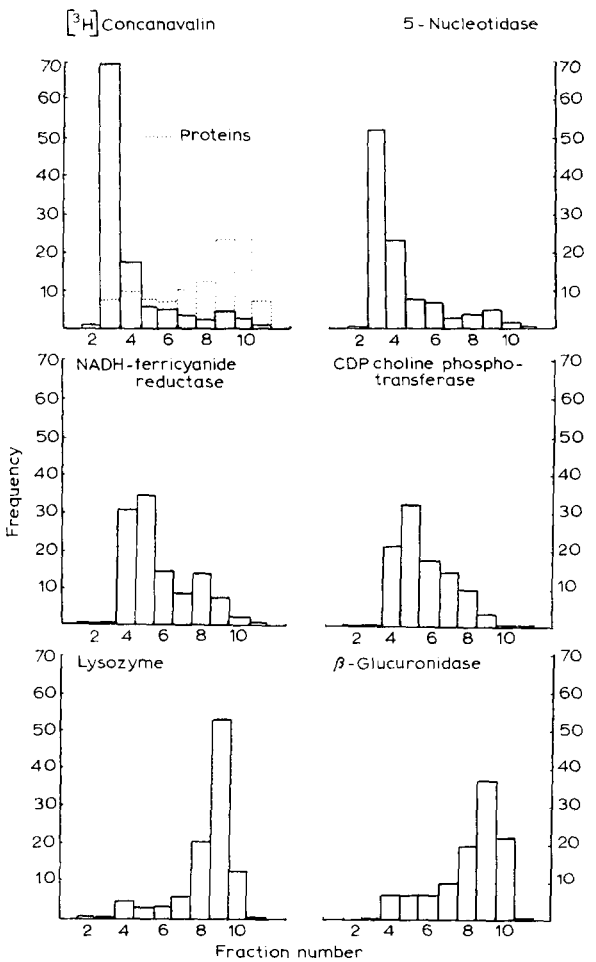

fig. 3. Typical fractionation experiment at pH 9.05. Distribution of markers on Percoll gradients.

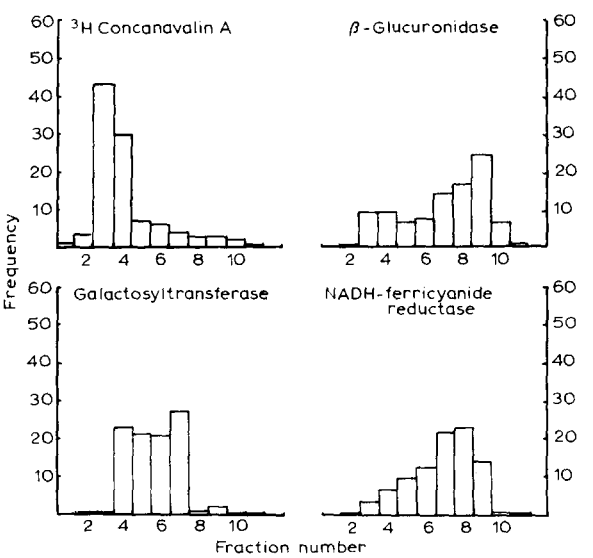

Fig. 4. Behaviour of galactosyltransferase activity on Percoll gradients at pH 9.05. Identical results were obtained in two separate experiments.

TABLE I

SPECIFIC ACTIVITIES OF MARKERS IN PLASMA MEMBRANE (FRACTIONS 3, 4: 4 ml): INTERMEDIATE (FRACTIONS 5, 6, 7: 6 ml) AND GRANULE FRACTIONS (FRACTIONS 8, 9, 10: 6 ml)

Results are means ± S.E. from three experiments. Specific activities are expressed in dpm/μg protein (a), nmol/h per mg protein (b), nmol/min per mg proteins (c), and μg/min per mg protein (d). Protein content of fractions (e) is in μg/ml. Numbers in parenthesis refer to enrichment ratios relative to cell lysate.

Markers	Cell lysate	Plasma membrane fractions	Intermediate fractions	Granule fractions
[^{3}H]Concanavalin A (a)	76 ± 13	434 ± 70 (5.7)	23 ± 11 (0.30)	4 ± 2 (0.05)
5′-Nucleotidase (b)	756 ± 215	4197 ± 1552 (5.6)	1677 ± 775 (2.2)	177 ± 70 (0.23)
NADH-ferricyanide reductase (c)	125 ± 42	85 ± 14 (0.68)	1771 ± 632 (14.2)	125 ± 37 (1.0)
NADH-cytochrome c reductase (c)	22 ± 7	68 ± 12 (3.1)	247 ± 58 (11.2)	13 ± 6 (0.59)
CDPcholine-diacylglycerolcholine phosphotransferase (b)	3 ± 1	3 ± 1 (1.0)	12 ± 5 (4.0)	2 ± 1 (0.67)
β-Glucuronidase (b)	1986 ± 196	500 ± 46 (0.25)	1055 ± 242 (0.53)	3145 ± 781 (1.6)
Lysozyme (d)	27 ± 5	2 ± 1 (0.07)	21 ± 4 (0.78)	75 ± 22 (2.8)
Proteins (e)	3680 ± 1212	182 ± 47	150 ± 21	566 ± 122

The plasma membrane fraction was similarly enriched in 5′-nucleotidase and [^{3}H]concanavalin A (5.6- and 5.7-fold, respectively), and only slightly contaminated by endoplasmic reticulum markers, which displayed maximal activities in the intermediate fraction. As for β-glucuronidase and lysozyme, significant enrichment ratios (1.6 and 2.8, respectively) were only observed in the granule fraction.

In order to better define the plasma membrane fraction isolated under our conditions, additional experiments were performed to detect galactosyltransferase on the Percoll gradients. As shown in Fig. 4, the Golgi membrane marker was the most abundant in the intermediate fractions, although appearing at a somewhat lower density than the endoplasmic reticulum enzyme, NADH-ferricyanide reductase. When considering the plasma membrane itself, the major fraction (fraction 3) was devoid of galactosyltransferase activity, whereas fraction 4 displayed some contamination by Golgi membranes.

Electron microscopy examination

Fig. 5A shows that fractions 3 and 4 from the Percoll gradient contained predominantly membrane vesicles of variable size. The section from fractions 5 to 7 (Fig. 5B) shows a general aspect very different from Fig. 5A. The material is more heterogeneous and structures similar to strands of rough endoplasmic reticulum are observed (arrows). However, their identity with authentic rough endoplasmic reticulum remains to be established. Only a slight cross-contamination by plasma membrane and small granules can be seen. The predominant structures in fractions 8–10 (Fig. 5C) are typical lysosomes with their electron-dense matrices, and limited by an intact membrane.

Discussion

This paper reports a simple and convenient procedure for rapid isolation of both human neutrophils and their plasma membrane using self-generating Percoll gradients.

The cellular yield obtained was similar to that provided by previously described methods, using Ficol-Hypaque medium. In our experience, the latter method resulted in a contamination of neutrophils by platelets in a ratio 1 : 10. Using the

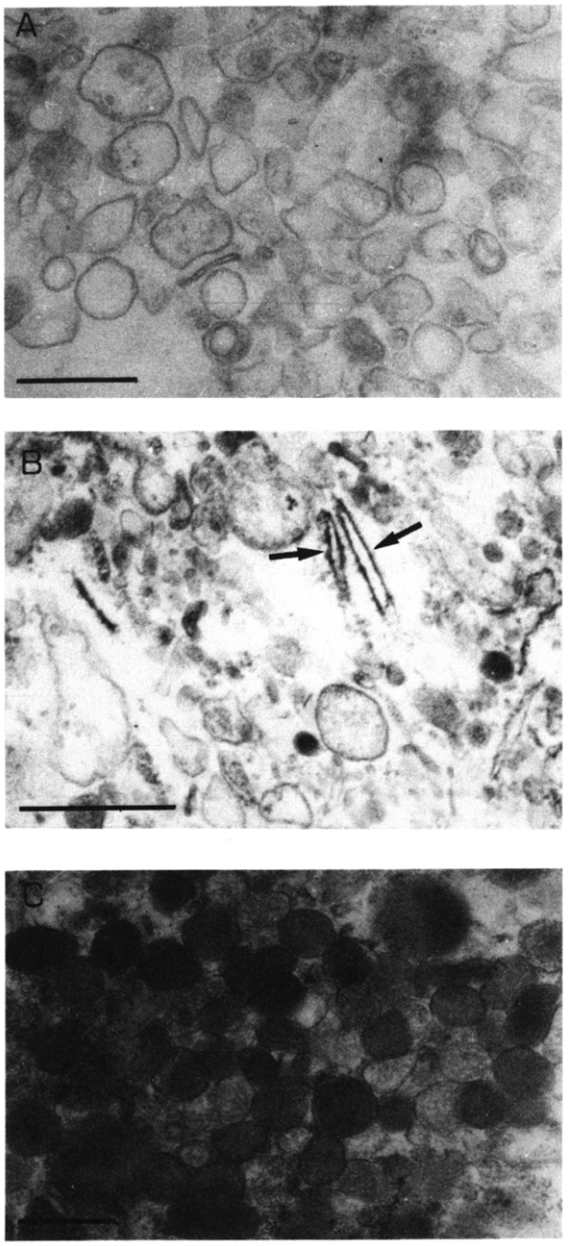

Fig. 5. Electron micrographs of neutrophil subcellular fractions: (A) plasma membrane fraction (×32000); (B) intermediate fractions (×44800), arrows indicate strands of endoplasmic reticulum; (C) granule fraction (×24000). The bar represents 0.5 μm in each case.

present procedure, this contamination was reduced to less that 1 platelet for 1000 neutrophils.

Prior incubation with Percoll substantially inhibits adherence of peritoneal macrophages from mice [19]. At 20–37°C, these cells ingest a large amount of Percoll particles [19]. However, neutrphils isolated by our procedure exhibited satisfactory chemotaxis and superoxide anion production. Moreover, electron micrographs showed they did not take up Percoll (data not shown).

These highly purified neutrophils were disrupted by the cavitation procedure. The N_2 pressure we used was higher than that generally reported for neutrophils, i.e. 350–450 lb/inch^2. However, we observed neither nuclear breakage with subsequent contamination of membranes by basic nuclear proteins [4], nor abnormal marker solubilization, which proves subcellular organelle integrity [9].

This almost total cell lysis and the composition of the lysis buffer probably account for the amount of membranous material recovered in the 1000 × *g* supernatant, as compared with other procedures [5].

Contrary to previous reports [6,20–22], our data give evidence for 5′-AMP phosphohydrolase activity in human neutrophils. Failure to detect such an enzyme could be explained by the alkaline pH required to support a maximal activity (Fig. 1), which remains higher compared to that reported by some authors [5,23]. Although 5′-nucleotidase can also display an intracellular localization in hepatocytes [24], in our case the distribution profile coincides exactly with that of the other membrane markers, borohydride labelling and concanavalin A fixation (Figs. 2, 3). So 5′-nucleotidase determined as described herein might represent a convenient tool to follow neutrophil plasma membrane during subcellular fractionation. Further studies dealing with various substrates are sill required to conclude that this activity corresponds to an authentic 5′-nucleotidase, different from non-specific alkaline phosphatase [25,26] also used as a plasma membrane marker [6]. However, the lack of significant enzyme inhibition by an excess of *p*-nitrophenyl phosphate fits with the data of Stewart et al. [27], who also described 5′-nucleotidase as a useful marker for plasma membrane from rabbit neutrophils.

In the present study, concanavalin A was used in a sucrose-free medium which prevented its removal from the plasma membrane receptors. Thus,

contrary to previous findings [20], no redistribution of the lectin was observed during cell disruption (Figs. 2, 3). We thus conclude that 5′-nucleotidase as well as [^{3}H]concanavalin A are suitable markers for plasma membranes in human neutrophils.

The enzyme NADH-ferricyanide reductase is present in human neutrophils at levels significantly higher than those reported in human erythrocytes. This enzyme has been localized by Badwey et al. [28] in the plasma membrane fraction. An NADH-oxidoreductase which reduces ferricyanide has also been described in the plasma membrane fraction of murine liver [29]. However, using exactly the same assay procedures as Badwey et al. [28], we found that this enzyme is associated with endoplasmic reticulum markers. The same results were obtained with cytochrome *c* as electron acceptor.

Depending on the experimental conditions (e.g. gradient pH), our data indicate that endoplasmic reticulum markers may contaminate either the plasma membrane or the granule fraction, the latter possibility being found in the literature [5].

For this reason, we consider that possible contamination by endoplasmic reticulum must be taken into account in studies on the metabolic processes occurring after neutrophil stimulation, such as translocation of oxido-reductase activities [6,20,30,31]. In this respect our data would seem to exclude any participation of NADH-ferricyanide and NADH-cytochrome *c* reductases in the respiratory burst occurring at the plasma membrane level, which is known to be supported by an NADPH oxidase [6,20,30,31]. The physiological importance of endoplasmic reticulum in neutrophils is also emphasized by the specific activity of an enzyme of the phospholipid metabolism, CDPcholine:diacylglycerolcholine phosphotransferase, whose level is similar to that observed in cells with well-characterized endoplasmic reticulum [13].

From the data of galactosyltransferase activity, it could be deduced that Golgi membranes display an intermediary density under our experimental conditions. However, some contamination occurs in the plasma membrane peak, which might require to select the lower fraction of the plasma membrane band in order to avoid contamination by this organelle. A similar dissociation of plasma and Golgi membranes from human neutrophils was successfully achieved by Jesaitis et al. [5] using isopycnic sucrose density gradients.

The granular fraction accounts for the most important part of the total cell protein content. This could explain the relatively low level of enrichment factors for the lysosomal markers. The low enrichment factor of β-glucuronidase activity may also be explained by the presence of trace amounts of Percoll, which was found to interfere with this enzymatic assay [6]. At variance with previous reports the granules concentrated in a single fraction, which did not discriminate between specific and azurophil granules [6]. However, this was not the purpose of the present study, which set out to isolate in a one-step procedure plasma membranes with good guaranties of a sufficient purity. As for mitochondria, even with sensitive techniques, we found no significant activity of the two enzyme markers tested, due to the scarcity of this organelle in mature granulocytes [32–34].

Acknowledgements

Thanks are due to Mrs. Y. Jonquières and Ms. N. Crowte for correcting the English manuscript.

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